Optimization of Culture Medium to Increase The Biomass Production of *Scenedesmus obliquus*: The Impact of Carbon Source

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Abstract

This study aimed to investigate the effect of adding an exogenous carbon source on the growth of *Scenedesmus obliquus*. For this purpose, the impact of three carbon sources, including sodium acetate (two concentrations of 0.01 and 0.02 g/l), sodium carbonate (two concentrations of 0.01 and 0.02 g/l), and aeration to supply carbon dioxide on the growth and production of *S. obliquus* biomass cultured on BBM medium were investigated. Based on the results, all three carbon treatments significantly increased biomass production and microalgae growth compared to the control group ($P < 0.01$). Among the carbon sources studied, the concentration of 0.02 g/l sodium acetate had the most significant effect on microalgae growth. Therefore, this treatment uses for the heterotrophic culture of *S. obliquus*. Aeration treatment also increased the biomass concentration of the microalgae compared to control ($P<0.01$). This assay showed that *S. obliquus* was capable of heterotrophic growth using acetate as a carbon source. The results also showed that carbon treatments significantly increased the content of photosynthetic pigments in microalgae ($P < 0.01$). Since pH may affect algal growth, an examination of the pH trend showed that acetate and carbonate treatments significantly increased the pH of the culture medium at the end of the experiment. In contrast, aeration treatment did not affect pH change compared to the control group.

Keywords: Aeration, Sodium acetate, Heterotrophic growth, microalgae, photosynthetic pigments.

Introduction

Since the advent of algal culture medium formulations, often since the 1950s efforts have been made to optimize culture media for achieving maximum biomass (Hughes et al., 1958; Kiyohara et al., 1960). Algae culture medium is a combination of salts of macro and microelements essential for the survival and growth of algal cells. Optimization of the culture medium allows researchers to reach quickly high levels of biomass, which is an important issue both in laboratory research and in the large-scale culture of algae for commercial purposes (Verma et

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al., 2020). The trend of the evolution of algal culture medium in recent decades confirms the claim that culture medium optimization is an essential concern in the biotechnology of microalgae (Karpagam et al., 2015). On the other hand, aquaculture of microalgae requires a culture medium that incorporates a combination of technical, economic, and environmental considerations; highlighting the significance of ingredients in the media (Khan et al., 2018).

Carbon source is one of the main components of the culture medium that significantly impacts the growth of microalgae cells. Like all photosynthetic organisms, algae need carbon (often in the form of carbon dioxide) to grow. No change occurs in the absence of carbon, and carbon deficiency is an important limiting factor in the growth of microalgae (Li et al., 2020). On the other hand, empirical evidence shows that carbon enrichment in the culture medium significantly improves the growth and speeds up biomass production both in the laboratory and large-scale culture (Jafari et al., 2021). Although CO_2 is often used as a carbon source for algal growth, this approach suffers from shortcomings. Based on the results of the analysis of the chemical composition of the biomass of microalgae, it is determined that to produce each gram of microalgae, 1.8 grams of carbon dioxide is needed. Therefore, the use of carbon dioxide in the air alone is not enough to achieve high levels of microalgae biomass (Sydney et al., 2010). On the other hand, in most laboratory studies that require sterile conditions, aeration may lead to contamination of the culture medium (Cordoba-Perez M and de Lasa, 2020). The use of air filters to prevent contamination of the culture medium also faces technical and handling problems. Therefore, the use of a carbon source formulated as part of the culture medium is an effective way to increase microalgae biomass and at the same time, prevent contamination in aseptic cultures. Accordingly, several studies have been conducted to find a suitable carbon source for microalgae cultivation (Gim et al., 2016; Le Gouic et al., 2021).

Scenedesmus obliquus is a green microalgae species widely used to generate bio-hydrogen and biodiesel (Agrawal and Verma, 2022).

Other applications of *S. obliquus* include, and are not limited to, phycoremediation of wastewater and production of fish meal (Patnaik et al., 2019). BBM (Bold Basal Medium) is one of the major culture media for *Scenedesmus* culture with no carbon source, which encourages photoautotroph growth of microalgae. Photoautotrophic culture of microalgae suffers from limitations such as low biomass accumulation; thus, *Scenedesmus* grows more slowly than many common microalgae such as *chlorella* sp. and *chlamydomonas reinhardtii* (Matsudo et al., 2017). Accordingly, enhancing the usual cultivation media of *S. obliquus*- such as BBM - with a carbon source that promotes heterotrophic growth has the potential to have a positive effect on the growth rate and biomass production of this microalga (Wang et al., 2015). In addition, heterotro-

phic growth has advantages such as better culture control, no need for continuous aeration, and increased biomass and lipid production (El-Sheekh et al., 2013). According to these propositions, the present study was conducted to modify BBM culture medium by adding a carbon source and investigating its effect on the concentration of *S. obliquus* biomass. Aeration treatment can increase the growth of microalgae by injecting carbon dioxide- a carbon source- from air into the culture medium. In large-scale cultural settings, aeration treatment is also widely used to supply carbon and improve the growth of microalgae (Zhu et al., 2017). Therefore, aeration was also applied as a carbon source in this research to monitor its impact on microalgae growth.

Material and methods

Microalgae culture and carbon treatments

Seed culture of *S. obliquus* (IBRC-M 50028) was provided by the Iranian Biological Resource Center and used for cultivation under laboratory conditions. The microalgae were cultivated in Bold Basal Medium (BBM) manually prepared according to standard guidelines (Vonshak, 2017). *S. obliquus* cells were grown under white light with a lux intensity of 80.5 µmol/s/m^2, the temperature was kept constant at 25°C, and the pH of BBM medium was set at 6.8. The automatic timer is at 14:10 (L/D). All cultures included 250ml of BBM, which added 50 ml of fresh microalgal; the initial biomass concentration was 0.04 g/L for each experiment (Abomohra et al., 2014).

Carbon treatments included a source of Sodium acetate ($C_2H_3NaO_2$) at two concentrations (0.01 and 0.02 g/l); 2) and Sodium carbonate (Na_2CO_3) in two concentrations (0.01 and 0.02g/l); 3) without aeration and aeration to supply CO_2 to the culture medium. All cultivation procedures and carbon treatments were performed under aseptic conditions and laminar airflow. Another set of group 0.02 g/L Sodium acetate was cultivated in the dark for heterotrophic growth, and the rest conditions were the same as the above.

Growth and physiological Measurements

According to the method proposed by Almomani and Mermeci (2018), to measure the fresh weight of algae, 50 ml of culture medium was added to the Falcon tube and then centrifuged. The supernatant was discarded entirely. Falcons were then measured with a digital scale. The difference between the weight of the empty Falcon and the Falcon containing algal pellets was reported as wet weight. The Falcons were placed in a 60 °C oven for 60 hours until the algal bullet was completely dry to determine the dry weight. The Falcons were then weighed again. The difference in weight of the falcons before and after placement in the oven was reported as dry weight.

Algal growth was observed by measuring light absorption at 680 nm. The pH of the culture medium was measured with a table-top pH meter. Chlorophylls content was determined by the method proposed Gim et al. (2016). For this purpose, 10 ml of the homogeneous algal suspension was first

centrifuged at 380 g for 10 minutes. The supernatant was then discarded. Then, 5 ml of 80% acetone was added to the precipitate and then extracted for 15 minutes at 10 °C. The concentration of chlorophylls in the extracted solution was determined by measuring the absorbance at 645 and 663 nm with a UV–visible spectrophotometer (NanoDrop One UV-Vis Spectrophotometer, USA) and then calculating with the following equation:

Chlorophylls(mg/L)=8.02×A_{663}+20.21× A_{645}.

This research was carried out in a completely randomized design with three replications. Duncan's mean comparison test was performed using SAS. 10 software for statistical analysis.

Results

The present study was carried out for 14 days. The measurement results at the end of the experiment are presented in Table 1.

The biomass production at sodium acetate concentrations of 0.01 g/l and 0.02 g/l were 0.589 and 0.704 g/l; respectively, significantly higher than the control group's ($p<0.01$). The biomass production at sodium carbonate concentrations of 0.1 g/l and 0.02 g/l were 0.305 and 0.372 g/l, respectively, significantly higher than that of the control group but lower than biomass produced under aeration treatment (0.601 g/l). Overall, the results obtained concerning biomass productivity under different treatments showed that sodium acetate at a concentration of 0.02 g/l had the best results, followed by aeration, sodium acetate (0.01 g/l), and sodium carbonate (0.02 g/l), sodium carbonate (0.01), and control; respectively.

To evaluate the heterotrophic growth potential of *S. obliquus* using acetate as a carbon source, the optimum acetate concentration (0.02 g/l) in mixotrophic culture was adopted for cultivation under dark conditions. The result showed that *S. obliquus* could grow under darkness in the presence of carbon acetate at a concentration of 0.02 g/l. At the end of the experiment (14th day), a biomass concentration of 0.158 g/l was achieved. Although this value is lower than those obtained under every mixotrophic treatment and there was no significant difference with

Table 1. Summary of measurements at the end of experiment (the day 14th)

	$C_2H_3NaO_2$ (g/l)		Na_2CO_3 (g/l)				Dark
	0.01	0.02	0.01	0.02	Aeration	Control	$C_2H_3NaO_2$ 0.02 (g/l)
Biomass (g/l)	0.589	0.704	0.305	0.372	0.601	0.149	0.158
OD_{680}	1.4	1.81	0.66	084	1.5	0.42	0.43
Chlorophyll a (mg/l)	4.06	4.58	3.31	3.53	4.11	1.32	0.52
Chlorophyll b (mg/l)	1.35	1.55	1.1	1.17	1.4	0.44	0.21
Carotenoid (mg/l)	0.63	0.77	0.54	0.59	0.71	0.21	0.17
pH	10.05	11.26	10.16	11.08	8.88	7.5	8.1

control, it is of great importance to observe the adequacy of carbon acetate for heterotrophic growth of *S. obliquus*.

Optical density is an indicator for evaluating the growth of microalgae. For this purpose, the algae growth rate was measured based on the OD_{680} value on days 0, 5, 10, and 14 (Figure 3). As the trend in the chart shows, after a 24-hour lag phase, the growth graph of carbon treatments started to rise. As observed for biomass, at the end of the experiment (day 14) the highest OD levels were observed in the treatments of sodium acetate, aeration, sodium carbonate, and finally the control group, respectively. Optical density values followed the same trend as biomass values.

Regarding photosynthetic pigments, sodium acetate treatment significantly increased the content of chlorophyll a, chlorophyll b, and carotenoids compared to the control ($p<0.01$). Similarly, the increase in photo-

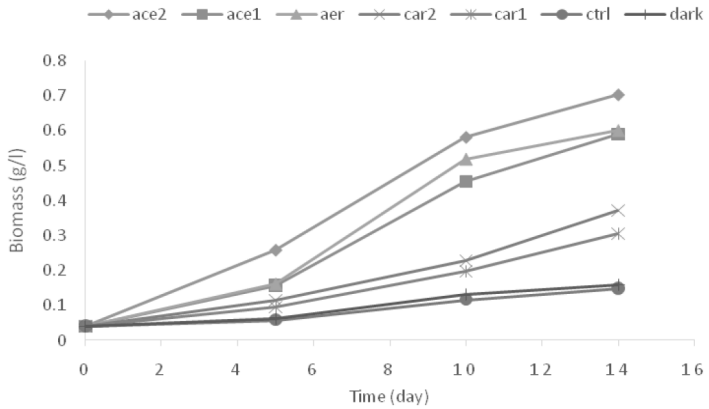

Fig. 1. Biomass concentration of *S. obliquus* under various treatments of carbon (ace2, ace1, aer, car2, car1 and ctrl stand for 0.02g/l acetate, 0.01g/l acetate, aeration, 0.02g/l carbonate, 0.01g/l carbonate, control; respectively)

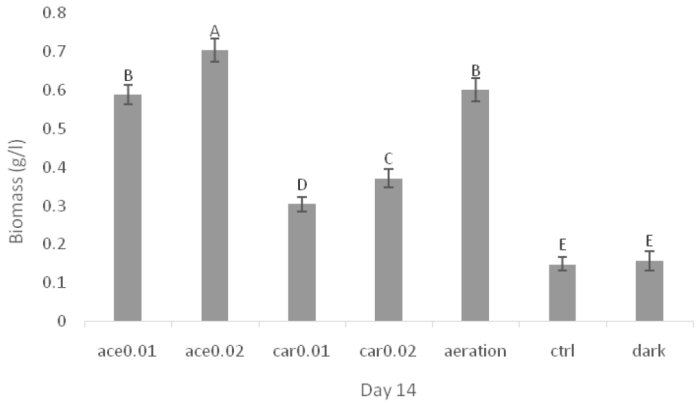

Fig. 2. Comparison of biomass production under various carbon treatments at day 14. (ace2, ace1, aer, car2, car1 and ctrl stand for 0.02 g/l acetate, 0.01g/l acetate, aeration, 0.02g/l carbonate, 0.01g/l carbonate, control; respectively)

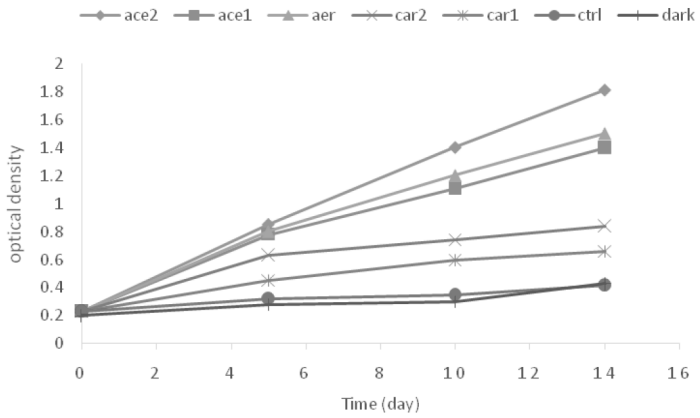

Fig. 3. Growth kinetics of *S. obliquus* under various treatments of carbon (ace2, ace1, aer, car2, car1 and ctrl stand for 0.02g/l acetate, 0.01g/l acetate, aeration, 0.02g/l carbonate, 0.01g/l carbonate, control; respectively)

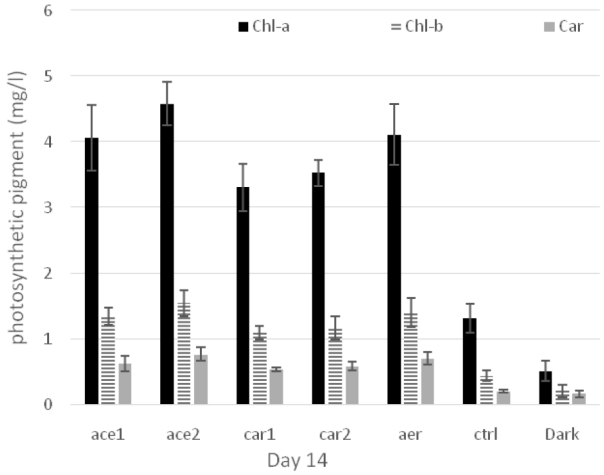

Fig. 4. Photosynthetic pigment concentration at various carbon treatments (ace2, ace1, aer, car2, car1 and ctrl stand for 0.02g/l acetate, 0.01g/l acetate, aeration, 0.02g/l carbonate, 0.01g/l carbonate, control; respectively)

synthetic pigments in sodium acetate treatment at 0.02 g/l was significantly higher than in other treatments. Also, aeration and sodium carbonate treatments (as a carbon source) significantly increased the content of photosynthetic pigments compared to the control group ($p<0.01$). In photosynthetic pigments, sodium acetate, aeration, and sodium carbonate treatments had the most significant effect on chlorophyll content, and increased chlorophyll b and carotenoids were in the next rank. Overall, the results of this assay showed that the use of an exogenous carbon source significantly increases photosynthetic pigments ($p<0.01$). The pH level at heterotrophic condition was also higher than that of control group under mixotrophic culture.

Microalgae have recently attracted considerable interest worldwide due to their exten-

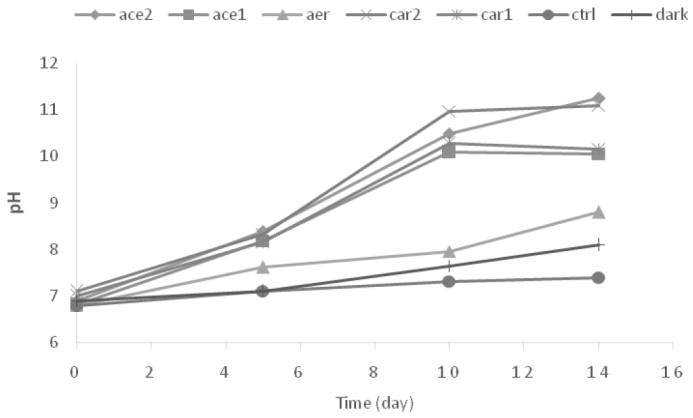

Fig. 5. pH variation as the result of carbon treatments (ace2, ace1, aer, car2, car1 and ctrl stand for 0.02g/l acetate, 0.01g/l acetate, aeration, 0.02g/l carbonate, 0.01g/l carbonate, control; respectively).

sive application potential in the renewable energy, biopharmaceutical, and nutraceutical industries. Microalgae are renewable, sustainable, and economical sources of biofuels, bioactive medicinal products, and food ingredients (Verma et al., 2020). Microalgae are mainly photoautotrophs and grow in a culture medium containing mineral salts; because microalgae can convert sunlight into chemical energy. However, autotrophic culture can't provide sufficient efficiency to produce microalgae biomass. Autotrophic culture increases production costs due to the constant need for light and technical considerations in the design of bioreactors (Lu et al., 2021). Therefore, switching to heterotrophic culture - which uses a carbon source - has been proposed as a suitable solution to increase the biomass production efficiency of microalgae. Some microalgae species, such as *S. obliquus*, can grow heterotrophically or mixotrophically (Cordoba-Perez et al., 2020).

Discussion

One of the main components of heterotrophic culture systems is using an external carbon source to meet the metabolic needs of microalgae. Therefore, the design of carbon source-based heterotrophic systems and determining the optimal concentration of carbon source to achieve a cost-effective level of biomass in such algae are of great importance (Gim et al., 2016, Jafari et al., 2021).

One of the remarkable results was that the amount of biomass produced with aeration is very close to sodium acetate, which shows the critical role of aeration. Because aeration is a cost-effective and easy method, this treatment can be used as a source to supply carbon to the microalgae culture medium. Previous studies have also highlighted the importance of aeration to improve the growth of microalgae. For example, Tang et al. (2016) found that aeration treatment enhances the development of both microorganisms (algae and bacteria) in a survey of

an algal-bacterial symbiosis system. Similarly, Liu et al. (2006) showed that artificial aeration has a positive effect on microalgae growth; these results support the findings of the present study.

Based on the results obtained in the present study, *S. obliquus* microalgae can grow mixotrophically in the presence of a carbon source in cultures containing acetate and carbonate. Even the microalgae could undergo heterotrophic growth when supplied with carbon acetate. This finding is of great practical importance, autotrophic and inability to heterotrophic growth have been considered limiting factors in algal biomass production in many studies (Scarponi et al., 2021). Heterotrophic growth significantly reduces cultivation costs and increases productivity (Karpagam et al., 2015). Heterotrophic culture is of great importance in producing microalgae biomass for several reasons. One of the biggest problems in autotrophic cultivation is the lack of light reaching the depths of the culture medium, which causes a significant drop in biomass production efficiency (Morales-Sánchez et al., 2015).

On the other hand, the phenomenon of light saturation is considered a factor is inhibiting the growth of microalgae in autotrophic culture. Problems of autotrophic cultivation are significant in the large-scale cultivation of microalgae because it reduces microalgae biomass production units (Ye et al., 2018). Many researchers consider many researchers have viewed the use of heterotrophic culture. First, in heterotrophic culture, more control is possible and thus can reduce the amount of potential contamination of the culture medium. Moreover, there is no need for continuous aeration in heterotrophic cultivation, which plays a vital role in reducing energy consumption and production costs (El-Sheekh et al., 2013).

Numerous reports have also suggested that the production of biomass and lipids can be increased in heterotrophic cultures (Sutherland et al.., 2021; Senet al. l, 2018). Accordingly, the ability of microalgae to heterotrophic growth is both technically and economically significant.

On the other hand, it has already been mentioned that one of the main challenges in the large-scale production of algal biomass is due to the inability of algal species to heterotrophic growth (El-Sheekh et al., 2013). Another finding of the present study is a significant increase in biomass production as a result of the application of carbon sources (sodium acetate and sodium carbonate). Because *S. obliquus* is a species with high commercial value, improving biomass production efficiency can be of great economic importance. Efforts have already been made to increase biomass production in *S. obliquus*. For example, Mandal and Mallick (2009) studied a combination of different factors to increase the biomass of *S. obliquus* and found that using an appropriate light regime and a combination of other minerals has a significant effect on increasing the growth of this microalgae. Abomohra et al. (2014) reported that temperature optimization and growth regulators significantly improved *S. obliquus* biomass. De Oliveira et

al. (2020) introduced the improvement of biomass production as a vital factor in the commercialization of *S. obliquus* cultivation. In general, the literature indicates the high importance of biomass production optimization methods for the large-scale cultivation of *S. obliquus*.

Since microalgae such as *S. obliquus* are photosynthetic organisms, studying their photosynthetic apparatus under different treatments is of great importance. For this purpose, in the present study, the effect of carbon treatments on photosynthetic pigments of *S. obliquus* was investigated. Based on the results, it was found that all three treatments of sodium acetate, sodium carbonate, and aeration significantly increased chlorophylls and carotenoids in microalgae. These findings indicate that using an external carbon source is a desirable factor to improve the growth and stimulate photosynthetic activity in microalgae. The results obtained in this assay are consistent with the findings reported by other authors. For example, Song & Pei (2018) said that using an exogenous carbon source increases the content of chlorophyll and carotenoids in the microalgae *Scenedesmus quadricauda*. Pancha et al. (2015) pointed out that optimizing the carbon composition in *Scenedesmus* sp. culture medium significantly increases photosynthetic pigments. Similarly, Vijay et al. (2021) reported that the *S. obtusus* could efficiently utilize the supplemented carbon source, increasing its photosynthetic efficiency.

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According to the results of this study, sodium acetate as a carbon source had the most significant effect on increasing the growth of *S. obliquus*. This finding suggests that sodium acetate can provide sufficient carbon for microalgae's growth and metabolic processes, thereby increasing its biomass. Some previous studies have also suggested the importance of sodium acetate as a carbon source for the development of microalgae. For example, enrichment of BBM medium with 7.5 g/l each of glucose and sodium acetate has been reported to produce dry biomass equal to 1.75 g/l in the microalgae *Neochloris oleoabundans* (Silva et al., 2016). Similarly, Wang et al. (2019) demonstrated that Coccomyxa subellipsoidea subjected to the feeding of 12 g/l sodium acetate achieved favorable biomass of 1.35 g/l and lipid content of 52.16%. The results obtained in this study are consistent with the results of other researchers on the adequacy of sodium acetate as a carbon source for the growth of microalgae. For example, Lacroux et al. (2021) showed that using sodium acetate with a nitrogen source significantly improved the development and production of biomass in *Chlorella sorokiniana.*

Similarly, Turon et al. (2015) showed that acetate is a good carbon source for the heterotrophic growth of microalgae. Jeon et al. (2006) also showed that the combination of light intensity and sodium acetate as a carbon source has a significant effect on the growth of *Haematococcus pluvialis*. Although these studies demonstrate the importance of acetate as a carbon source in the heterotrophic growth of microalgae, acetate has been studied less concerning with *S. obliquus*. The literature shows that researchers have explored other carbon sources in heterotrophic growth.

In previous studies, other carbon sources other than sodium acetate or sodium carbonate have been introduced as promoting factors for growth and biomass production in *S. obliquus*. Yang et al. (2014) reported

the utility of xylose as a carbon source for the mixotrophic growth of *S. obliquus*. Matsudo et al. (2017) investigated ethanol as a complementary carbon source in *S. obliquus* cultivation. They concluded that alcohol has a positive impact on biomass production of the microalgae. More recently, Scarponi et al. (2021) recommended the application of organic solid waste digest as the carbon source for the growth and cultivation of *S. obliquus*. Abomohra et al. (2014) called for introducing a new and cost-effective alternative for large-scale commercial cultivation of *S. obliquus*. According to our results, in addition to acetate, sodium carbonate as a carbon source was able to improve the growth of *S. obliquus* significantly. Since sodium carbonate is a relatively inexpensive carbon source that can use for the heterotrophic growth of *S. obliquus* microalgae, some previous studies also show that sodium carbonate is sufficient to supply the carbon required to develop microalgae (Elvira-Antonio et al., 2013; Duan et al., 2020). The results are consistent with the findings of the present study.

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