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Research article

Effect of different abiotic conditions on biomass and fucoxanthin content of *Amphora capitellata*

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Abstract

The aim of the study was to investigate the influence of physical conditions such as aeration rate (1, 3, 5 L/min) as well as chemical conditions including sodium nitrite ($NaNO_2$), urea (CH_4N_2O) and ammonium chloride (NH_4Cl) on the biomass productivity and fucoxanthin concentration of *A. capitellata*. The optimum cultures were cultivated in f/2 medium using sodium nitrate ($NaNO_3$) in 2 L bubbling bottle photobioreactors under the light intensity of 100 µE/ m^2s with aeration rate of 2 L/min. All the bottles were then incubated at 22.0±2°C, under the light intensities of 300 µE/m^2s with three different airflow rates of 1, 3, 5 L/min for 16 days. And then, culture medium was prepared with three different nitrogen sources to achieve higher biomass productivity. During the production of *A. capitellata*, the maximum specific growth rate of 0.166 day^{-1}, which conformed to the doubling time of 4.166 day, was achieved at the light intensity of 300 µE/m^2s with an aeration rate of 1 L/min when sodium nitrate was used. Chlorophyll-a and fucoxanthin contents were also at the highest level in the same light intensity. Dry biomass amount reached the maximum level of 0.66±0.17 g/L in case of $NaNO_2$. In this study, it was defined that the airflow rate of 1 L/min, the light intensity of 300 µE/m^2s and sodium nitrate ($NaNO_3$) were the optimum values not only for the growth of *A. capitellata* cells but also for the production of biomass and a higher fucoxanthin concentration.

Keywords: *Amphora capitellata; biomass; fucoxanthin; growth conditions; growth rate*

1. Introduction

Microalgae have achieved much attention as an up-and-coming material because of being a source for high feedstock value industrial products such as food additives, biological fertilizers, aquaculture feedstocks, biofuels etc. that are manufactured from their cell mass. Additionally, microalgae are easily produced compared to terrestrial plant-derived products due to both short life cycle and requirement for a lower amount of land area. However, the limitation of microalgae production is its low mass productivity (Bayu et al., 2020; Nigam et al., 2022). Microalgal biomass productivity depends on nitrogen-rich nutrients included in the growth media and their valuable metabolites are important for biofuels, health care and foodstuffs (Li et al., 2019).

Diatoms (Bacillariophyta) as eukaryotic microalgae are considered a crucially valuable source of chemicals, especially fatty acids, and carotenoids, in the aquaculture feedstock, food, pharmaceutical and nutraceutical industries. In addition, diatom frustules (silica cell walls) are the most interesting material to be used in nanotechnology. Diatoms are generally found widespread in aquatic ecosystems such as rivers, lakes, oceans, and marine areas which makes them frequently investigated sources for diatoms. Diatoms are either unicellular or multicellular microalgae with cell surfaces creating a siliceous skeleton including the frustule composed of amorphous silica [$(SiO_2)n(H_2O)$]. They are aquatic organisms that can move on their own in the water column (planktonic) and

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lie at the bottom of the underwater bed (benthic) (Brinkmann et al., 2011; Jamali et al., 2012; Cointet et al., 2019). The pennate diatom, *Amphora capitellata*, with a bilaterally asymmetrical valve, sized about 12-18 µm in length and nearly 3-6 µm in width is an isolated halophilic diatom at Izmir Bay, Aegean Sea (Demirel, 2016). Amphoroid taxa are acknowledged in the bright field microscope by their robustly dorsiventral shell outline; their linear ventral margin and raphe system are settled nearby place the ventral margin, and their girdle-band is a distinguishing characteristic group on the dorsal margin (Sato et al., 2013).

Environmental and other affecting factors may be curial on the algal biomass productivity, biomass compositions, and algal photosynthetic performance (Markou and Muylaert, 2016). Nitrogen can serve with a significant nutrient supply for the diatom growth and reproduction. It can have an effect on microalgae growth, subject to the amount, suitability, and type of the nitrogen source (Li et al., 2019).

Nitrogen is the second most vital element required for the cultivation of microalgae after carbon and performs a nitrogen cycle in the cellular processes-containing lipid and fucoxanthin production (Wang et al., 2018a). The major resources of nitrogen for the microalgae are inorganic nitrogen as nitrate, nitrite, ammonium, and many forms of organic nitrogen including urea, amino acids, are utilized for the synthesis of amino acids and proteins (Ruckert and Giani, 2004).

A large fraction of total ammonia nitrogen which consists of unionized ammonia (NH_3) and ammonium ion (NH_4^+) is unprotonated at higher pH values whereas ammonia strongly drops pH at high temperatures. The toxicity of NH_3 in algae has also been reported (Markou and Muylaert, 2016; Ayre et al., 2017; Berg et al., 2017). Therefore, ammonium (NH_4^+ or NH_3) is the preferential nitrogen supply for different strains of microalgae, but the high level of ammonium nitrogen is toxic, inhibiting microalgae productivity. The reason for NH_3 toxicity at alkaline pH in microalgae may be that the algae take in carbon dioxide due to the triggering of photosynthesis as a result of the inhibited photosystem due to high pH (Ayre et al., 2017) or it may perform photophosphorylation separately to reach a low pH to enable the conversion of ADP to ATP (Kumar and Bera, 2020). For microalgae cultivation, ammonium-based fertilizers at cheaper prices are preferred to other nitrogen sources fertilizers (Li et al., 2019).

The purpose of this study was to observe the influence of physical conditions such as aeration rate (1, 3, 5 L/min) in $NaNO_3$ containing f/2 medium as well as chemical conditions including sodium nitrite ($NaNO_2$), urea (CH_4N_2O) and ammonium chloride (NH_4Cl) on the biomass productivity and fucoxanthin concentration of *A. capitellata*. The growth rate, doubling time, biomass dry weight and fucoxanthin amount were detected at a continuous light intensity of 300 µE/m^{2}s at the late phase of the batch production process. Variations of the aeration rate and nitrogen sources were used to measure the fucoxanthin content and cell morphology of diatom.

2. Materials and methods

2.1. Diatom and growth conditions

The strain was supplied from Ege University Microalgae Culture Collection with collection number of EGE MACC2 on agar-solidified f/2 medium for autotrophic cultivation.

The optimum cultures (control cultures) were cultivated in f/2 medium using sodium nitrate ($NaNO_3$) at the light intensity of 100 µmol/m^{2}s with aeration rate of 2 L/min. The f/2 medium was prepared by Guillard's recipe and contained (per liter) 20 g of sea salt (Guillard and Ryther, 1962). The inoculum for all experiments was set at 10% volume in 2 L bubbling bottle photobioreactors. Cultures were then incubated at 22.0±2°C, continuously under the light intensities of 300 µE/m^{2}s with three different air at rates of 1, 3, 5 L/min for 16 days.

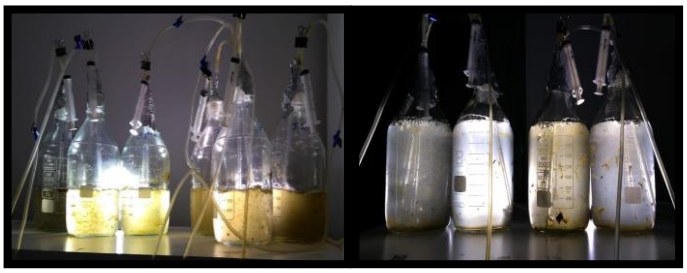

Fig. 1. Cultivation of *A. capitellata* in prepared different nitrogen resources in f/2 medium.

After, different nitrogen sources (sodium nitrite ($NaNO_2$)), urea (CH_4N_2O) and ammonium chloride (NH_4Cl) were added to the culture media containing the same amount of nitrogen to evaluate their effects on *A. capitellata.* These were cultivated with airflow at rate of 1 L/min under the light intensities of 300 µE/m^{2}s (Fig. 1). The optimum culture was cultivated in f/2 medium. The growth of diatom was observed by cell counting using an improved Neubauer haemocytometer. Diatom densities were measured every two days for 16 days using UV-Vis spectrophotometry at 600 nm. The observed maximal growth rate (µ) was calculated using Equation 1 (Sener et al., 2022). Based on the data for each experiment which contained three analytical replicates, the mean values with standard deviations were calculated.

$$\mu = (lnXb - lnXa) \,/\, (Tb - Ta) \qquad \text{Eq. 1}$$

where µ=specifc growth rate, Xa=cell concentration at time Ta, and Xb=cell concentration at time Tb. Doubling time=In 2/µ.

Diatom cultures were precipitated by centrifugation (about 6000 rpm for 10 min) and then, the pellet was dried using lyophilization and dried pellet was placed in the freezer at -20°C.

2.2. Fucoxanthin extraction and HPLC-DAD determination

For the extraction of fucoxanthin, a modified protocol reported in our previous study was applied. For this purpose, 0.20 g of dry biomass was weighed and 0.20 g of $CaCO_3$ was added (Erdogan et al., 2022).

The mixture was treated using an ultrasonic bath for 15 minutes at 40°C (40 kHz, 300 W), and then the extracted sample was separated by centrifugation for 2 minutes at 5000 rpm. The supernatant was kept, and the precipitated was re-extracted (3 times) with fresh ethanol until decolourization of the biomass. Finally, all extraction solutions were combined and filtered by vacuum filtration using 47 mm of 0.20 µm nylon filter paper. The mixed solution was evaporated using a rotary evaporator. The residue was resolved in chloroform stabilized with ethanol and kept at -20°C prior to HPLC analysis. Fucoxanthin determination was preformed with HPLC-DAD using YMC Carotenoid C_{30} column (5 µm particle size, L×ID 250×4.6 mm) at 450 nm with a flow rate of 1 mL/min according to the previous work performed by (Erdogan et al., 2016;

Erdogan et al., 2022).

3. Results and discussion

3.1. Microalgae growth optimization

Amphora capitellata culture was used to explore the proper conditions for aeration, and light intensity, and also for revealing the effect of different nitrogen sources on enhanced biomass concentration, specific growth rate, and fucoxanthin content. In this study, an experimental approach was applied to maximize biomass concentration and fucoxanthin content in a bubbling bioreactor.

Fucoxanthin, a primary marine carotenoid, constitutes, over 10% of the approximate whole production of carotenoids on earth, and is the major carotenoid pigment existing in chloroplasts of microalgae, especially Bacillariophyta and Haptophyta, and macroalgae brown seaweeds. Fucoxanthin can harvest the light and transfer furthest 60% of the energy to chlorophyll-a in diatoms (Li et al., 2018; Roychoudhury et al., 2021). Physiological and biological properties of fucoxanthin are reported in lots of studies, such as having anticancer, antioxidant, antihypertensive, antiinflammatory, radio-protective, antiobesity, hepatoprotective activities (Maeda, 2013; Sun et al., 2018; Wang et al., 2018b; Mohamadnia et al., 2022).

Diatoms of microalgae, with nearly two-fold higher fucoxanthin amount than brown seaweed, are much more encouraging for fucoxanthin production. Bacillariophyta samples including *Phaeodactylum tricornutum*, *Odontella aurita* and *Cyclotella cryptica* were studied at an industrial scale for utilization of their commercial productions of fucoxanthin (Wang et al., 2018b). The present study aims to survey the influence of various incident light intensities, aeration rates and nitrogen sources on biomass and fucoxanthin productivity in *A. capitellata*.

In the first experimental group, diatom was grown in f/2 medium using sodium nitrate ($NaNO_3$) at the light intensity of 100 μE/m^{2}s with an aeration rate of 2 L/min. The growth of diatom was not increased fucoxanthin content and then, diatom culture was exposed to increasing light intensity and various aeration rates. The highest irradiance treatment (300 μE/m^{2}s) exposited the most fruitful growth rates and increased the fucoxanthin concentration of *A. capitellata*. It was found that four diatom culture treatments resulted in similar cell densities during the growth (Fig. 2). However, treatments were determined to double increase the fucoxanthin content from 6.36±0.12 to 19.66±0.39 mg/g at 300 μE/m^{2}s with different aeration rates. Considering the light-harvesting antennas of fucoxanthin chlorophyll binding proteins, it can be concluded that the production of this pigment and proteins enhanced the cultivation of the diatoms. Therefore, for the high-quality production of this precious material, optimal cultural conditions may be determined.

Adequate light intensity and nitrogen sources are required for the efficient production of microalgae. Because microalgal production is a process as a result from the transfer of the energy harvested from absorbed light to the photosynthesis complex (Mata et al., 2010), in theory, increased growth rate can be achieved with higher light intensities. However, both the growth and cell mass yield were diminished with the highest light intensity under phototropic conditions which may be explained by the photo-inhibition (Wang et al., 2018a). Increasing light intensity, the biomass and fucoxanthin concentration do not inhibit photosynthetic activity of *A. capitellata*. Meanwhile, the excessive light intensity cause photoinhibition and diminish the growth conditions. For this reason, light intensity could be calibrated to optimum conditions by the cell turbidity. Besides, several studies were reported that low light intensities were better than the relatively high light intensity on fucoxanthin yield and biomass concentration (Guo et al., 2016; Gómez-Loredo et al., 2016).

During the production, the maximum specific growth rate (0.166 day^{-1}) was reached with doubling time of 4.166 day, at the light level of 300 μE/m^{2}s at an airflow rate of 1 L/min in $NaNO_3$ as presented in Table 1. The highest biomass concentration (0.78 g/L) could be reached at airflow of 1 L/min in $NaNO_2$. On the other hand, the minimum fucoxanthin content (6.36±0.12 mg/g) was determined at airflow of 2 L/min and the light intensity of 100 μmol photons/m^{2}s in $NaNO_3$. Chlorophyll-a and fucoxanthin amounts were also at the highest level in the same light intensity $NaNO_3$. In this study, it was identified that the airflow rate of 1 L/min and the light level of 300 μE/m^{2}s were the optimum values for the growth of *A. capitellata* cells and production of biomass.

Nitrogen supply has a vital influence on biomass and fucoxanthin concentration. In the culture with the enhanced light level of 300 μE/m^{2}s, $NaNO_3$ is consumed rapidly in f/2 medium. Light intensity and nitrogen supply affects not only cell mass productivity but also the quality of cells both physiologically

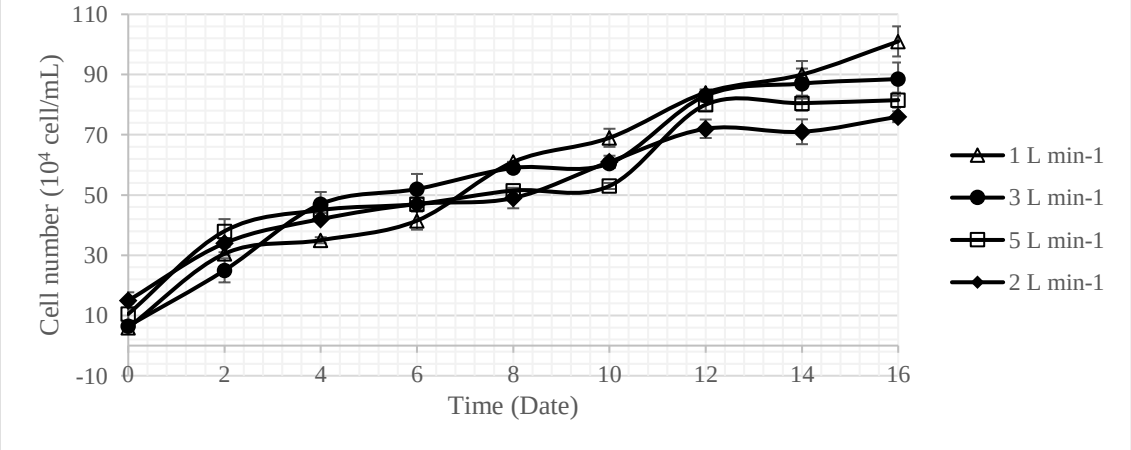

Fig. 2. Growth curves of *A. capitellata* in a photobioreactor under the light intensity of 300 μE/m^{2}s at different aeration rates.

Table 1
Specific growth rate, biomass, fucoxanthin concentration and cell number of diatom cultivated with various nitrogen sources and different aeration rates.

	Fucoxanthin (mg/g)	Doubling time (day)	Growth rate (day^{-1})	Biomass (mg/L)	Cell number (10^6 cell/mL)
$NaNO_3$ 2 L/min	6.36±0.12	4.864	0.143	0.31±0.05	7.1±0.2
$NaNO_3$ 1 L/min	19.66±0.39	4.166	0.167	0.36±0.17	10.1±0.9
$NaNO_3$ 3 L/min	17.97±0.35	4.416	0.157	0.29±0.15	8.9±0.6
$NaNO_3$ 5 L/min	19.29±0.38	4.659	0.149	0.28±0.18	8.2±0.3
$NaNO_2$ 1 L/min	9.64±0.19	2.7046	0.256	0.78±0.12	9.3±0.3
CH_4N_2O 1 L/min	10.11±0.20	6.7836	0.102	0.29±0.01	7.4±0.2
NH_4Cl 1 L/min	11.88±0.24	16.1845	0.043	0.17±0.02	3.6±0.6

and morphologically. Alterations in cell morphology were observed with SEM photography analysis of diatom cells in sodium nitrite ($NaNO_2$), urea (CH_4N_2O), ammonium chloride (NH_4Cl) and nitrate seem to be in correlation with the observed alterations in chemical compositions (Figure 3-6). For instance, Kaspar et al. (2014) defined that the wide variety in cell size, and the changeable cell surface of *Chaetoceros calcitrans* grown in the culture were attributed to nutrient depletion in growth medium. This experiment proposed that the cell surface alteration could be a result of nutrient starvation. However, the relationship between the cell morphological state and the concentration of the fucoxanthin has not yet been well studied.

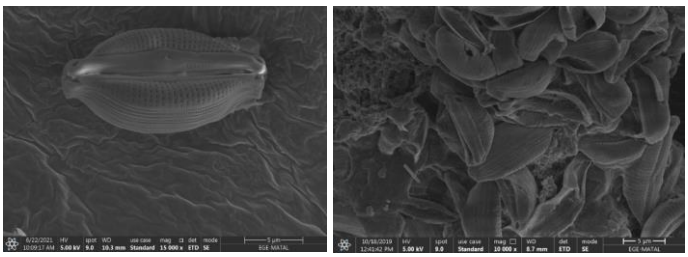

Fig. 3. SEM images of *A. capitellata* cultivated in f/2 medium prepared using sodium nitrate.

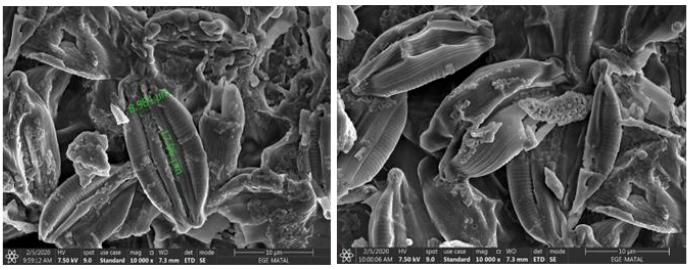

Fig. 4. SEM images of *A. capitellata* cultivated in f/2 medium prepared using sodium nitrite.

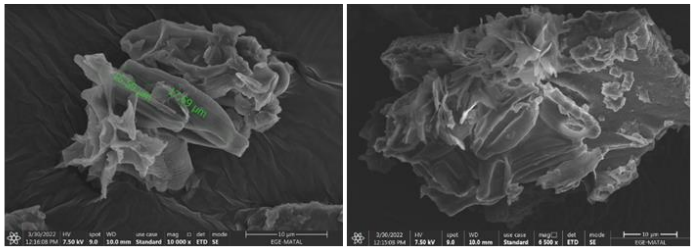

Fig. 5. SEM images of *A. capitellata* cultivated in f/2 medium prepared using urea.

Silica skeletons are the diatom walls existing as an envelope of the cell surface, called frustule/shell. The shells of diatoms are characteristic structure with a size and shape indicative of the morphological features of diatoms (Roychoudhury et al., 2016; Legalov and Reshetnikov, 2020). As seen in Figs. 3-6, *A. capitellata* frustule alteration was observed with different nitrogen sources in f/2 media at an airflow rate of 1 L/min and light intensity of 300 μE/m^{2}s. This study aims to measure morphological frustule changes in the monoalgal cultures of *A. capitellata* by cultivation in four different nitrogen sources (Fig. 3, 4, 5, 6). Amphora silica frustules are elliptic in shape with specific adornment and cells possess size pores hierarchically organized in curved bands in the cultivation both with $NaNO_3$ and $NaNO_2$. Nevertheless, cultivated diatoms in urea and ammonium chloride do not clear frustule pores and shapes as seem in the Fig 5, and 6. The presence of macronutrients and micronutrients or pollutants is valuable in inducing frustule morphology changes. In both marine and freshwater diatoms changes in salinity alter the morphological traits of the silica valve (Hervé et al., 2012). In accordance with the results of these studies, our study indicated that the volume of silica frustule decreased in *Amphora* cell cultures grown in urea and ammonium.

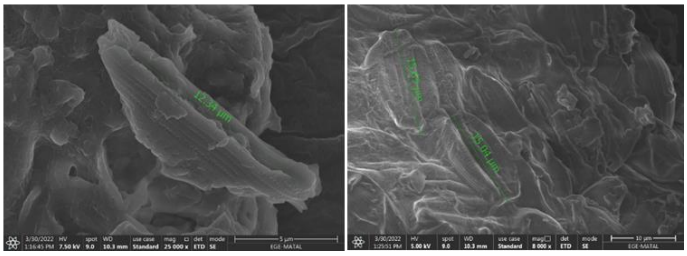

Fig. 6. SEM images of *A. capitellata* cultivated in f/2 medium prepared using ammonium chloride.

Diatoms absorb nitrogen from supplies such as nitrate, ammonia, and urea in the growth medium. When ammonium is utilized as the primary nitrogen source at high concentrations for algal cultivation, it causes a decrease in cell viability and may induce cell inhibition by diminishing the pH of the medium (Li et al., 2018, Kumar and Bera, 2020).

Among all treatments, the lowest fucoxanthin content (9.64±0.19 mg/g) was obtained in the same $NaNO_2$ containing medium where the maximum biomass concentration (0.78±0.12 mg/L) and growth rate (0.256 day^{-1}) were achieved in bubbling photobioreactor. Compared to the other nitrogen sources, $NaNO_3$ significantly enhances the specific growth rate and fucoxanthin content. Diatom cells absorb nitrogen to grow at comparable rates nitrogen from supplies other than ammonium and urea. In a study, cultures of *Phaeodaetylum tricornutum* were cultivated in nitrate ($NaNO_3$), nitrite ($NaNO_2$), ammonium sulphate ($(NH_4)_2S0_4$) or urea ($(NH_2)_2CO$), and all of them were prepared at 4 mg atom N/L concentration in media. Percentages of nitrogen in various media converted into cellular-nitrogen were determined by measuring productivities in dry weight of biomass nitrate, nitrite, urea and ammonia in cultures (Fidalgo

Paredes et al., 1995).

It was suggested that intracellular oxidative stress induced by increased concentration of ammonium could affect the specific activities of some enzymes and even cause deterioration of lipid peroxidation in cellular level. The primary function of microalgal pigments was to collect and convert light energy in the photosystems to generate the chemical energy required for the cultivation process. For this reason, inadequate energy induced by ammonia toxicity has been shown to block the photosynthesis of microalgal carotenoids (Li et al., 2019). Although the culture medium containing H_2O_2/NaOCl is known to cause oxidative stress by the formation of reactive oxygen species, the microalgal pigment of fucoxanthin was increased as 41.83±0.92 mg/g (Erdogan et al., 2022).

The nitrate transport metabolism pathway is similar to that of environmental plants, but their transport system was interesting to prove that diatoms have a metazoan-like urea cycle. Ammonia can be transported inside the cell by the nine ammonium transporters (AMTs). The nitrate transport mechanism by chloroplast using GSII-GOGAT and urea transport mechanism by mitochondrial using GSIII-GOGAT produce glutamine and glutamate, which must be transported from these organelles to replace nitrogen elsewhere in the cell (Smith et al., 2019).

Indrayani et al. (2020) reported that diatom *Amphora* sp. MUR 258 was investigated to grow over various temperatures and salinities with the aim of lipids and fatty acid profiles. The diatom obtained its highest specific growth rate (0.607 day^{-1}) at 7% NaCl at 35°C, and its peak lipid concentration (57.69%) was observed at 7% NaCl at 25°C. In another study, *Cylindrotheca closterium*, *P. tricornutum*, *Amphora* sp., and *Thalassiosira weissflogii* were growth in photobioreactors under different physical conditions. The maximum growth rate of *C. closterium* was substantially enhanced compared to that of the other three species at 20±1°C and a 12:12 light: dark by fluorescent light of 80 μE/m^2s. Besides, the cultures of *C. closterium* was found to have increased fucoxanthin content (21 mg/g) compared to the other three diatoms (Wang et al., 2018b).

4. Conclusion

In conclusion, diatoms are unicellular phytoplankton that is responsible for primary production in marine plants and plays an important role in biogeochemical cycling. The nutraceutical and cosmetic industries using diatom products have dealt with an investigation into the utilization of value-added compounds, especially fucoxanthin in the past few decades. Current study evaluated the influence of physical conditions such as aeration rate (1, 3, 5 L/min) in $NaNO_3$ containing f/2 medium as well as chemical conditions including sodium nitrite ($NaNO_2$), urea (CH_4N_2O) and ammonium chloride (NH_4Cl) on the biomass productivity and fucoxanthin concentration of *A. capitellata*. It is found that sodium nitrate is the potential chemical of the growth medium for *A. capitellata* to replace the nutrient supplies and growth conditions enhanced fucoxanthin productivity in the diatom with the light level of 300 μE/m^2s and an airflow rate of 1 L/min.

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