

Development of a Mixotrophic Strategy for Efficient Cultivation of a Coccolithophore, *Chrysotila roscoffensis*

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Abstract Coccolithophores have unique ability of dual carbon fixation (photosynthesis and biocalcification) and can accumulate bioactive compounds effectively, thereby showing huge potential for CO₂ bioremediation and simultaneous production of value-added substances. However, biotechnological exploration of these microalgae is still very limited. In order to develop efficient cultivation strategies of these organisms, we tested the abilities of *Chrysotila roscoffensis*, a coccolithophore, to grow under mixotrophic and heterotrophic cultivation conditions. It was confirmed that only glycerol stimulated the cell growth of this microalga in the presence of light. To study the physiological significance of mixotrophy, we conducted cell culture experiments both autotrophically and mixotrophically, the only difference of which was optimal amount of glycerol was added under mixotrophic conditions. The results showed that the addition of glycerol substantially enhanced the lipids and monounsaturated fatty acids accumulation of *C. roscoffensis*, but imposed slight influences on the photosynthetic capacity, pigments content, polyunsaturated fatty acids content, and biocalcification. We concluded that mixotrophy might be an appropriate strategy for exploration of this species for production of high value molecules.

Key words coccolithophores; *Chrysotila roscoffensis*; photoautotrophy; mixotrophy; glycerol

1 Introduction

The increased emission of CO₂ has led to various environmental issues, including global warming and ocean acidification. Microalgae, as a vast group of photosynthetic microorganisms widely distributed in diverse habitats, are well known for their major roles in carbon recycling in natural ecosystem. Additionally, they have a remarkable ability to convert CO₂ into various high-value bioproducts, such as carotenoids, polyunsaturated fatty acids (PUFAs), vitamins and proteins, which can be widely used as biofuels, functional foods, and pharmaceuticals (Xu *et al.*, 2023). Hence, the cultivation of microalgae has emerged as an exceptional strategy for both mitigating CO₂ and producing bio-based materials with diverse applications.

Coccolithophores, belonging to the Haptophyta, are a group of unicellular flagellate microalgae known for their unique capacity to form intricate inorganic calcite scales (termed as coccoliths) around their cells, made of calcium carbonate (CaCO₃). Their dual carbon fixation systems,

combining photosynthesis and biocalcification, make them significant contributors to marine carbon sink, thereby attracting considerable attention in the field of marine carbon cycling (Claxton *et al.*, 2022). Recent studies have highlighted the efficient accumulation of various bioactive compounds by several coccolithophores species. For example, *Emiliania huxleyi*, one of the most abundant coccolithophores, was found to accumulate a high percentage (60.8%) of polyunsaturated fatty acids (PUFA) of the total fatty acid (TFA) and substantial total alkenone content (2.5%) of the total biomass (Vicente *et al.*, 2021). In another study, Zhang *et al.* (2023) reported that the biomass content of *E. huxleyi* reached 864.2 mg L⁻¹ with a fucoxanthin concentration around 0.2 mg L⁻¹ when this microalga was cultivated using red light. In addition, studies have shown that coccoliths from coccolithophores could be utilized as novel feedstock for biomaterials production and have diverse applications in the biomedical field (Moheimani *et al.*, 2012). All these studies indicate that coccolithophores can be considered a promising feedstock of the bioactive substances.

Chrysotila (formerly known as *Pleurochrysis*) is another representative genus of Haptophyta that has been extensively studied in the field of global biogeochemical

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cycle (Meng *et al.*, 2022). Research has also indicated the significant potential of *Chrysotila* as a resource for biofuels and bioactive compounds. For example, one study has shown that *Chrysotila* spp. can accumulate a substantial amount of PUFA, accounting for 30%–60% of the TFA (Moreira *et al.*, 2022). Additionally, studies also reported that fucoxanthin is the most abundant carotenoid in *Chrysotila*. *C. carterae*, when cultured in a semi-continuous cultivation mode, exhibited a high fucoxanthin content up to 6.88% of dry cell weight (DCW) (Okcu *et al.*, 2021). Except exhibiting high growth rates and rich bioactive substances, *Chrysotila* spp. show additional characteristics essential for large-scale cultivation. Long-term (13 months) outdoor raceway pond cultivation of *C. carterae* in Western Australia demonstrated a wide range of temperatures tolerance (3–41°C) and an average biomass productivity of 0.19 g L⁻¹ d⁻¹, with significant lipid and CaCO₃ contents (Moheimani and Borowitzka, 2006). In a studied conducted by Rezvani *et al.* (2019) the biomass of *C. carterae* was found to contain 6%–11% coccoliths and around 34% lipids. The authors also assessed the economic viability of *C. carterae* cultivation based on various biorefinery strategies (including bio-oil based on total lipids, biogas from wastes, animal feed and fishmeal from proteins and carbohydrates, biomaterial from coccoliths, *etc.*), and concluded that the multiproduction of fishmeal, bio-oil, and biomaterial based on *C. carterae* biomass is economically attractive. Collectively, these studies demonstrate that *Chrysotila* is a versatile genus with great potential for both commercial application and CO₂ bioremediation.

To enhance the commercial exploitation of *Chrysotila* spp., the development of efficient cultivation strategy is essential. Microalgae can be cultivated under three major modes, photoautotrophy, mixotrophy, and heterotrophy, each with distinct metabolic pathways. In general, achieving optimal light supply in photoautotrophy poses a significant challenge in mass cultivation of microalgae, making mixotrophy and heterotrophy more favorable due to higher cell growth rates, biomass accumulation rates, and productivities of bioactive substances (Hu *et al.*, 2018). Although heterotrophic cultivation offers high growth rates, it heavily relies on the assimilation of organic carbon, making it less suitable for mitigating global CO₂ emissions. On the other hand, mixotrophy allows for simultaneous assimilation of both organic carbon and inorganic carbon, making it a preferred process for microalgae-based high-value substances production.

At an earlier time, we isolated a strain of *C. roscoffensis* from coastal waters in Xiangshan Bay, China, and performed comprehensive studies relevant to its biological properties. This strain demonstrated the ability to bloom under stress conditions and exhibited a remarkable capacity for accumulating fucoxanthin and PUFA (Zhou *et al.*, 2022). Furthermore, we successfully developed a bacteria-removal method, through which an axenic *C. roscoffensis* strain was obtained (Liu *et al.*, 2023). The axenic strain allows us to study the influences of external organic carbon on microalgae without the interference of

other organisms. Recognizing the potential advantages of *C. roscoffensis* in both CO₂ mitigation and bioactive compounds production, we conducted a series of trials with the following objectives: 1) to verify the mixotrophic and heterotrophic capacities of *C. roscoffensis* by testing its response to three organic carbon sources; 2) to compare the growth performances and bioactive substances productivities among different cultivation modes. Based on these trials, we aim to gain a comprehensive understanding about the growth potential and bioactive compounds production of *C. roscoffensis* under different cultivation modes. This information will be valuable for optimizing the cultivation strategy of this microalgae and exploring its potential application in CO₂ mitigation and bio-based materials production.

2 Materials and Methods

2.1 Microalgae Strain and General Cultivation Conditions

Both xenic and axenic *C. roscoffensis* NMBjih026-8 strains were preserved in the Microalgae Collection Center of Ningbo University using antibiotic-free medium. For all experiments, axenic cells in the early stationary phase (the optical density at 750 nm, OD₇₅₀, at around 0.15) were inoculated into fresh medium with an inoculation ratio of 1:9 (cultures volume:medium volume). The initial cell density was maintained at around 0.015 of OD₇₅₀. In photoautotrophy experiments, cells were cultured using NMB3# liquid medium (each liter containing 100.0 mg KNO₃, 10.0 mg KH₂PO₄, 10.0 mg EDTA-Na₂, 2.5 mg FeSO₄·7H₂O, 250.0 µg MnSO₄·H₂O, 6.0 µg VB₁, and 0.05 µg VB₁₂) prepared with artificial seawater (Liu *et al.*, 2023). The initial pH and salinity were adjusted to 8.30 and 25‰, respectively (Meng *et al.*, 2022). The temperature was then maintained at 23°C ± 1°C, and the illumination intensity and the light/dark cycle were set to 60 µmol photons m⁻² s⁻¹ and 12 h:12 h, respectively.

2.2 Determination of Optimal Organic Carbon Under Mixotrophic and Heterotrophic Conditions

To screen the suitable organic carbon source for *C. roscoffensis*, sterilized glucose, glycerol, and sodium acetate (NaAc) at gradient concentrations (carbon concentration: 0.1, 0.2, 0.3, 0.4, and 0.5 mol L⁻¹) were supplemented into NMB3# liquid medium to prepare organic carbon-containing media. Before inoculation, the axenicity of *C. roscoffensis* was confirmed using SYBI Green I, based on a previously reported method (Liu *et al.*, 2023). For the heterotrophy experiments, cells were cultivated in dark conditions, while for mixotrophy, the light intensity was same as the photoautotrophy. For this batch, all experiments were conducted in 100 mL conical flasks containing 30 mL of medium, and the photoautotrophic *C. roscoffensis* was taken as the control group. During the cultivation, the cell density (represented by OD₇₅₀) was measured every two days by Thermo Fisher Scientific

Microplate Reader (Varioskan LUX, Finland). At the final day of cultivation, 10 mL of *C. roscoffensis* cultures were collected using pre-weighted Whatman GF/F microfiber filter (pore size 0.7 μm). The collected cells were then washed with 30 mL of ultrapure water and dried to a constant weight to calculate the biomass content.

2.3 Comparative Analysis of *C. roscoffensis* Under Different Cultivation Modes

To comprehensively evaluate the effects of organic carbon on *C. roscoffensis*, additional cultivation trials of photoautotrophy and mixotrophy were conducted with the axenic strain.

Throughout the entire cultivation period of 12 d, OD_{750} were determined every two days, and photosynthetic parameters, including the maximal photosystem II (PSII) quantum yield (F_v/F_m), relative electron transfer rate at PSII (ETR), and photochemical quenching coefficient (qP), were measured by WATER-PAM (WALZ, Germany). The biomass content was determined at the end of cultivation. The specific growth rate (μ , d^{-1}) was calculated based on OD_{750} using the Eq. (1):

$$\mu (\text{d}^{-1}) = (\ln N_{t_1} - \ln N_{t_2}) / t_1 - t_2, \quad (1)$$

in which t_1 and t_2 are the initial and ending time of the cultivation period, and N_{t_1} and N_{t_2} are the corresponding OD_{750} values at those time points.

Biomass compositions (refers to lipid, protein, carbohydrates, and coccoliths), fatty acids (FAs) and pigments were measured at day 6 and day 12. The coccolith was determined based on 10 mL of microalga cultures, following the protocol reported by Moheimani and Boro-

witzka (2007). The carbohydrate was extracted using phenol-sulfuric acid method (DuBois *et al.*, 2002), and the content was calculated based on a glucose standard curve. With homogenized microalga powder, the lipid and protein were determined using chloroform-methanol method (Bligh and Dyer, 1959) and Coomassie brilliant blue staining method (Sedmak and Grossberg, 1977), respectively. For FA analysis, lyophilized microalga powder was used for lipid extraction, and FA components were determined by gas chromatography-mass spectrometry (GC-MS). Pigments were extracted using 5 mL of microalga cultures with chloroform-methanol method, and then quantified by liquid chromatography-mass spectrometry (LC-MS). The experimental procedures and related details for FAs and pigments were the same as our previous reports (Sun *et al.*, 2022; Zhang *et al.*, 2022).

3 Results and Discussion

3.1 Identifying the Heterotrophic and Mixotrophic Capacities of *C. roscoffensis*

It is well known that certain microalgae under heterotrophy and mixotrophy can achieve much higher growth and biomass accumulation rates than autotrophy. Studies also showed that both the carbon concentration and carbon type are important factors underlying cultivation efficiency (Castillo *et al.*, 2021). Hence, three organic carbon sources with gradient concentrations, all of which have been widely used for microalgae cultivation, were tested to verify the performance of axenic *C. roscoffensis* (Fig.1) under heterotrophic and mixotrophic conditions.



Fig.1 Axenicity verification of *Chrysotila roscoffensis* based on SYBR Green I. Red and orange arrows indicate the microalga and bacteria, respectively.

In darkness, the OD_{750} of *C. roscoffensis* in all treatments displayed almost even curves during the whole cultivation period, whereas that of the photoautotrophy group increased from 0.015 to 0.170 (Figs.2a–c). Such

results proved that *C. roscoffensis* could not grow solely on external organic carbon. Due to no observable cell density enhancement in heterotrophic cultures, the biomass was not measured further.

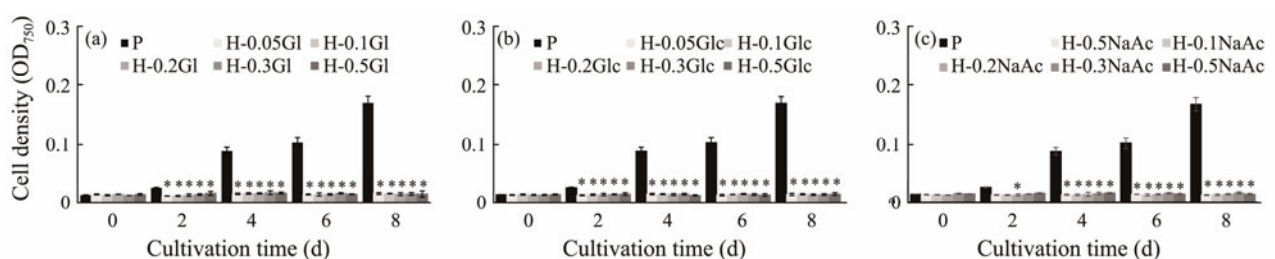


Fig.2 Growth performance of *Chrysotila roscoffensis* under heterotrophic conditions based on glycerol (a), glucose (b), and NaAc (c). “*” indicates significant differences. P, photoautotrophy; H, heterotrophy; Gl, glycerol; Glc, glucose; NaAc, sodium acetate.

Under illuminated condition, the OD₇₅₀ values of *C. roscoffensis* varied great along with both carbon sources and carbon concentrations (Figs.3a–c). The final OD₇₅₀ of autotrophy group was 0.146, while those of the glycerol addition groups varied from 0.189 (0.05 mol L⁻¹) to 0.303 (0.5 mol L⁻¹) (Fig.3a), significantly higher than that of the autotroph. With regard to glucose groups, all concentrations resulted in OD₇₅₀ values ranging from 0.146 to 0.168, close to the photoautotrophy group, suggesting that the addition of external glucose had no appreciable influence on *C. roscoffensis* growth (Fig.3b). Contrary to glucose and glycerol, the addition of NaAc (even at 0.05 mol L⁻¹) induced growth inhibition of *C. roscoffensis*, and when the NaAc was further increased to 0.5 mol L⁻¹, the final OD₇₅₀ sharply reduced to 0.098, around 30% lower than that of the autotrophy group (Fig.3c). The influences

of external organic carbon on *C. roscoffensis* growth were further consolidated by biomass data (Figs.3d–f). The autotrophy group reached a biomass content of 210.0 mg L⁻¹ on the final day, while those of the glycerol addition groups ranged from 226.7 mg L⁻¹ to 323.3 mg L⁻¹, around 7.9%–54.0% higher than the autotrophy group (Fig.3d). The DCW values of glucose groups (ranging from 196.7–213.3 mg L⁻¹) showed no significant differences compared to that of the autotrophy group (Fig.3e). The lowest DCW of 126.7 mg L⁻¹ was obtained from the group supplemented with 0.5 mol L⁻¹ NaAc, accounting for only 60% of the autotrophy group (Fig.3f). All the results above demonstrated that only glycerol significantly promoted *C. roscoffensis* growth under illuminated conditions, and the facilitating effects enhanced with the increase of glycerol concentrations.

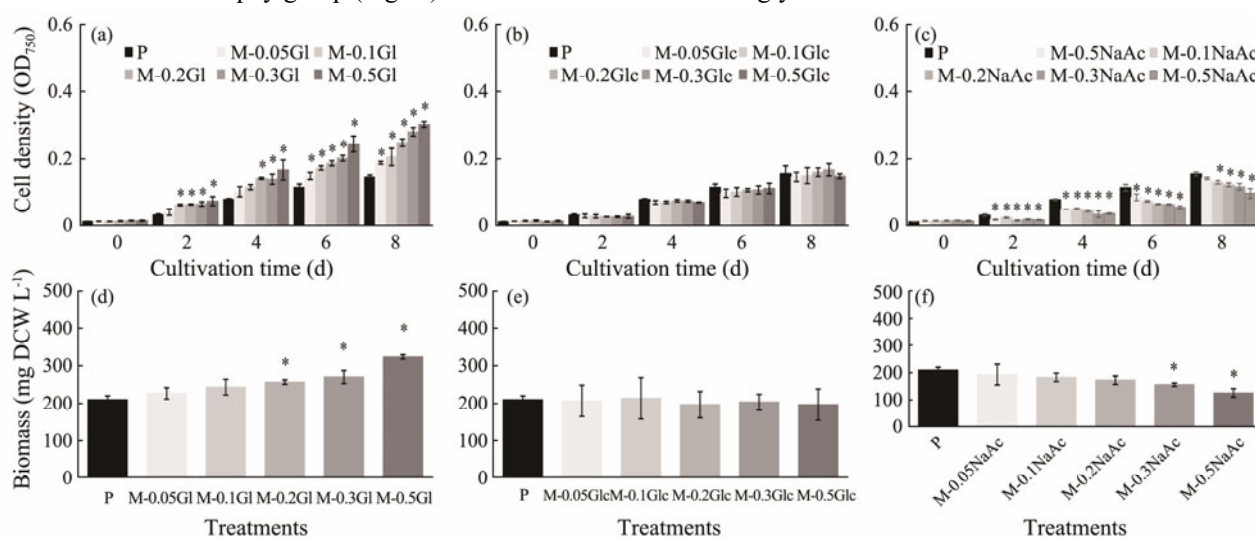


Fig.3 Cell densities (a–c) and biomass (d–f) of *Chrysolita roscoffensis* under mixotrophic conditions based on glycerol (a, d), glucose (b, e), and NaAc (c, f). ‘*’ indicates significant differences. P, photoautotrophy; M, mixotrophy; Gl, glycerol; Glc, glucose; NaAc, sodium acetate.

It has been reported that only some microalgae (*e.g.*, *Chlorella* spp., *Nitzschia laevis*, *Cyclotella cryptica*) can grow by assimilating organic carbon in the absence of light (Perez-Garcia *et al.*, 2011). For obligate autotrophic microalgae, the lack of heterotrophic capacity can be attributed to various reasons, including the absence of sugar transporter in the outer membrane (Zhang *et al.*, 2023), the low levels or insufficiency of enzymes related to carbon dissimilation (Perez-Garcia *et al.*, 2011), and the inhibition of carbon assimilation pathway in the dark (Wang *et al.*, 2023).

The transmembrane uptake of glucose is hexose transporter-dependent. In this study, the addition of glucose did not result in any variation in the growth performance of *C. roscoffensis*, either under heterotrophic or mixotrophic conditions, demonstrating that this microalga lacks glucose transporters. Such an observation also has been reported in other microalgae, *e.g.*, *Phaeodactylum tricornutum* and *Volvox carteri* (Hallmann and Sumper, 1996; Zaslavskaya *et al.*, 2001).

With respect to acetate, the failure of heterotrophy based on external NaAc might not be simply attributed to the lack of monocarboxylic acid/proton transporter be-

cause a concentration dependent inhibition effect can be observed in the mixotrophic groups. It is clear that the addition of NaAc can lead to increased pH, which may affect the growth of *C. roscoffensis* in turn. Indeed, the NaAc groups did show elevated pH in this study, albeit the initial pH had been adjusted to 8.3. Moheimani and Borowitzk (2011) have reported that another species of *Chrysolita* (*C. carterae*) can grow fast within a diurnal pH range of 8.3–10.9, suggesting that the potential influence of elevated pH on *C. roscoffensis* may be negligible. Studies have found that acetate can suppress the ammonium uptake and nitrogen assimilation by *Scenedesmus*, which can impose negative effects on the growth and biomass accumulation rates (Combres *et al.*, 1994; Ogbonna *et al.*, 2000). Nevertheless, related information has never been reported in *Chrysolita* or other closely related species, thus more research is still needed to disclose the mechanism behind the inhibition effect of acetate on *C. roscoffensis* in future.

Glycerol can enter cells by passive diffusion and almost show no toxicity even at high concentrations. However, the addition of glycerol resulted in no growth enhancement of *C. roscoffensis* in darkness, suggesting this

microalga can only utilize glycerol in the presence of light. Such an observation has been reported in many microalgae species, *e.g.*, *Nannochloropsis* sp., *Chlorella* sp., and *Cylindrotheca* sp. (Poddar *et al.*, 2018; Wang *et al.*, 2023). However, it is worthy to note that the mechanism underlying the incapacity of these microalgae to metabolize glycerol in the dark is still elusive.

3.2 Comprehensive Analysis of Organic Carbon on *C. roscoffensis*

3.2.1 Growth performance and biomass accumulation

In this section, for the purpose of obtaining sufficient

microalgae biomass for comprehensive analyses, we conducted experiments with an increased cultivation volume of 300 mL and an extended cultivation period to 12 d. Despite these changes, the results obtained from this batch of experiments were basically in line with those observed in the previous batches (Fig.4). For example, the OD₇₅₀ values of mixotrophy were higher than those of corresponding photoautotrophy throughout the entire cultivation period (Fig.4a). The specific growth rate and biomass content of mixotrophy were 40% and 60% higher than those of photoautotrophy, respectively (Figs.4b, c). These data once again confirmed that the addition of external glycerol could significantly stimulate the cell density and biomass accumulation of *C. roscoffensis*.

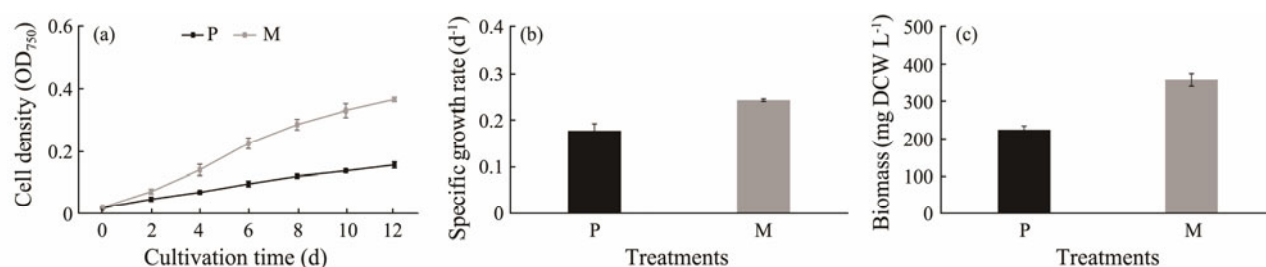


Fig.4 Cell density (a), specific growth rate (b), and biomass (c) of *Chrysotila roscoffensis* under different cultivation modes. P and M represent photoautotrophy and mixotrophy, respectively.

3.2.2 Chlorophyll fluorescence parameters

Previous studies have reported that the addition of external organic carbon can disturb the formation of thylakoids, leading to a significant decrease of photosynthetic capacity of microalgae (Grama *et al.*, 2016; Ma *et al.*, 2022). However, other reports indicated that microalgae cells cultivated under optimal conditions can achieve enhanced photosynthetic activities, generating increased biomass levels (Villanova *et al.*, 2021). To reveal the actual situation of external organic carbon induced in *C. roscoffensis*, photophysiological parameters such as F_v/F_m , qP, and ETR, which have been extensively used to investigate the physiological status of microalgae cells, were investigated and compared between photoautotrophy and mixotrophy.

F_v/F_m indicates the photosynthetic activity and corresponds to the maximum quantum efficiency of PSII. In this study, the F_v/F_m of photoautotrophy increased from 0.43 at the initial cultivation day to around 0.55 at the fourth day, and then began to decrease gradually to 0.41 at the end of cultivation (Fig.5a). As for mixotrophy, its F_v/F_m presented similar values and followed the same variation pattern as the photoautotrophic group during the early stage of cultivation (Fig.5a), implying that the addition of glycerol displayed no negative effect on the photosynthetic apparatus of *C. roscoffensis*. However, the F_v/F_m of glycerol addition group began to drop sharply from the eighth day and achieved a value of 0.28 at the final day, around 30% lower than that of the photoautotrophy. The observation that mixotrophy reached much higher cell densities and biomass contents than photo-

autotrophy suggested that microalgal cells of the former group consumed more nutrients during cultivation. Additionally, our previous report has demonstrated that the nitrate in the NMB3# can be rapidly consumed up by microalgae within 5–7 d, thereby generating a nutrients deficiency condition (Zhang *et al.*, 2023). Thus, it can be speculated that the low F_v/F_m values of mixotrophy at the later cultivation phase are probably caused by severe nutrient deficiency. Such an observation was consistent with previous studies, in which the F_v/F_m of microalgae dropped under nutrient limitation conditions and rose back to normal level when nutrients were supplemented (Young and Beardall, 2003; Tan *et al.*, 2019).

ETR indicates the electrons transported for energy production. Under autotrophic conditions, an increase in ETR often means an enhancement of biomass accumulation, while a decline in ETR represents a reduction of biochemical processes related to photosynthesis (Mogany *et al.*, 2022). Under autotrophic conditions, the ETR remained between 31.5 $\mu\text{mol electrons m}^{-2} \text{s}^{-1}$ (day 12) and 39.2 $\mu\text{mol electrons m}^{-2} \text{s}^{-1}$ (day 6), displaying a relative flat curve during the whole cultivation period (Fig.4b). However, the glycerol addition group exhibited a dramatic decline in ETR since the sixth day (34.3 $\mu\text{mol electrons m}^{-2} \text{s}^{-1}$) and minimized at the final day (16.7 $\mu\text{mol electrons m}^{-2} \text{s}^{-1}$) (Fig.5b). The observation of the low ETR and high biomass showed in mixotrophic group indicated that glycerol assimilation played an important role in the biomass accumulation of *C. roscoffensis*.

qP reflects the probability that a singlet-excited chlorophyll in PSII drives photochemistry and has been widely used as an important parameter describing the percentage of light energy absorbed by the PSII for photo-

synthetic electron transfer (Yu *et al.*, 2022). In this study, all of qP values of different groups were around 0.8 during days 0–8, suggesting that external glycerol did not influence the capacity of cells to direct light energy to photochemistry (Fig. 5c). However, mixotrophy group displayed downward qP values at days 10 and 12, implying that the electron transport activity of *C. roscoffensis* cells was restrained, probably due to nutrient deficiency (Mo-

gany *et al.*, 2022).

In summary, the chlorophyll fluorescence parameters obtained in this study demonstrated that the addition of glycerol showed no negative effects on the photosynthetic capacity of *C. roscoffensis* under nutrients depletion conditions. These observations were consistent with Villanova *et al.* (2021) but contrary to others (Grama *et al.*, 2016; Ma *et al.*, 2022).

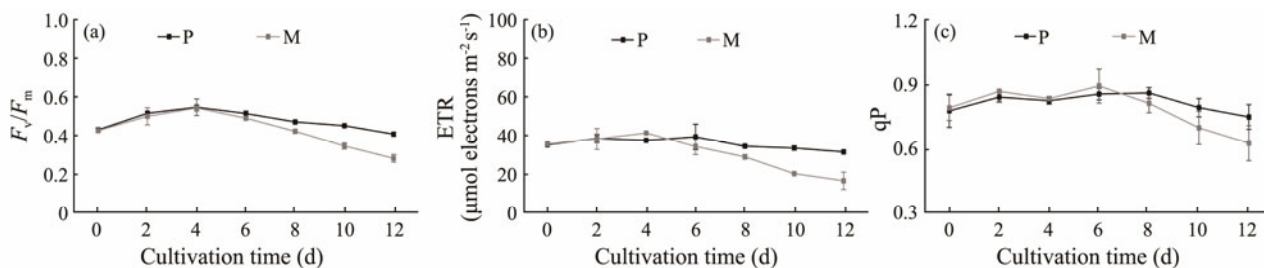


Fig. 5 Chlorophyll fluorescence parameters of *Chrysolita roscoffensis* under different cultivation modes. P and M represent photoautotrophy and mixotrophy, respectively.

3.2.3 Biomass composition analysis

Coccolithophores are well known for their unique ability of forming CaCO_3 scales, whereas their coccolith contents varied great among different species. Using an outdoor raceway pond, Moheimani and Borowitzka (2006) conducted a long-term cultivation trial of *C. carterae* and found the coccolith contents of this microalgae were 6.8%–7.9%, showing no significant seasonal change. In another study, Moheimani *et al.* (2011) tested the feasibility of producing *C. carterae*, *Emiliania huxleyi*, and *Gephyrocapsa oceanica* in different types of closed photobioreactors and illustrated that the coccoliths of different species range between 1.0% and 18.5%. In this study, the coccoliths content of *C. roscoffensis* under photoautotrophy increased from 6.37% at day 6 to 9.73% at day 12. The situation of mixotrophy group, increasing from 5.79% to 10.21 along with the cultivation period extension, resembled that of photoautotrophy (Table 1). Moreover, no significant differences were observed between different treatments. All these results demonstrated that coccoliths biosynthesis in *C. roscoffensis* is inducible, while nutrients level rather than external organic carbon maybe the key regulatory factor.

Although coccolithophores are generally considered as lipid-rich microalgae (Fernández *et al.*, 1994; Moheimani *et al.*, 2011; Rezvani *et al.*, 2019), other studies have suggested that the lipid contents of coccolithophorids are only around 10.0% (Kanda *et al.*, 2020; Moreira *et al.*, 2022). In this study, the lipid contents of *C. roscoffensis* varied

substantially between different cultivation phases (Table 1). Under photoautotrophic conditions, the lipid content at day 6 was only 15.00%, while reached as high up to 32.26% at day 12. The same variation trend was also observed from mixotrophic group. Availabilities of nitrogen and phosphorous are important factors affecting the lipid contents of microalgae. Under nutrients-deficient conditions, most microalgal species tend to promote lipid biosynthesis by modulating biochemical pathways (Khoo *et al.*, 2023). Our results suggested that this was also the case for *C. roscoffensis*. Additionally, it has been widely reported that the addition of external organic carbon will lead to an increase in lipid contents of microalgae, such as *Phaeodactylum tricornutum*, *Nitzschia laevis*, and *Chlorella sorokiniana* (Cecchin *et al.*, 2018; Lu *et al.*, 2019). The lipid contents of mixotrophic *C. roscoffensis*, ranging between 20.21% and 39.67%, were significantly higher than those of corresponding photoautotrophy and aligned well with previous reports (Cecchin *et al.*, 2018; Lu *et al.*, 2019).

In comparison with rich lipid contents, *C. roscoffensis* contained relatively small amounts of carbohydrates. For example, the carbohydrate content of photoautotrophy at day 6 was only 17.85%, which further declined to 10.62% at day 12. In the presence of external glycerol, the carbohydrate content increased only at the end of cultivation (Table 1). These values agreed with previous reports, in which the carbohydrate contents of *Chrysolita* spp. were found to be around 10%–20% (Rezvani *et al.*, 2019; Moreira *et al.*, 2022;).

Table 1 Biochemical compositions of *Chrysolita roscoffensis* under different modes

Compositions	DCW% (day 6)		DCW% (day 12)	
	P	M	P	M
Coccolith	6.37 ± 0.59 ^b	5.79 ± 0.56 ^b	9.73 ± 1.19 ^a	10.21 ± 0.78 ^a
Lipid	15.00 ± 1.35 ^d	20.21 ± 1.84 ^c	32.26 ± 2.12 ^b	39.67 ± 2.78 ^a
Carbohydrate	17.85 ± 1.07 ^a	16.76 ± 1.28 ^{ab}	10.62 ± 0.65 ^c	14.33 ± 1.60 ^b
Protein	34.74 ± 2.48 ^a	33.23 ± 3.00 ^a	26.05 ± 1.61 ^b	14.04 ± 1.01 ^c

Notes: Results were displayed as mean ± SD ($n=4$). Different letters of superscripts in the same row mean statistically significant difference between groups ($P<0.05$). DCW, dry cell weight. P and M represent photoautotrophy and mixotrophy, respectively.

Contrary to the lipid, *C. roscoffensis* exhibited a decrease in protein along with cultivation process. For instance, the protein of photoautotrophic group decreased from 34.74% at day 6 to 26.05% at day 12. Mixotrophic group displayed the same variation trend (Table 1). Additionally, significant differences in protein contents were only observed from the ending time of cultivation period between different treatments.

3.2.4 Fatty acids profile and content

The FA profiles of *Chrysotila* species other than *C. roscoffensis* have been investigated in several studies, revealing that the palmitic acid (PA, C16:0), oleic acid (OA, C18:1), α -linolenic acid (ALA, C18:3), and stearic acid (SA, C18:4) are commonly predominant FAs of

Chrysotila, with proportions of PUFAs ranging between 30% and 60% (Kanda *et al.*, 1996; 2020; Moreira *et al.*, 2022). In this study, a total of 11 types of FAs were identified in *C. roscoffensis*, with the proportions of PUFA, monounsaturated fatty acid (MUFA), and saturated fatty acid (SFA) found to be 14.12%–28.12%, 15.54%–30.10%, and 55.78%–59.54%, respectively (Table 2). FAs such as ALA, OA, PA, linoleic acid (LA, C18:2), and SA (5.88%–19.50%) were relatively abundant (Table 2). Additionally, *C. roscoffensis* also accumulated considerable amount of docosahexaenoic acid (DHA, C22:6) (7.07%–7.96%). These data suggest that the FAs profile of *C. roscoffensis* is generally consistent with that of other *Chrysotila* species (Kanda *et al.*, 1996, 2020; Moreira *et al.*, 2022).

Table 2 Fatty acid profiles and contents of *Chrysotila roscoffensis* under different modes

Fatty acids (TFA%)	TFA% (day 6)		TFA% (day 12)	
	P	M	P	M
C14:0	0.51 ± 0.04 ^b	0.31 ± 0.01 ^b	0.97 ± 0.11 ^a	0.80 ± 0.17 ^a
C16:0	25.29 ± 1.20 ^a	18.51 ± 1.45 ^b	14.34 ± 1.40 ^c	12.00 ± 0.76 ^c
C16:1	1.75 ± 0.03 ^a	0.93 ± 0.09 ^b	1.49 ± 0.21 ^a	1.68 ± 0.08 ^a
C16:2	3.70 ± 0.53 ^a	1.91 ± 0.18 ^b	0.96 ± 0.10 ^c	1.69 ± 0.09 ^b
C18:0	2.32 ± 0.20 ^a	1.50 ± 0.48 ^b	3.66 ± 1.14 ^a	1.32 ± 0.50 ^b
C18:1	13.80 ± 0.68 ^c	22.79 ± 1.67 ^b	20.00 ± 0.73 ^b	28.42 ± 0.96 ^a
C18:2	7.25 ± 0.84 ^b	11.47 ± 2.54 ^a	13.23 ± 0.98 ^a	15.08 ± 0.66 ^a
C18:3	15.74 ± 0.29 ^c	21.32 ± 1.47 ^b	26.14 ± 2.05 ^a	21.93 ± 0.58 ^b
C18:4	19.34 ± 2.23 ^a	10.48 ± 0.73 ^b	9.84 ± 1.19 ^b	7.27 ± 0.35 ^b
C20:5	3.23 ± 0.31 ^a	2.77 ± 0.27 ^{ab}	1.64 ± 0.12 ^c	2.40 ± 0.13 ^b
C22:6	7.07 ± 0.27 ^a	7.96 ± 0.88 ^a	7.73 ± 0.80 ^a	7.41 ± 0.44 ^a
SFA	28.12 ± 0.98 ^a	20.32 ± 0.97 ^b	18.97 ± 0.21 ^b	14.12 ± 0.30 ^c
MUFA	15.55 ± 0.67 ^c	23.71 ± 1.77 ^b	21.49 ± 0.93 ^b	30.10 ± 0.88 ^a
PUFA	56.33 ± 1.64 ^a	55.96 ± 0.80 ^a	59.54 ± 0.72 ^a	55.78 ± 0.73 ^a
TFA [#]	99.32 ± 2.23 ^d	124.93 ± 2.29 ^c	167.74 ± 5.43 ^b	232.84 ± 11.08 ^a

Notes: Results were displayed as mean ± SD ($n=3$). SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; TFA, total fatty acid. Different letters of superscripts in the same row mean statistically significant difference between groups ($P < 0.05$). [#] indicates the fatty acids contents (mg g^{-1} DCW), other values reflect the proportions of specific fatty acids accounting for the total fatty acids (%TFA). P and M represent photoautotrophy and mixotrophy, respectively.

It has been reported that external organic carbon can modify the FAs contents and compositions of microalgae. In one study conducted by Su *et al.* (2020), the SFA ratio and TFA content of *Phaeodactylum tricornutum* were found to increase under mixotrophic conditions, but the proportion of unsaturated fatty acid (UFA) declined largely. Similar conclusions have also been reached in other microalgal species, such as *Thalassiosira pseudonana*, *Chlamydomonas reinhardtii*, and *Micractinium reisseri* (Baldiasserotto *et al.*, 2021; Cao *et al.*, 2022). Unexpectedly, our data revealed a different pattern from above observations. At day 6, the SFA ratio of photoautotrophy group was 28.12%, remarkably higher than that of mixotrophy (20.32%). However, the photoautotrophic group displayed much lower MUFA proportion (15.54%) than the glycerol addition group (23.71%). As for PUFA, the ratios showed no significant differences between different cultivation modes. The significant influences of glycerol on FA were further evident in specific FAs. At day 6, C16:0 with a proportion of 25.29% was the most dominant FA in photoautotrophy. However, its ratio de-

creased to 18.51% in mixotrophy. On the contrary, C18:3 increased from 15.74% to 21.32%, becoming the predominant FA in mixotrophy group. Besides that, the addition of glycerol also significantly enhanced TFA contents. For example, the TFA content of the photoautotrophy group at day 6 was 99.32 mg g^{-1} DCW, whereas that of mixotrophy group was 124.93 mg g^{-1} DCW, showing a remarkable increase of around 25%. Considering that all groups showed no significant differences related to the chlorophyll fluorescence parameters at day 6, it can be concluded that the reduced ratios of SFA as well as the enhanced TFA contents and MUFA ratios during the early cultivation stage were a result of glycerol addition rather than nutrient deficiency. Additionally, day 12 displayed a similar variation pattern to that of day 6, featured by increased TFA and MUFA ratios, as well as decreased MUFA ratio in mixotrophic conditions. Taking the significant enhancements of mixotrophy on biomass accumulation, TFA production and proportion of PUFAs into consideration, mixotrophy displays enormous advantages in prompting FAs productivities in *C. roscoffensis*.

Our results also indicated that both the FAs compositions and contents of *C. roscoffensis* were heavily dependent on the cultivation stages. For example, with the prolonged cultivation time, the PA and SA ratios in the photoautotrophic group declined from 25.29% and 19.34% to 14.34% and 9.84%, respectively. In contrast, OA and ALA ratios increased from 13.80% and 15.74% to 20.00% and 26.14%, respectively. With regard to TFA contents, photoautotrophy displayed TFA value of 167.74 mg g⁻¹ DCW at day 12, around 1.5-times higher than that of day 6. In comparison with the photoautotrophy group, mixotrophic *C. roscoffensis* exhibited the same variation trend but with much higher variation magnitude, ranging from 124.93 mg g⁻¹ DCW at day 6 to 232.84 mg g⁻¹ DCW at day 12. Researchers have claimed that nutrients limitation is capable of inducing an increase in TFA contents of microalgae such as *Chlamydomonas* sp., *Nannochloropsis* sp., *Scenedesmus* sp., and *Chlorella* sp. (Mandal and Mallick, 2009; Can *et al.*, 2017). As mentioned in Section 3.2.2, a phenomenon of nutrients deficiency reflected by the declining values of F_v/F_m and ETR was observed during the later stage of cultivation period, which possibly contributed to the remarkable increase of TFA contents of *C. roscoffensis* at day 12.

3.2.5 Pigments contents

To further clarify the effects of glycerol on photosynthesis system and evaluate the potential of *C. roscoffensis* for fucoxanthin production, three types of pigments with relatively high amounts were analyzed by LC-MS. At day 6, the chlorophyll *a* (Chl-*a*) of two groups remained

around 20.0 mg g⁻¹ DCW, showing no significant differences (Table 3). These findings further supported the conclusions deduced from the chlorophyll fluorescence parameters, suggesting that the glycerol addition imposed no significant impacts on photosynthetic capacity of *C. roscoffensis*. However, as the cultivation time was prolonged, the Chl-*a* of two groups decreased sharply. Among them, the mixotrophic group exhibited relatively large descending values, resulting in much lower Chl-*a* contents (8.32 mg g⁻¹ DCW) compared to photoautotrophic group (13.05 mg g⁻¹ DCW). Previous studies demonstrated that the Chl-*a* content of microalgae is closely related to nutrient availability. For example, Ikaru *et al.* (2015) investigated the effect of nitrogen limitation on the physiology of *Chlorella vulgaris* and found that the cellular Chl-*a* content was twice as high under nitrogen repletion conditions compared to nitrogen depletion conditions. Studies conducted with other microalgae such as *Chlamydomonas* sp., *Coccomyxa* sp., and even *Spirulina* sp. (Li *et al.*, 2018; Seth *et al.*, 2021) also reached the same conclusion. The trends in fucoxanthin closely resembled those of Chl-*a*. Compared to day 6, the values of day 12 displayed a sharp decrease of 26.57% in autotrophic group and 58.16% in mixotrophic group, respectively (Table 3).

Fucoxanthin is attracting intensive attention due to its multiple health benefits, and majority of previous studies related to its industrial production focused on diatoms (Seth *et al.*, 2021). Until recently, several studies have evaluated the feasibility of producing fucoxanthin in coccolithophores (Ishika *et al.*, 2017; Okcu *et al.*, 2021).

Table 3 Pigments of *Chrysolita roscoffensis* under different modes

Contents (mg g ⁻¹ DCW)	Day 6		Day 12	
	P	M	P	M
Chlorophyll <i>a</i>	20.30 ± 2.06 ^a	21.32 ± 1.91 ^a	13.05 ± 1.05 ^b	8.35 ± 0.67 ^c
Fucoxanthin	7.00 ± 1.15 ^a	7.72 ± 0.43 ^a	5.14 ± 0.49 ^b	3.23 ± 0.19 ^c
Diadinoxanthin	0.63 ± 0.10 ^a	0.57 ± 0.08 ^{ab}	0.37 ± 0.06 ^b	0.45 ± 0.08 ^{ab}

Notes: Results were 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. P and M represent photoautotrophy and mixotrophy, respectively.

In this study, the fucoxanthin of *C. roscoffensis* peaked at day 6 with a value of around 7.0 mg g⁻¹ DCW. Compared to other known microalgae species, such a value of *C. roscoffensis* seems moderate (Seth *et al.*, 2021). Additionally, it has been reported that the specific growth rate and biomass productivity of coccolithophores can be largely enhanced when cultivated using photobioreactors with a semi-continuous cultivation method (Moheimani *et al.*, 2011). Thus, it can be expected that mixotrophic *C. roscoffensis* cultivated using semi-continuous cultivation mode, in which microalgal cells can be maintained at exponential phase with relatively high fucoxanthin content, maybe the optimal choice for the purpose of producing fucoxanthin from *C. roscoffensis*.

4 Conclusions

Under heterotrophic cultivation conditions, none of

glucose, glycerol, or NaAC was found to be able to promote cell growth, confirming that *C. roscoffensis* lacks the ability of heterotrophy. Under illuminated conditions, the addition of glucose showed no effects on *C. roscoffensis* either, demonstrating the absence of a hexose transporter in this microalga. External NaAC resulted in reduced cell density of *C. roscoffensis* even in the presence of light supply, whereas the underlying mechanism behind this phenomenon requires further exploration. On the other hand, only glycerol, when added under illuminated conditions, facilitated *C. roscoffensis* growth, and its promoting effects were enhanced with the increase of carbon concentrations (within the range of 0.05–0.5 mol L⁻¹). Moreover, mixotrophy significantly modified the biochemical composition of *C. roscoffensis* biomass, characterized by increased lipids and TFA contents as well as altered FAs profile. However, the photosynthetic capacity, biocalcification process, and pigments biosynthesis of *C.*

roscoffensis seemed unaffected by glycerol addition. Considering the multiple biotechnological advantages of *C. roscoffensis*, such as its unique biocalcification ability, rich lipids content with high PUFA ratios, moderate fucoxanthin contents, and high growth rates under mixotrophy, it can be concluded that this microalga deserves more attention for the purpose of CO₂ mitigation and high-value substances production.

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Author Contributions

Luyao Zhang: methodology, raw material preparation, writing-original draft. Zhichao He: methodology, data curation, raw material preparation. Yan Sun: methodology, software, surveys. Jian Li and Pengfei Cheng: software, data analysis. Spiros N. Agathos: conceptualization, investigation. Chengxu Zhou and Lin Zhang: administration and supervision, conceptualization. Jichang Han: writing-review and editing, funding acquisition, project administration.

Data Availability

The data and references presented in this study are available from the corresponding author upon reasonable request.

Declarations

Ethics Approval and Consent to Participate

This article does not contain any studies with human participants or animals performed by any of the authors.

Consent for Publication

Informed consent for publication was obtained from all participants.

Conflict of Interests

The authors declare that they have no conflict of interests.

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