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Research Paper

An axenic strain reveals the responses of *Phaeodactylum tricornutum* to external organic carbon*

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Abstract The model diatom *Phaeodactylum tricornutum* is considered a promising source of various high value bioproducts, and developing cultivation processes is crucial for its commercialization. Although mixotrophy and heterotrophy have been recommended as effective strategies for microalgal cultivation, previous studies on *P. tricornutum* have yielded conflicting results in terms of cultivating this microalga. To verify the capacity of this microalga utilizing external organic carbon, both heterotrophic and mixotrophic cultivation with varied carbon sources were performed using an axenic strain. The results demonstrate that glycerol was the only organic carbon that substantially stimulated the growth of *P. tricornutum* in the presence of light. Sodium acetate (NaAc) at low concentrations could also promote growth, while at high concentrations led to severe inhibition under mixotrophic conditions. The addition of glucose imposed no appreciable impact on either cell density or biomass concentration, confirming that *P. tricornutum* cannot metabolize external glucose. Subsequently, a comparative analysis between mixotrophy and autotrophy was performed to reveal the influences of glycerol on the cellular metabolism based on growth performances, biochemical compositions, and chlorophyll fluorescence parameters. Results also indicate that the addition of glycerol did not have detrimental effects on the capacity of either pigments biosynthesis or photosynthesis, but enhanced the saturated fatty acids and reduced the unsaturated fatty acids.

Keyword: *Phaeodactylum tricornutum*; autotrophy; mixotrophy; fucoxanthin; glycerol

1 INTRODUCTION

Due to their high photosynthetic efficiency and rich bioactive substances, microalgae are being increasingly recognized as promising organisms of bioexploration for value-added compounds, such as polyunsaturated fatty acids (PUFAs), carotenoids, and polysaccharides (Marella et al., 2021). *Phaeodactylum tricornutum* is one of the most extensively studied microalgal species and it is well known for its outstanding ability on accumulation significant amounts of PUFAs and fucoxanthin. One notable PUFA that *P. tricornutum* accumulates is eicosapentaenoic acid (EPA, C20∶5), which exhibits multiple benefits in brain and eye development and cardiac diseases prevention (Song et al., 2020). In *P. tricornutum*, EPA accounts for 30%–40% of the total fatty acids (TFAs) and varies within the range of 1.7%–4.4% of the dry cell weight (DCW) (Song et al., 2020; Su et al., 2020). Fucoxanthin, with

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prices exceeding $40 000 per kilogram (Abu-Ghosh et al., 2016), is the most dominant carotenoid in *P. tricornutum*. Under optimal cultivation conditions, the fucoxanthin in *P. tricornutum* can reach as high as to 60 mg/g, approximately one or even two orders of magnitude higher than that of macroalgae (Wang et al., 2021). In addition, versatile molecular tools have been developed in this microalga, making it possible to be utilized as a chassis for the biosynthesis of a variety of both natural and genetically engineered products (Butler et al., 2020). Consequently, interests in developing *P. tricornutum* as a biofactory for valuable bioproducts have been on the rise.

However, several obstacles remain to be overcome for *P. tricornutum* to become a viable source of valuable products. One of the greatest challenges is the low biomass productivity in large-scale cultivation. The limitation of light supply, especially in high-density cultures where shelf-shading occurs, makes it challenging for microalgae to achieve high biomass concentrations under autotrophic conditions. Although microalgae are generally regarded as autotrophs, some species can thrive on exogenous organic carbon in the presence or absence of light, a phenomenon known as mixotrophy or heterotrophy, respectively. Compared to autotrophy, microalgae grown under mixotrophic and heterotrophic conditions can overcome the constraints of light availability, resulting in significantly higher maximum biomass concentrations (Cerón-García et al., 2013; Marella et al., 2021). For instance, it has been observed that heterotrophic and mixotrophic microalgae exhibit growth rates and biomass concentrations that are several times higher than those cultured under autotrophic conditions (Castillo et al., 2021). As a result, both heterotrophy and mixotrophy are considered promising strategies for the commercialization of microalgae.

Heterotrophic and mixotrophic studies in *P. tricornutum* have been extensively conducted, with glycerol, glucose, fructose, and acetate being the most frequently used organic carbon sources (Perez-Garcia et al., 2011; Marella et al., 2021). However, the conclusions drawn from these studies differed significantly. For instance, Wang et al. (2012) reported that sodium acetate (NaAc) when added at 0.5 g/L enhanced the biomass concentration of *P. tricornutum* from 0.66 g/L (under autotrophic conditions) to 0.89 g/L (under mixotrophy). Conversely, other researchers have demonstrated that the addition of NaAc had a negative impact on *P. tricornutum* growth (Hayward, 1968; García et al., 2005). Studies related to glucose and fructose have also yielded conflicting results. Zaslavskaia et al. (2001) showed more than 20 years ago that *P. tricornutum* lacks a hexose uptake protein and cannot utilize glucose, while the introduction of a gene encoding a glucose transporter enabled this microalga to thrive on exogenous glucose in the dark. Recent independent studies have successfully reproduced these findings, leading to genetically engineered *P. tricornutum* strains with heterotrophic capability using glucose (Hamilton et al., 2016; Krämer et al., 2022; Zhang et al., 2023). In another study by Krämer et al. (2022), attempts to generate heterotrophic strains by heterologously expressing the hexose uptake protein from *Chlorella kessleri* into *P. tricornutum* were unsuccessful. However, the transgenic strains exhibited a significant enhancement in biomass when cultivated under mixotrophic conditions with glucose as the organic carbon source. All these studies collectively suggest that natural *P. tricornutum* cannot metabolize external glucose as an organic carbon source. Nevertheless, there is evidence from numerous reports indicating the stimulatory effects of external glucose and fructose on the growth rates and biomass concentrations of *P. tricornutum* under mixotrophic conditions (García et al., 2005; Wang et al., 2012; Cerón-García et al., 2013; Zheng et al., 2013; Patel et al., 2019; Penhaul Smith et al., 2020). In theory, the absence of hexose transporter in the outer membrane of *P. tricornutum* determines its incapacity of utilizing glucose under either heterotrophic condition or mixotrophic condition. This gives rise to the question how the external glucose contributes to the enhancement of cell density and biomass concentration of mixotrophic *P. tricornutum*. Irrespective of the undefined effects of specific organic carbon sources on *P. tricornutum* growth, another important question that remains to be clarified is whether the addition of external organic carbon under mixotrophic conditions has a negative impact on the accumulation of cellular pigments. Several studies have suggested that mixotrophy can lead to increased biomass concentration and cell density but reduced fucoxanthin contents and photosynthetic activities in *P. tricornutum* (Liu et al., 2009a, b). However, there have also been reports indicating that *P. tricornutum* cells cultivated under optimal conditions can exhibit improved respiration and photosynthetic activities, leading to enhanced biomass concentration and fucoxanthin production (Villanova et al., 2021).

As mentioned above, although there have been

previous attempts to explore mixotrophic and heterotrophic cultivation in *P. tricornutum*, several important questions remain unanswered. It is also important to note that many previous studies used xenic *P. tricornutum* strains for mixotrophic trials, where co-existing bacteria may introduce interference and lead to misleading conclusions. In this study, to eliminate the potential interference from other microorganisms, an axenic strain of *P. tricornutum* was firstly obtained for this research. With this strain, we investigated the growth performance of *P. tricornutum* under both heterotrophic and mixotrophic conditions using various organic carbon sources. Subsequently, we conducted cell culture experiments to uncover the effects of organic carbon sources on *P. tricornutum*, including cell growth, biomass composition, photosynthetic capacity, pigment content, and fatty acids (FAs) profiles. These results achieved in this study might be very constructive for developing commercially viable processes to produce valuable bioproducts from *P. tricornutum*.

2 MATERIAL AND METHOD

2.1 Microalgae strain and cultivation conditions

Xenic *P. tricornutum* NMBguh002, cultivated using NMB#3 liquid medium (each liter medium contains 100.0-mg KNO_3, 10.0-mg KH_2PO_4, 10.0-mg EDTA-Na_2, 2.5-mg $FeSO_4 \cdot 7H_2O$, 250.0-μg $MnSO_4 \cdot H_2O$, 6.0-μg VB_1, and 0.05-μg VB_{12}) prepared with artificial seawater (Zhang et al., 2020), was obtained from the Microalgae Collection Center of Ningbo University. The salinity of fresh NMB#3 liquid medium was set to 30. Cells were cultivated at 23±1 ℃ with an illumination intensity of 60 μmol photons/($m^2 \cdot s$), and the light/dark cycle was set as 12 h/12 h. The initial pH was adjusted to 8.3.

2.2 Bacteria removal with antibiotics

Five types of antibiotics (i.e., ampicillin, gentamycin sulfate, kanamycin, neomycin, and streptomycin), each with a working concentration of 600 mg/L, were used to scavenge the bacteria present in the microalga cultures. After being treated by antibiotics for three days, 1 mL of microalga cultures were inoculated into 9 mL of antibiotics-free medium to let the cells recover from antibiotics stress. Four days later, 100 μL of recovered cells were plated onto solid NMB3# plate (containing five types of antibiotics with a concentration of 300 mg/L). After around four weeks, *P. tricornutum* colony was picked and inoculated into antibiotics-free NBM3# medium to cultivate for two weeks. Then, two methods: nucleic acid staining based on SYBR Green I (Solarbio®, China) and 16S rRNA gene amplification based on universal primers of 27F and 1492R, were adopted to verify the axenicity of *P. tricornutum*. Procedure details were the same as in our previous report (Han et al., 2016a).

2.3 Verification of heterotrophic and mixotrophic capacity of *P. tricornutum*

Glucose, glycerol, and NaAc, at gradient carbon concentrations (0.01, 0.05, and 0.1 mol/L), were applied to test if *P. tricornutum* has the capacity of utilizing external organic carbon sources. Before conducting heterotrophic and mixotrophic experiments, the axenicity of *P. tricornutum* was confirmed using the methods described in Section 2.2. For heterotrophy, cultures were conducted in darkness, while for mixotrophy, the light intensity was kept at the same level as that of autotrophy. In this section, *P. tricornutum* was cultivated with a volume of 40 mL in 100-mL conical flasks. Other cultivation conditions were the same as those described in Section 2.1. During cultivation, cell density (indicated by optical density at 750 nm, OD_{750}) was measured every two or three days using Thermo Fisher Scientific Microplater Reader (Varioskan LUX, Finland) and the quantum yield (F_v/F_m) using WATER-PAM (WALZ, Germany). At the end of cultivation, 10 mL of cultures were sampled and filtered using pre-weighted Whatman GF/F microfiber filter (pore size 0.7 μm), and then the filters with collected cells were rinsed with 30 mL of Milli-Q pure water and dried to a constant weight to calculate the biomass concentrations.

2.4 Comparative analysis of *P. tricornutum* under different cultivation modes

To evaluate the impacts of glycerol on the biomass composition of *P. tricornutum* comprehensively, additional experiments of both autotrophy and mixotrophy (based on glycerol with carbon concentration of 0.1 mol/L) were conducted in an enlarged cultivation volume (300 mL of cultures in 500-mL conical flasks). The cultivation conditions and inoculation ratio were the same as those described in Section 2.3. During cultivation, the OD_{750} and photosynthetic parameters, such as F_v/F_m and relative electron transfer rate (ETR) at photosystem II (PSII), were tested every two days, and the biomass concentrations were determined at the final day of cultivation.

Biomass composition, including proteins, carbohydrates, lipids, pigments, and FAs, were analyzed at the middle (Day 6) and end (Day 12) of cultivation. For FAs and pigments, procedures related to the extraction and quantification were the same with our previous reports (Sun et al., 2022; Zhang et al., 2022).

As for proteins, a modified Coomassie brilliant blue staining method based on 10-mg microalgae powder was adopted (Sedmak and Grossberg, 1977). Briefly, microalgae powder was mixed with 2 mL of solution (prepared using 50-mmol/L Tris-HCl (pH 7.4), 150-mmol/L NaCl, 1-mmol/L PMSF, 1-mmol/L EDTA, and 1% Triton X-100) and homogenized at a speed of 12 000 r/min for 15 min to facilitate protein extraction. After homogenization, the mixture was placed in an ice bath for 3 h to ensure complete protein extraction. Subsequently, the mixture was centrifuged, and 500 μL of the resulting supernatant was collected. Then, the supernatant was mixed with 2.5 mL of Coomassie brilliant blue reagent, and the OD_{595} was measured and used to calculate the protein concentration.

The carbohydrate content was quantified with a modified phenol-sulfuric acid method (DuBois et al., 1956). In brief, an enzyme complex composed of 0.5 mL of papain and 0.5 mL of cellulase were added to the microalgae powder firstly. Then, the mixture was incubated in a 40 °C water bath for 30 min, followed by a boiling water bath for 10 min, and then placed in a 70 °C water bath for 4 h. After the incubation, the mixture was centrifuged at 4 °C for 15 min, and the supernatant was collected. Subsequently, the supernatant was mixed with 4.5 mL of 95% ethanol, and the mixture was left at room temperature for 12 h to precipitate carbohydrates. Then, the mixture was centrifuged at 4 °C for 15 min, and the generated pellet was resuspended in 2 mL of deionized water. Ten minutes later, the mixture was centrifuged again at 4 °C for 15 min, and the supernatant was collected and then mixed with phenol-sulfuric acid to react for 5 min. Afterward, the mixture was boiled for 20 min and cooled with an ice bath for 10 min. Finally, OD_{490} values measured by a spectrophotometer (Philes, T-6, China) were used to calculate the carbohydrate content based on a glucose standard curve.

The lipid content was determined using a modified chloroform-methanol method (Bligh and Dyer, 1959). The procedures were briefly summarized as follows. Firstly, 25 mg of microalgae powder (W_1) were mixed with 2.5 mL of methanol and 1.25 mL of chloroform, and then the mixture was homogenized at 12 000 r/min for 15 min. Then, the homogenized mixture was transferred into a shaker and subjected to lipid extraction for 12 h at 200 r/min. After centrifugation, the supernatant was collected and transferred into a new tube. Subsequently, 2.5 mL of chloroform and 4.5 mL of sodium chloride (1%) were mixed with the supernatant, and the mixture was further vortexed for 15 min. After centrifugation, the lower phase containing extracted lipids was transferred into a weighted tube (W_2), and then dried with nitrogen. The tube with extracted lipids was weighted again and recorded as W_3. Finally, the lipid content (in %DCW) was determined using Eq.1 below:

$$\text{Lipid content}=(W_3-W_2)/W_1\times 100. \quad (1)$$

3 RESULT AND DISCUSSION

3.1 Obtaining axenic culture of *P. tricornutum*

After treating with an antibiotic cocktail, two methods were employed to verify the removal of bacteria: nucleic acid staining using SYBR Green I and 16S rRNA gene amplification using universal primers. As shown in Fig.1, xenic *P. tricornutum* cultures exhibited two types of fluorescence signals: one was relatively large and displayed a fusiform shape, indicating the microalgal cells, while the other was considerably smaller, representing the bacteria (Fig.1a). In the case of axenic *P. tricornutum* cultures, no bacterial fluorescence signals were observed (Fig.1b). Additionally, the Sanger

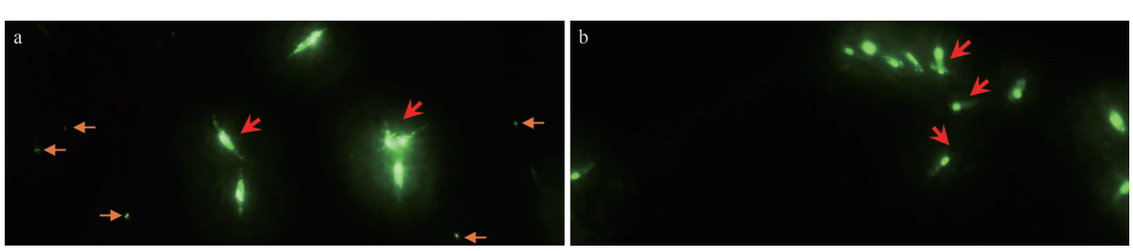

Fig.1 Fluorescence observation of xenic (a) and axenic (b) *P. tricornutum*

The red and orange arrows indicated the microalga cells and bacteria, respectively.

sequencing result of the PCR band exhibited single-peak signals (Supplementary Fig.S1), confirming that the PCR product originated from a single DNA template. The BLAST result further demonstrated that the sequence (GenBank accession No. OR416135) obtained in this study shared a 99.56% identity with the 16S rRNA gene of *P. tricornutum* chloroplast (GenBank accession No. MN937452.1). All these observations indicated that the bacteria present in the *P. tricornutum* cultures had been thoroughly removed.

3.2 The capacity of *P. tricornutum* to utilize external organic carbon

The type and concentration of carbon sources play a significant role in affecting the growth performance of microalgae. In this study, we used three types of carbon sources with gradient concentrations to investigate the capacity of *P. tricornutum* to utilize external organic carbon.

After 18 days of cultivation, the OD_{750} of *P. tricornutum* under autotrophic conditions increased from 0.02 to 0.36. However, none of the tested glucose treatments in the dark displayed appreciable OD_{750} enhancements (Fig.2a). These results confirmed previous findings that *P. tricornutum* cannot grow on glucose in the dark (Zaslavskaia et al., 2001; Hamilton et al., 2016; Krämer et al., 2022). In the mixotrophic trials, the glucose-added group exhibited final OD_{750} values of 0.41–0.46 (Fig.2b), with biomass concentrations ranging from 200 to 206.7 mg/L (Fig.2c). These values are quite similar to those observed in the autotrophic group. F_v/F_m reflects photosynthetic activity and corresponds to the maximum quantum efficiency of PSII. In this study, both the glucose-free and glucose-added groups exhibited similar F_v/F_m values throughout the cultivation period, which remained around 0.6 from Days 1 to 8 and gradually declined to approximately 0.4 on Day 14 (Fig.2d). These results indicated that *P. tricornutum* could not utilize glucose under either heterotrophic or mixotrophic conditions. It has been reported that primary reason attributing to the obligate autotrophy of some microalgal species is the absence of specific sugar transporters (Chen and Chen, 2006; Hu et al., 2018). Both results shown in this study and previous reports (Zaslavskaia et al., 2001; Hamilton et al., 2016; Krämer et al., 2022) confirm that this might be the case for *P. tricornutum* not being able to utilize glucose. It is worth noting that many studies have observed stimulatory effects of glucose on *P. tricornutum* under mixotrophic conditions (García et al., 2005; Liu et al., 2009a, b; Cerón-García et al., 2013; Patel et al., 2019; Penhaul Smith et al., 2020). The discrepancy between these reports and our results may be attributed to xenic culture of *P. tricornutum*. Indeed, the presence of co-cultured bacteria can

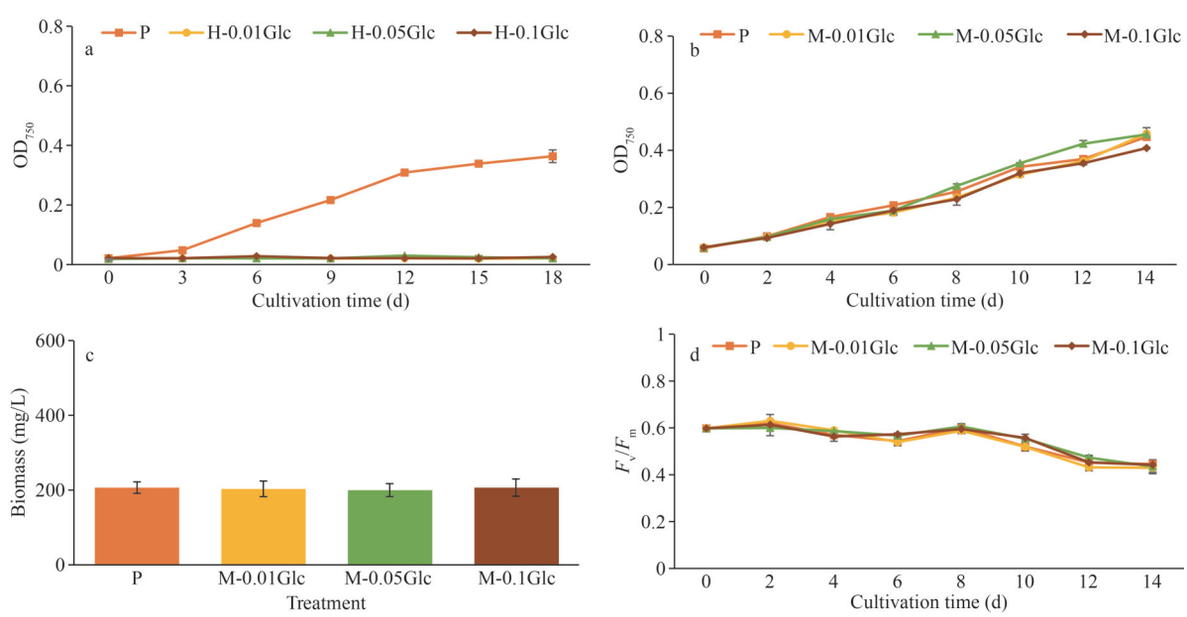

Fig.2 Performance of *Phaeodactylum tricornutum* under heterotrophic (a) and mixotrophic (b–d, OD_{750}, biomass, and F_v/F_m, respectively) conditions based on glucose

Results are displayed as mean±SD (n=3). P, H, and M: autotrophy, heterotrophy, and mixotrophy, respectively; Glc: glucose. Cell density is represented by OD_{750}, and the biomass was determined on the final day of cultivation.

significantly improve the growth performance and biomass accumulation of microalgae under mixotrophic conditions, as observed in various microalgal species such as *Tetradesmus obliquus*, *Scenedesmus obliquus*, *Tetraselmis chuii*, and *Cylindrotheca fusiformis* (Han et al., 2016b; Li et al., 2021; Perera et al., 2022). However, it is important to note that the bacterial community structure in xenic microalgal cultures can vary greatly among microalgal strains, cultivation periods, and cultivation conditions, making it challenging to establish a stable mixotrophic strategy based on xenic microalgae that consistently demonstrates stimulatory performance.

Although NaAc can be transported across the cell membrane through passive diffusion (Kim et al., 2021), it still failed to support the heterotrophic cultivation of *P. tricornutum* (Fig.3a). However, NaAc did influence the growth performance of *P. tricornutum* under mixotrophic conditions (Fig.3b). Generally, only NaAc at a concentration of 0.01 mol/L displayed a higher final OD_{750} than that of the autotrophic conditions. At 0.05 mol/L, an inhibitory effect of NaAc on *P. tricornutum* began to appear although it seemed that this effect only occurred during the early cultivation stage. When the NaAc concentration was further increased to 0.1 mol/L, the growth of *P. tricornutum* was completely inhibited. The final biomass concentrations of cell cultures with NaAc at 0.01 mol/L and 0.05 mol/L are similar to each other (284 and 290 mg/L), both are significantly higher than that of the autotrophic culture (206.7 mg/L). In contrast, the NaAc concentration at 0.1 mol/L resulted in an extraordinarily low biomass concentration of 126.7 mg/L (Fig.3c). These results are consistent with the observation that NaAc has the ability to buffer pH at low concentrations, but a high level of addition can change the pH to an unsuitable value for *P. tricornutum* (Perez-Garcia et al., 2011), which in turn can negatively impact microalgal growth. The F_v/F_m values displayed a similar trend to those of cell density and dropped drastically with NaAc addition at 0.1 mol/L (Fig.3d), suggesting that the photosynthetic activity of *P. tricornutum* was severely suppressed under this condition. These findings broadly align with several previous studies (Hayward, 1968; García et al., 2005) but differ from the study reported by Wang et al. (2012), who found that the addition of NaAC at high concentration also displayed stimulatory effects on *P. tricornutum* growth.

Similar to glucose and NaAc, glycerol did not induce any observable increase in cell density of *P. tricornutum* in the dark (Fig.4a). Glycerol can enter cell through simple diffusion (Perez-Garcia et al., 2011), so the inability of *P. tricornutum* to grow heterotrophically on glycerol cannot be attributed to

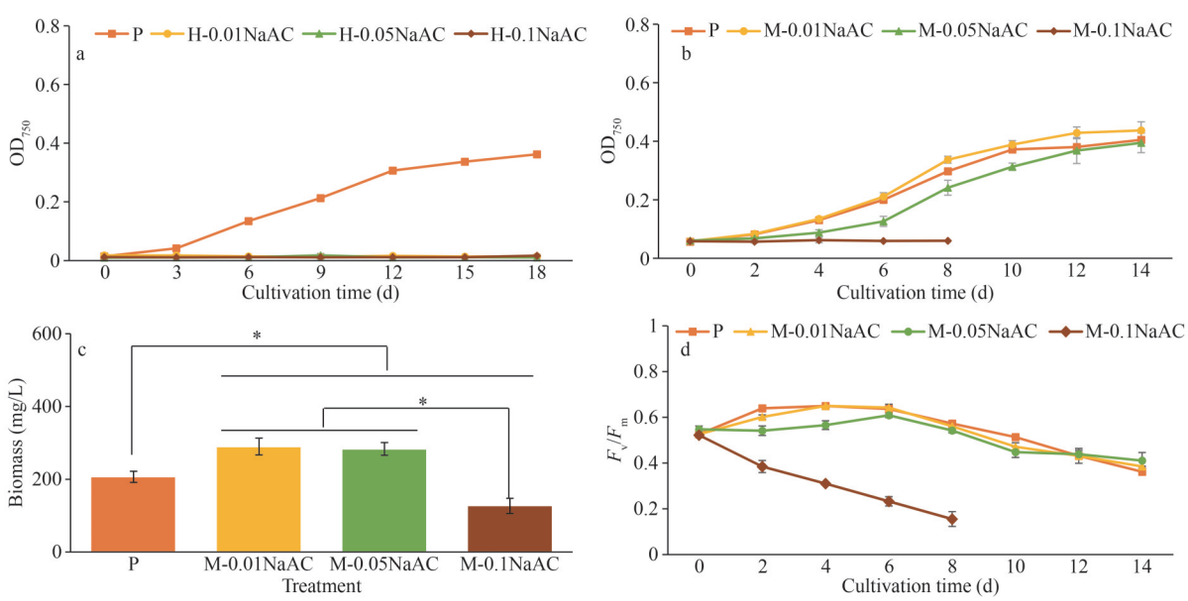

Fig.3 Performance of *P. tricornutum* under heterotrophic (a) and mixotrophic (b–d, OD_{750}, biomass, and F_v/F_m, respectively) conditions based on NaAc

*: significant difference. All results are displayed as mean±SD (n=3). P, H, and M: autotrophy, heterotrophy, and mixotrophy, respectively; NaAc: sodium acetate. Cell density is represented by OD_{750}, and the biomass was determined on the final day of cultivation.

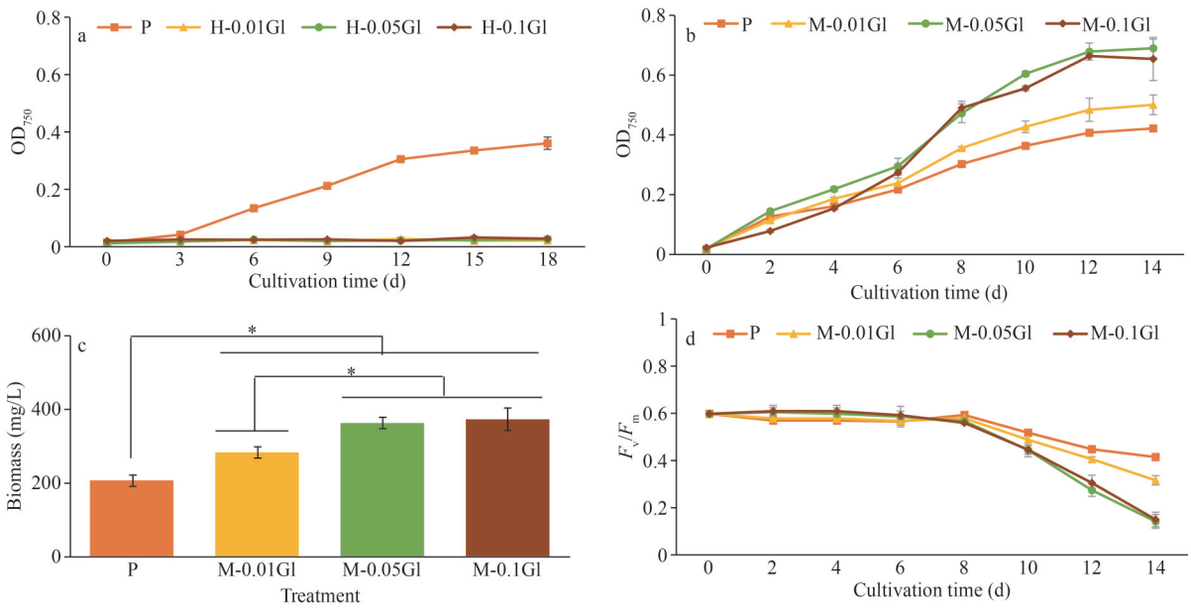

Fig.4 Performances of *P. tricornutum* under heterotrophic (a) and mixotrophic (b–d, optical density, biomass, and F_v/F_m, respectively) conditions based on glycerol

*: significant difference. All results are displayed as mean±SD (n=3). P, H, and M: autotrophy, heterotrophy, and mixotrophy, respectively; Gl: indicates glycerol. Cell density is represented by OD_{750}, and the biomass was determined on the final day of cultivation.

the absence of a sugar transporter. The deficiency of an efficient or complete carbon metabolizing pathway has been widely regarded as another important reason for the obligate autotrophy of some microalgae (Hu et al., 2018). This likely accounts for the failure of *P. tricornutum* to grow on glycerol in darkness. In fact, such a phenomenon has been widely reported in many microalgal species such as *Nannochloropsis salina*, *Haematococcus pluvialis*, and *Cylindrotheca* sp. (Poddar et al., 2018; Dechatiwongse and Choorit, 2021; Wang et al., 2023), while the mechanism behind this phenomenon has not been clearly elucidated.

Under mixotrophic conditions, glycerol, when added even at 0.01 mol/L, displayed a significant promoting effect on cell growth of *P. tricornutum*, resulting in a final OD_{750} of 0.50, approximately 18% higher than that of the autotrophic culture (Fig.4b). When the glycerol concentration was increased to 0.05 mol/L, the final OD_{750} of *P. tricornutum* further rose to 0.69, around 65% higher than the autotrophic culture. However, further increasing the glycerol concentration to 0.1 mol/L displayed a similar promotional effect to that of 0.05 mol/L. Biomass concentration data corroborated these findings (Fig.4c). Glycerol at 0.1 mol/L exhibited the highest biomass value of 373.3 mg/L, followed by 363.3 mg/L at 0.05 mol/L and 283.3 mg/L at 0.01 mol/L. All of them are significantly higher than the biomass concentration of the autotrophic culture (206.7 mg/L). Previous studies have reported similar conclusions, where the addition of glycerol at a concentration of 0.04 mol/L resulted in a 60% increase in *P. tricornutum* biomass productivity over that under autotrophic conditions (Su et al., 2020), and similar results were reported by other researchers (García et al., 2005; Liu et al., 2009b; Cerón-García et al., 2013).

During the early and middle cultivation stages, the addition of glycerol only had slight influences on F_v/F_m. However, in the late cultivation period, glycerol-added groups at all tested concentrations displayed much lower F_v/F_m values than the autotrophic group (Fig.4d). For example, the F_v/F_m values of *P. tricornutum* cultivated with 0.05-mol/L glycerol remained similar to those of the autotrophic group during Days 1–8 but declined sharply starting from Day 10, ultimately reaching a quite low value of around 0.15 on Day 14. Studies have found that F_v/F_m values are closely related to nutrient availability (Zhao et al., 2017; Carstensen et al., 2018). Under nutrient-deficient conditions, microalgae often display low F_v/F_m values, and when transferred to nutrient-replete medium, their F_v/F_m values gradually return to normal levels (Young and Beardall, 2003; Tan et al., 2019). Moreover, studies also indicated that the nutrient concentrations declined along with the extension of cultivation period, thereby

generating quite low F_v/F_m values at the end of cultivation period (Huang et al., 2019; Kadalag et al., 2022). In this study, the glycerol-added groups reached much higher cell density and biomass concentration compared to the autotrophic group, suggesting that the mixotrophic microalgal cells in the late cultivation period experienced more severe nutrient deficiency than those in autotrophic conditions. This likely explains the significantly reduced F_v/F_m values starting from Day 10. Based on these results, it can be inferred that the addition of glycerol does not have negative effects on the photosynthetic capacity of *P. tricornutum*, and the decreased F_v/F_m values in mixotrophy during the late cultivation period are primarily due to nutrient limitation (Huang et al., 2019; Kadalag et al., 2022).

3.3 Comprehensive analysis of glycerol on *P. tricornutum*

3.3.1 Growth performance and chlorophyll fluorescence parameters

In order to conduct a comprehensive analysis of *P. tricornutum* under different cultivation modes, additional trials with increased cultivation volumes were performed. Despite the differences in cultivation volumes, the results observed in this batch of trials were generally consistent with those of previous batches. For instance, axenic *P. tricornutum* under mixotrophic conditions reached a final OD_{750} of 0.57, approximately 47% higher than that of autotrophy (Fig.5a), confirming the stimulatory effects of glycerol on microalgal growth once again. The biomass concentration data supported the same conclusion as those of cell density (Fig.5b). The mixotrophy group achieved biomass concentration of 380.0 mg/L, which doubled that of autotrophy (192.5 mg/L). Regarding the F_v/F_m values, both treatments remained around 0.6 from Day 2 to Day 6. However, the values of the mixotrophy group began to drop drastically from Day 8 onwards, generating an extraordinarily low value of 0.30 at the end of cultivation (Fig.5c). ETR, which represents the electrons transported for energy production and is another important parameter indicating the photosynthetic capacity (Figueroa et al., 2019), showed a similar trend with F_v/F_m (Fig.5d). During the early cultivation period (Days 1–6), both treatments displayed similar ETR values ranging from 65.2 to 80.2 μmol electrons/(m^2·s). However, the mixotrophic group showed a drastic decline in ETR since the eighth day and reached much lower values than those of the autotrophy on Day 12.

3.3.2 Biomass composition

Glycerol imposed substantially influences on the biomass composition of *P. tricornutum*. For the total lipid, its content increased with the addition of

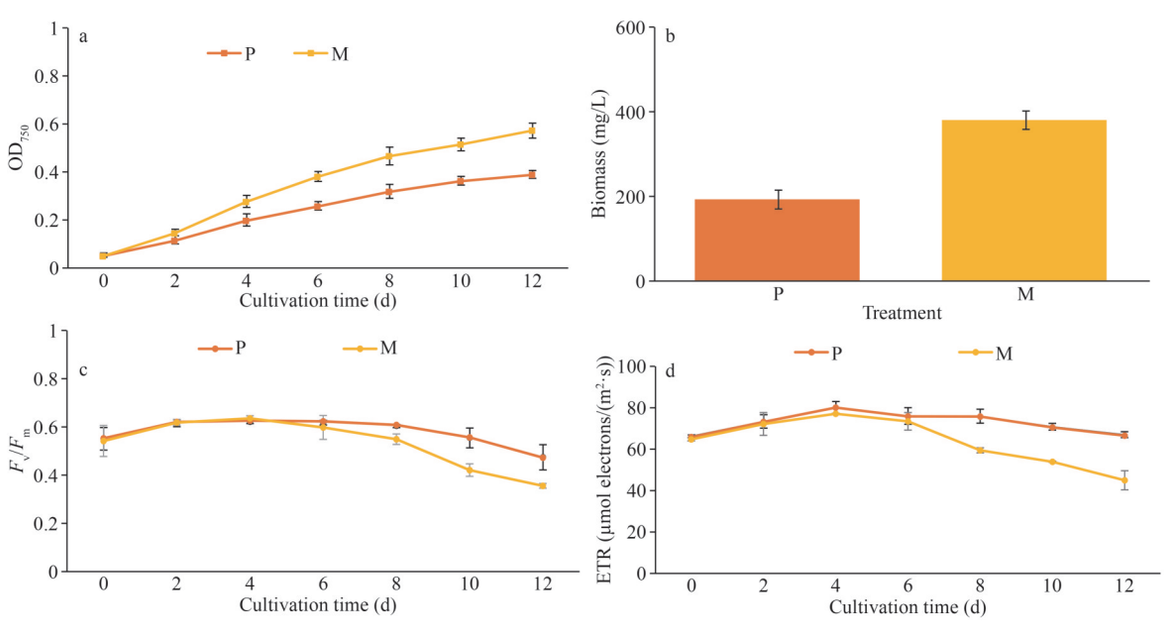

Fig.5 Autotrophic and mixotrophic (glycerol with carbon concentration of 0.1 mol/L) performances of *P. tricornutum* cultivated in enlarged volumes

a–d: cell density, biomass, F_v/F_m, and ETR, respectively. All results are displayed as mean±SD (n=3). P and M: autotrophy and mixotrophy, respectively. Cell density is represented by OD_{750}, and the biomass was determined on the final day of cultivation.

glycerol throughout the cultivation process (Table 1). For an example, on the sixth day, the lipid content of the autotrophy group was 13.17% of DCW, while that of the mixotrophy was 16.06% of DCW, around 20% higher than that of autotrophy. It has been reported that glycerol entering the microalgal cells can be catalyzed into glycerol phosphate by glycerol kinase, which can further serve as the precursor for lipid synthesis (Celi et al., 2022). Both the results obtained in this study and those previously reported suggest that the presence of glycerol boosted lipid accumulation by providing the glycerol backbone required for triacylglycerols synthesis (Villanova et al., 2017, 2021). As the cultivation progressed, both treatments showed substantially enhanced lipid contents. For example, the lipid content of the axenic autotrophy was 23.56% of DCW on Day 12, while that of the mixotrophy group increased to 31.27%, more than 30% higher than that of the former group. Such enhancements of lipids can be attributed to the deficiency of nutrients at the end of the cultivation period. *P. tricornutum* which uses lipids as an important compound for energy storage, tends to form abundant lipids under nutrient-limitation conditions (Yang et al., 2013; Villanova et al., 2017; Sajjadi et al., 2018; Huang et al., 2019). Glycerol also led to an increase in carbohydrates (Table 1), suggesting that the glycerol added to the cultures drove the metabolic flux partially towards carbohydrate accumulation. Under specific conditions, the simultaneous increase in carbohydrates and lipids has been observed in many microalgal species, including *P. tricornutum* (Li et al., 2011; Msanne et al., 2012; Huang et al., 2019). Our results are consistent with these reports. In contrast to carbohydrates and lipids, glycerol addition resulted in a significant decrease in protein content throughout the cultivation period (Table 1). Particularly during the late cultivation period, the protein content of mixotrophy on Day 12 was 12.23% of DCW, representing for only around half of that in autotrophy. These results support the previous viewpoint that microalgal metabolism responds to glycerol addition in a manner similar to the response to nutrient deficiency, characterized by enhanced lipids and decreased proteins (Su et al., 2020).

Table 1 Macronutrient composition (% DCW) of *P. tricornutum* under autotrophic and mixotrophic (glycerol with carbon concentration of 0.1 mol/L) conditions

Composition	Day 6		Day 12	
	P	M	P	M
Lipid	13.17±0.61^{d}	16.39±0.81^{c}	23.56±0.70^{b}	31.27±0.35^{a}
Carbohydrate	10.48±0.71^{c}	13.15±0.34^{b}	14.98±0.52^{b}	17.69±0.76^{a}
Protein	34.46.±0.67^{a}	31.06±0.59^{b}	19.59±0.62^{c}	12.23±0.47^{d}

Results are displayed as mean±SD (n=3). Different letters of superscripts in the same row mean statistically significant difference between groups (P<0.05). DCW: dry cell weight. M and P: mixotrophy and autotrophy, respectively.

The impact of mixotrophy on the FA profile of *P. tricornutum* has been investigated previously, but contrasting results have been reported. Wang et al. (2012) used NaAc, glucose, and starch as organic carbon sources and discovered that the FA composition of *P. tricornutum* was relatively stable in different cultivation modes. However, based on a genetically modified strain, Hamilton et al. (2016) investigated the FA composition of *P. tricornutum* in autotrophy, mixotrophy, and heterotrophy and found that the EPA contents decreased in mixotrophic conditions with glucose as the organic carbon source. In another study conducted by Patel et al. (2019), the PUFAs of *P. tricornutum* in autotrophy were always lower than those of mixotrophic groups-while the SFAs displayed an opposite variation trend. In this study, the addition of glycerol had a significant impact on the FA profile. For autotrophic group, EPA was the most abundant FA, followed by palmitoleic acid (PLA, C16∶1) and palmitic acid (PA, C16∶0) (Table 2). These results were consistent with those of Su et al. (2020), who analyzed the FA profiles of *P. tricornutum* cultivated with different modes and found that the predominant FA components of this microalga in autotrophy were EPA, PLA, and PA. Regarding the mixotrophic group, its EPA and PLA contents decreased sharply, while saturated fatty acids (SFAs) such as PA, myristic acid (MA, C14∶0), and stearic acid (SA, C18∶0) increased substantially. For instance, the EPA ratio of mixotrophy decreased to less than 20% on Day 12, which was around 45% lower than that of the autotrophic group. On the contrary, MA contents in mixotrophic *P. tricornutum* reached to as high as 12.79% at the end of cultivation, which was about three-fold higher than that of the autotrophic group. Moreover, oleic acid (OA, C18∶1) was the only unsaturated fatty acid (UFA) showing an increased content under mixotrophic condition. All these observations resulted in the decreases of monounsaturated fatty acids (MUFAs) and PUFAs and the increase of SFAs in mixotrophic *P. tricornutum*. Additionally, it is worth noting that,

Table 2 Fatty acid composition (% total fatty acid) of *P. tricornutum* under autotrophic and mixotrophic (glycerol with carbon concentration of 0.1 mol/L) conditions

Fatty acid	Day 6		Day 12	
	P	M	P	M
C14:0	5.61±1.49^{c}	8.95±0.69^{b}	4.91±0.27^{c}	12.79±0.68^{a}
C16:0	21.30±1.16^{c}	27.69±1.40^{b}	17.90±0.99^{d}	35.45±0.51^{a}
C16:1	27.07±1.55^{b}	18.88±1.96^{c}	34.66±0.47^{a}	15.51±2.04^{c}
C18:0	2.12±0.51^{c}	4.76±0.08^{a}	0.68±0.01^{d}	4.14±0.01^{ab}
C18:1	2.67±1.06^{c}	4.98±0.11^{b}	3.29±0.60^{c}	8.66±0.25^{a}
C18:2	2.93±0.31^{ab}	2.73±0.31^{ab}	1.85±0.04^{ab}	1.66±0.25^{ab}
C18:3	0.54±0.58^{a}	0.72±0.54^{a}	0.52±0.07^{a}	0.71±0.15^{a}
C20:4	0.43±0.14^{a}	0.49±0.07^{a}	0.40±0.01^{a}	0.48±0.02^{a}
C20:5	36.21±1.90^{ab}	30.15±0.86^{c}	34.92±0.17^{bc}	19.73±1.97^{d}
C22:6	0.79±0.18^{a}	0.66±0.20^{a}	0.87±0.05^{a}	0.86±0.01^{a}
SFA	29.03±1.22^{c}	41.40±2.08^{b}	23.48±0.92^{d}	52.38±0.47^{a}
MUFA	29.75±2.57^{b}	23.85±1.86^{c}	39.95±1.07^{a}	24.17±2.22^{c}
PUFA	40.90±1.88^{ab}	34.75±1.26^{b}	36.57±0.30^{b}	23.44±1.87^{c}

Results are displayed as mean±SD (n=3). SFA: saturated fatty acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid. Different letters of superscripts in the same row mean statistically significant difference between groups (P<0.05). M and P: mixotrophy and autotrophy, respectively.

considering the remarkably increased biomass accumulation of *P. tricornutum* in mixotrophic condition, mixotrophy mode is still competitive for PUFAs production compared to autotrophy.

Previous studies have shown that the pigment contents of *P. tricornutum* decline in mixotrophic conditions, leading to a negative influence on photosynthetic activity (Liu et al., 2009a, b; Patel et al., 2019). In this study, the contents of chlorophyll *a* (Chl *a*), fucoxanthin, and diadinoxanthin of both treatments remained close to each other on Day 6 (Table 3). These results were consistent with the data on F_v/F_m (Figs.4c & 5c), confirming that the addition of glycerol only had slight influences on pigment composition and photosynthetic capacity of *P. tricornutum* during the early cultivation period. However, at the end of cultivation, the Chl *a* and fucoxanthin levels in the mixotrophic group were significantly lower than those in the autotrophic group. For instance, the fucoxanthin of the mixotrophy group on Day 12 was 4.65 mg/g, which was approximately 30% lower than that of autotrophy. The trends in Chl *a* closely mirrored those of fucoxanthin. Various factors can induce a decrease in carotenoids and chlorophylls. For an example, Ikaran et al. (2015) studied the impact of nitrogen availability on *Chlorella vulgaris* and indicated that Chl-*a* content was twice as high in cultures under nitrogen-replete conditions compared to nitrogen-deprived cultures. Similar observations have been reported in *P. tricornutum*. Alipanah et al. (2015, 2018) investigated the responses of *P. tricornutum* to nitrogen deprivation and phosphorus deprivation, demonstrating that nutrient deficiency played a pivotal role in the degradation of pigments. Considering the results of chlorophyll fluorescence parameters, pigments, and lipids together, it can be inferred that the sharp decreases in Chl *a* and fucoxanthin in mixotrophy on Day 12 might be mainly caused by nutrient deficiency rather than the addition of glycerol (Huang et al., 2019; Kadalag et al., 2022).

Table 3 Pigment content (mg/g of DCW) of *P. tricornutum* under autotrophic and mixotrophic (glycerol with carbon concentration of 0.1 mol/L) conditions

Pigment	Day 6		Day 12	
	P	M	P	M
Chlorophyll *a*	25.87±1.65^{a}	27.92±1.66^{a}	13.02±0.94^{b}	9.86±1.10^{c}
Fucoxanthin	11.42±1.19^{a}	10.84±1.31^{a}	6.88±0.40^{b}	4.65±0.69^{bc}
Diadinoxanthin	1.75±0.14^{a}	1.77±0.13^{a}	0.59±0.14^{b}	0.55±0.10^{b}

Results are displayed as mean±SD (n=3). Different letters of superscripts in the same row mean statistically significant difference between groups (P<0.05). M and P: mixotrophy and autotrophy, respectively.

4 CONCLUSION

Phaeodactylum tricornutum lacks the ability to grow heterotrophically and cannot utilize glucose in a mixotrophic manner either. NaAc can potentially serve as an organic carbon source for the mixotrophic cultivation of *P. tricornutum*, and its addition at low concentrations is beneficial for biomass accumulation. However, when NaAc is applied at high concentrations, pH adjustment may be necessary to maintain high growth rates. Glycerol, which significantly enhances the growth of *P. tricornutum*, appears to be a feasible organic carbon source for mixotrophic cultivation.

Mixotrophy can significantly alter the FA profile, characterized by reduced PUFAs and MUFAs and increased SFAs. Biomass composition analysis and chlorophyll fluorescence parameters confirm that the addition of glycerol has minor effects on pigment biosynthesis and photosynthetic capacity under nutrient-replete conditions, suggesting that mixotrophy is an efficient strategy for biomass and some high-value product production from *P. tricornutum*.

5 DATA AVAILABILITY STATEMENT

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Electronic supplementary material

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