Research Article

Enhanced Biomass Productivity and β-cryptoxanthin Content of *Chlorococcum* sp. through Optimization Via Central Composite Design (CCD)

Sirawit Chuechomsuk
Department of Agro-Industry Technology and Management, Faculty of Digital Agro-Industry, King Mongkut's University of Technology North Bangkok, Prachinburi, Thailand

Vilai Rungsardthong*
Department of Agro-Industrial, Food and Environmental Technology, Faculty of Applied Science, Food and Agro-Industrial Research Center, King Mongkut's University of Technology North Bangkok, Bangkok, Thailand

Sonia Mohamadnia and Irini Angelidaki*
Department of Chemical and Biochemical Engineering, Technical University of Denmark, Lyngby, Denmark

* Corresponding author. E-mail: iria@kt.dtu.dk; vilai.r@sci.kmutnb.ac.th DOI: 10.14416/j.asep.2025.07.004
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Abstract
β-cryptoxanthin is one of the most commercially valuable carotenoids, which is rare in nature and costly to synthesize. Microalgae is a promising alternative and renewable source for β-cryptoxanthin production. This study aimed to optimize the cultivation of the microalgae, *Chlorococcum* sp. TISTR 8266, in BG–11 medium, to achieve the highest yield of β-cryptoxanthin. Therefore, central composite design (CCD) was employed to optimize the addition of organic carbon and nitrogen sources under mixotrophic and heterotrophic conditions combined with aeration and agitation. The results showed that under the mixotrophic conditions, the BG–11 medium with 1.6 g/L of glucose and 0.16 g/L of urea enhanced the biomass of *Chlorococcum* sp. to 4.90 ± 0.14 and 4.85 ± 0.07 g/L with aeration and agitation, respectively. Furthermore, under the optimized conditions, β-cryptoxanthin, β-carotene, and lutein content increased to 4.02 ± 0.49, 4.50 ± 0.71, and 12.76 ± 0.26 mg/g dry cell weight (DCW), respectively. In contrast, β-carotene presented the highest content of 5.05 ± 0.52 mg/g DCW for the control (non-modified BG–11 medium). Hence, the cultivation time was 50% decreased (from 14 days to 7 days) while the biomass increased from 2.50 g/L to 4.9 g/L and β-cryptoxanthin content increased from 0.064 mg/g cell dry weight to 4.02 mg/g cell dry weight when compared to the control conditions in our previous study. Overall, these findings offer new and economically feasible perspectives for β-cryptoxanthin production by the selected microalgal strain.

Keywords: Central composite design (CCD), *Chlorococcum* sp., Microalgal biomass, Mixotrophic, β-cryptoxanthin

1 Introduction

Carotenoids are commonly used in the food and nutraceutical industries as colorants or dietary supplements. Their popularity in food, supplements, and cosmetics is growing largely due to their antioxidant properties [1]. Human serum has six major carotenoid types: lycopene, α-carotene, β-carotene, lutein, zeaxanthin, and β-cryptoxanthin. Three of these carotenoids, namely, α-carotene, β-carotene, and β-cryptoxanthin are converted to vitamin A in the human body [2]. β-Cryptoxanthin has gained particular interest in recent years because it can exhibit higher bio-accessibility and bioavailability than lycopene and β-carotene in human serum and tissues [3].

β-Cryptoxanhin (beta-cryptoxanthin; $C_{40}H_{56}O$) is a xanthophyll carotenoid with chemical structure and bioactivity almost like β-carotene. However, β-

cryptoxanthin presents a higher polarity than β-carotene due to its extra hydroxyl group at the third carbon atom of the β-ring. Conjugated double bonds (chromophore) in β-cryptoxanthin structure not only lead β-cryptoxanthin to light absorption but also provide both color and photoprotection in plants [4]–[6]. β-Cryptoxanthin is found only in some fruits and vegetables. The highest concentration of β-cryptoxanthin was detected in butternut squash at 34.71 μg/g sample. Commercially available natural β-cryptoxanthin is the product from the extraction of satsuma mandarin orange (18.00 μg/g sample) [7], [8].

In recent years, microalgae cultivation has attracted extensive attention due to its advantages in carotenoid production [9]. Compared with the plants that can produce carotenoids, microalgae have the advantages of a fast growth rate, high unit area carotenoid yield, less land use, potential cultivation in non-agricultural land, and so on [10]. Hence, microalgae are one of the most promising sources of carotenoid production. Some outstanding commercial microalgae to produce carotenoids are the production of lutein by *Desmodesmus* sp., astaxanthin by *Haematococcus pluvialis*, fucoxanthin by *Tisochrysis lutea* and β-carotene by *Dunaliella salina* [11]–[14]. However, the biomass yield and carotenoid production using commercial microalgae are still low and have a high production cost [15]. Hence, many researchers have attempted to use other microalgae strains that can offset commercial microalgae to fix the cost of cultivation, increase the biomass and carotenoids production, and decrease the period of microalgae cultivation [16], [17].

Chlorococcum sp. is one of the choices for high-value-added products because *Chlorococcum* sp. is fast-growing, produces large quantities of biomass, and can be cultivated both indoors and outdoors [18]. Moreover, *Chlorococcum* sp. can be a good feedstock to produce biodiesel and other high-value products [19]. There are various value-added products from *Chlorococcum* sp., such as omega-3 fatty acids, chlorophylls, lutein, zeaxanthin, β-carotene, and β-cryptoxanthin [20]–[23]. In addition, β-cryptoxanthin can also be produced by several microorganisms such as *Kocuria marina* DAGII, *Pseudomonas* sp. strains Akiakane and *Pantoea anthophila FL1_IS5* can produce 0.0012 mg/g, 4.76 mg/L and 34.67 mg/L of β-cryptoxanthin, respectively [24]–[27].

A commonly used technique for promoting microalgae to produce carotenoid pigments is to treat them with stress conditions during cultivation. Even though microalgae produce comparatively less biomass, the production of carotenoids can be enhanced under stress conditions [9]. A two-stage culture approach is a general remedy for the issue of cell development and carotenoid production. While the second stage is set aside for the accumulation of carotenoids under diverse stress situations, the first stage is devoted to the best growing circumstances to achieve maximal biomass output [28]. Therefore, to obtain bioactive compounds of high value-added at a commercial level, it is necessary to sustainably produce biomass at a large scale.

Conventional approaches to improve the biomass of microalgae mainly involve the manipulation of environmental factors (e.g. temperature, light, and salinity) and nutrition (e.g. carbon and nitrogen). We have reported the production of β-cryptoxanthin by the microalgal strain, *Chlorococcum* sp., and studied the effects of light spectrum and intensity on its microalgal biomass and β-cryptoxanthin production. Our previous results show that the accumulation of the biomass and β-cryptoxanthin production of *Chlorococcum* sp. at 14 days was 2.50 ± 0.11 g/L and 0.06 mg/g cell dry weight without optimized conditions [22], [23]. The product, β-cryptoxanthin, in the cultivation was confirmed by liquid chromatography-high resolution mass spectrometry (LC-HRMS/MS) using the standard chemical. This work aimed to enhance the production of β-cryptoxanthin using BG–11 medium with different organic carbon and nitrogen sources, and study the effect of mixotrophic and heterotrophic conditions, agitation, and aeration. Accordingly, the optimal conditions will be applied to achieve the highest carotenoid production, especially β-cryptoxanthin, by *Chlorococcum* sp.

2 Materials and Methods

2.1 *Chemicals*

Standards of β-cryptoxanthin, β-carotene, lutein, and glucose were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile and methanol were high-performance liquid chromatography (HPLC) grade, while all other reagents and chemicals were analytical grade, also purchased from Sigma-Aldrich (Brøndby, Denmark).

2.2 *Microalgal strain and culture medium*

Chlorococcum sp. TISTR 8266 was kindly provided by the Algae Library of the Thailand Institute of Scientific and Technological Research (TISTR). The microalga was pre-cultured at 25 °C with 120 mL of BG–11 medium, pH 7.5–7.8, in a 250 mL Erlenmeyer flask. The cultivation was performed in a shaking incubator at 110 rpm for 5 days before use in the experiment.

2.3 *BG–11 medium enriched with organic carbon and nitrogen sources*

To investigate the influence of organic carbon sources on *Chlorococcum* sp., the following compounds were used: glucose, glycerol, acetate (sodium acetate), and sucrose. The organic carbon sources and their concentrations that can promote algal growth and biochemical production under mixotrophic conditions were chosen. Since there are limited studies on *Chlorococcum* sp., carbon sources, and their concentrations were selected based on some research on related microalgae [29]–[35]. In addition, the effect of the nitrogen source, including urea, ammonium chloride, and sodium nitrate, was investigated with the selected organic carbon concentration. Table 1 presents the range of independent variables and their levels. Then, central composite design (CCD) was used to optimize glucose (X_1, g/L), and urea (X_2, g/L) concentrations on biomass production by *Chlorococcum* sp. A total of nine experimental runs, with three replicates at each point, were carried out. Using experimentally observed biomass yields, a mathematical model that describes the impact of the two components on biomass yield was developed. Equation 1 summarizes the model as a second-order polynomial.

$$Y = \beta_0 + \sum_{i=1}^{2} \beta_i x_i + \sum_{i=1}^{2} \beta_{ii} x_i^2 + \sum_{i=1}^{1} \sum_{j=1}^{2} \beta_{ij} x_i x_j \quad (1)$$

where β0, βi, βii, and βij are coefficients for intercept, linear, quadratic, and interaction terms, respectively. Once the data was collected, the polynomial coefficients were determined by the method of the least squares using Design-Expert 13 software.

Table 1: The range of independent variables.

Variables Level	Glucose (g/L)	Glycerol (g/L)	Sodium Acetate (g/L)	Sucrose (g/L)	Urea (g/L)	Ammonium Chloride (g/L)	Sodium Nitrate (g/L)
1	5.0	5.0	0.5	2.0	0.1	0.5	0.5
2	7.5	15.0	5.0	6.0	0.5	1.5	1.5
3	10.0	30.0	10.0	10.0	1.0	3.0	3.0

2.4 *Growth of Chlorococcum* sp. *using modified BG–11 under different light modes*

The light modes were investigated using a factorial design with 4 factors, including mixotrophic cultivation, heterotrophic cultivation, aeration, and agitation. BG–11 medium was used for both mixotrophic and heterotrophic cultivation. Pure microalga culture of *Chlorococcum* sp. (approx. 10% w/v, OD of 0.10 at 680 nm) was inoculated in a 1000 mL laboratory bottle containing 400 mL of modified BG–11, incubated in the culture room at 25 ℃. The mixotrophic culture was operated under an illuminated condition (light source, LED lamp; photosynthetic photon flux density, approximately 50 μmol m^{-2} s^{-1}; light: dark cycle, 12:12 h). To block the light effect, the heterotrophic culture was covered with aluminum foil. Filtered air was provided at a rate of 2.5 L/min and 0.5% of CO_2 for the aeration conditions. The agitation was performed in an orbital shaker at 110 rpm.

2.5 *Growth analysis*

The microalgal growth was measured using in vivo autofluorescence (IVF), optical density (OD), and dry cell weight (DCW). IVF signals at 440 nm excitation, 690 nm emission, and 100 nm bandwidth were compared to a Coulter Counter count of cells/mL, and biomass was monitored using OD measures at 680 nm read in a BIOTEK Synergy microplate reader. The standard deviation of the blank (n = 8) was used to calculate the instrument detection limits. Whatman GF/C filter papers (47 mm in diameter and 1.2 μm in pore size) were dried in a hot air oven (BINDER ED 56, Germany) at 80 °C for the duration of the night to measure DCW. An analytical balance was used to measure the empty weights after filter sheets were left in a vacuum desiccator for 30 minutes. Until

consistent weights were achieved, the drying and weighing processes were repeated. The pre-weighed and pre-dried filter papers were used to filter the grown microalgal cells. Then, the samples were dried at 80 °C for 24 h to constant weight. Samples were cooled in a desiccator and then weighed. The biomass unit was reported in grams per liter (g/L).

2.6 *Determination of pigment content*

The microalgal cells were ruptured and then extracted with acetone: methanol (3:7 v/v), with 0.5 mm silica beads, pulsed in an ultrasonic bath (Branson M2800H, Mexico) for 15 min at a frequency of 35 KHz. The supernatants were collected after being centrifuged at 12,000 g for 5 min. The total amounts of carotenoid and chlorophyll in the extracts were ascertained by measuring absorbances with a UV/vis spectrophotometer and applying Equations (2)–(4) from the study of Lichtenthaler *et al*. [36].

$$\text{Chlorophyll } a = (12{:}25 \times A663) - (2.79 \times A647) \quad (2)$$

$$\text{Chlorophyll } b = (21.50 \times A647) - (5.10 \times A663) \quad (3)$$

$$\text{Total carotenoids} = [(1{,}000 \times A470) - (1.82\text{Chl}a) - (85.02\text{Chl}b)]/198 \quad (4)$$

where A = absorbance at 663 nm, 647 nm, and 470 nm, Chl*a* = chlorophyll *a*, and Chl*b* = chlorophyll *b*.

2.6.1 Carotenoid extraction and saponification

Carotenoid compounds were exhaustively extracted from each freeze-dried sample (0.2 g) with acetone and methanol using a mortar and a pestle, followed by centrifugation at 9,000 g, 10 °C for 15 min, until the supernatant turned colorless [37]. The extract was filtered through a 0.22 μm polyethylene membrane and concentrated using a vacuum rotary evaporator (BUCHI R-114, Fawil, Switzerland) at 30 °C. The concentrated extract was further suspended in a mixture of petroleum ether: diethyl ether (1:1 v/v), and saponified with 10% (w/v) methanolic KOH for 16 h at room temperature. Alkali in the sample was removed by washing with 10% (w/v) sodium chloride, then filled with N_2 and kept at -20°C in the dark until analysis.

2.6.2 Carotenoid analysis

β-cryptoxanthin, β-carotene, and lutein content were analyzed by high-performance liquid chromatography, UHPLC with an UV-VIS detector, Dionex Ultimate 3000 Series system (Thermo Fisher Scientific, Waltham, MA, USA) and a Phenomenex C18 column, 150 × 4.6 mm, 5 μm. The mobile phase was composed of methanol and acetonitrile (96:4 v/v). The sample injection was 20 μL, with the flow rate of the mobile phase at 0.7 mL/min. The detection was performed at a wavelength of 450 nm for β-cryptoxanthin standard and 445 nm for β-carotene and lutein standards, and the calibration curves for individual standards were made. The chromatogram data were processed using the Chromeleon version 7 software (Thermo Fisher Scientific, Waltham, MA, USA).

2.7 *Determination of glucose*

High performance liquid chromatography (HPLC) with a Bio-Rad HPX-87H (300 mm × 7.8 mm) column and a refractive detector was used to evaluate glucose. The eluent had a flow rate of 0.60 mL/min and contained 12 mM H_2SO_4. The column oven's temperature was set at 63 °C. A calibration curve with different dilutions of glucose solution was performed for quantification of the compound [38].

2.8 *Statistical analysis*

The results were reported as the mean ± SD. IBM SPSS software (SPSS Inc.) version 28 for Windows, one-way analysis of variance (ANOVA) and post-hoc Duncan's test with p-value < 0.05 were used to determine the significance of the variables. A minimum of three replications were conducted for each experiment.

3 Results and Discussion

3.1 *Effect of organic carbon sources on growth*

IVF, as a representative of the photosynthetic performance, was used to monitor and measure the growth and health of these microalgal strains in real-time without disrupting their natural environment. The measurement typically involves the use of fluorescent probes or markers that can bind to specific molecules or structures within cells or a measurement of photosynthetic pigments when exposed to the light of a certain wavelength [39], [40]. IVF of the

Chlorococcum sp. cultures in modified BG–11 with different organic carbon sources (glucose, glycerol, sodium acetate, and sucrose) and different concentrations is presented in Figure 1. The IVF values in the modified medium with three different concentrations of glucose are significantly higher than the control and the other organic carbon sources (p-value < 0.05). In all *Chlorococcum* sp. cultures enriched with glucose, the IVF values started to increase from day 2. The IVF values for the glucose concentrations at 5, 7.5, and 10 g/L increased and reached the highest IVF of 2,859 ± 393, 3,183 ± 260, and 2,989 ± 177, respectively, on day 6. Simultaneously, the modified medium with glycerol, sodium acetate, and sucrose did not show any significant difference compared to the control, while the modified medium with both 5 and 10 g/L of sodium acetate showed a decreased IVF after 8 days of cultivation.

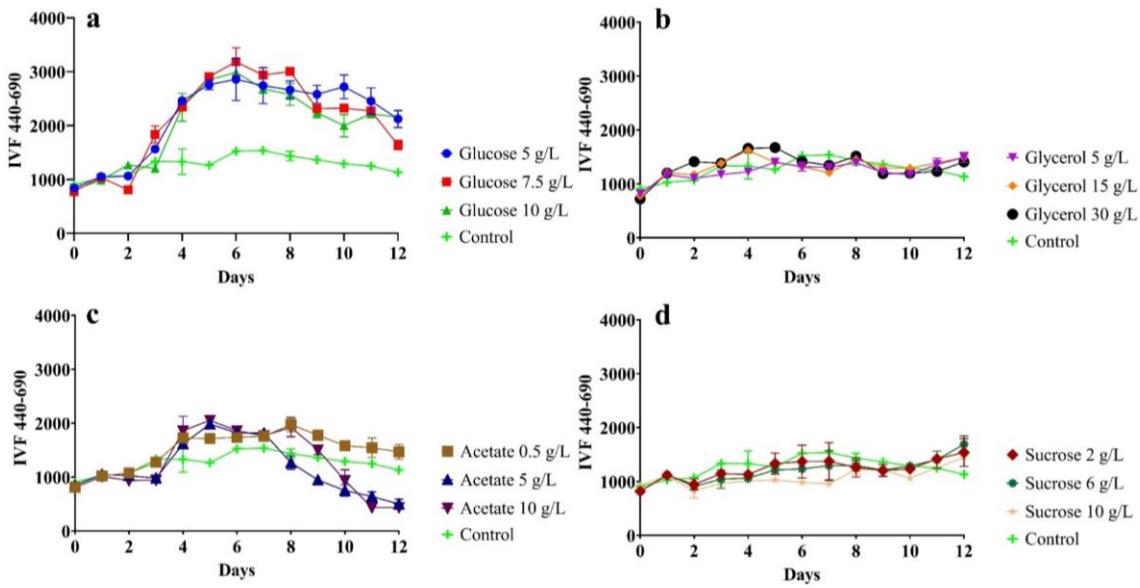

Figure 1: Effect of different concentrations of glucose (a), glycerol (b), acetate (c), and sucrose (d) on IVF of *Chlorococcum* sp. during the cultivation.

The biomass production of *Chloroccum* sp. by the modified medium with different organic carbon sources is presented in Figure 2. The results showed that the addition of glucose in modified BG–11 increased the biomass of the alga compared to the control. The biomass of the microalga was 2.67 ± 0.01 g/L, 2.12 ± 0.05 g/L, and 1.39 ± 0.01 g/L, with the glucose concentration of 5, 7.5, and 10 g/L, respectively. However, the biomass of the microalgae significantly decreased with an increase in the glucose concentration from 5 g/L to 10 g/L. The addition of glycerol at 5, 15, and 30 g/L showed an increased biomass for day 6 of the cultivation to 0.44 ± 0.08 g/L, 0.83 ± 0.10 g/L, and 1.36 ± 0.03 g/L, respectively. Harvested biomass from the BG–11 enriched with 0.5 g/L of acetate (0.27 ± 0.01 g/L) was not significantly different from the control (0.20 ± 0.07 g/L). The biomass collected from the BG–11 enriched with 5 g/L acetate (0.64 ± 0.05 g/L) was higher than that of acetate at 10 g/L (0.47 ± 0.01 g/L) (p-value < 0.05) as presented in Figure 2. *Chlorococcum* sp. grown by the modified media with sucrose in all concentrations (2, 6, and 10 g/L) showed that the biomass increased after a long lag phase of 7 days.

The IVF usually reflects the potential photochemical efficiency of photosynthesis in microalgae and is directly related to biomass [41]. An optimum concentration of organic carbon sources in the medium, such as glucose, is beneficial to the photosynthetic process and microalgae growth. However, the excessive glucose concentration further inhibits microalgae from performing photosynthetic processes. Lv *et. al.*, [32] described that with the high glucose concentration, the genes coding throughout the tricarboxylic acid cycle (TCA), namely citrate synthase, aconitate hydratase, isocitrate dehydrogenase,

oxoglutarate dehydrogenase, succinate dehydrogenase, and malate dehydrogenase, were notably downregulated. The TCA cycle was significantly blocked by the high concentration of glucose, which resulted in decreased biomass. In conclusion, 5 g/L of glucose was selected for enriching BG–11 medium for further experiments.

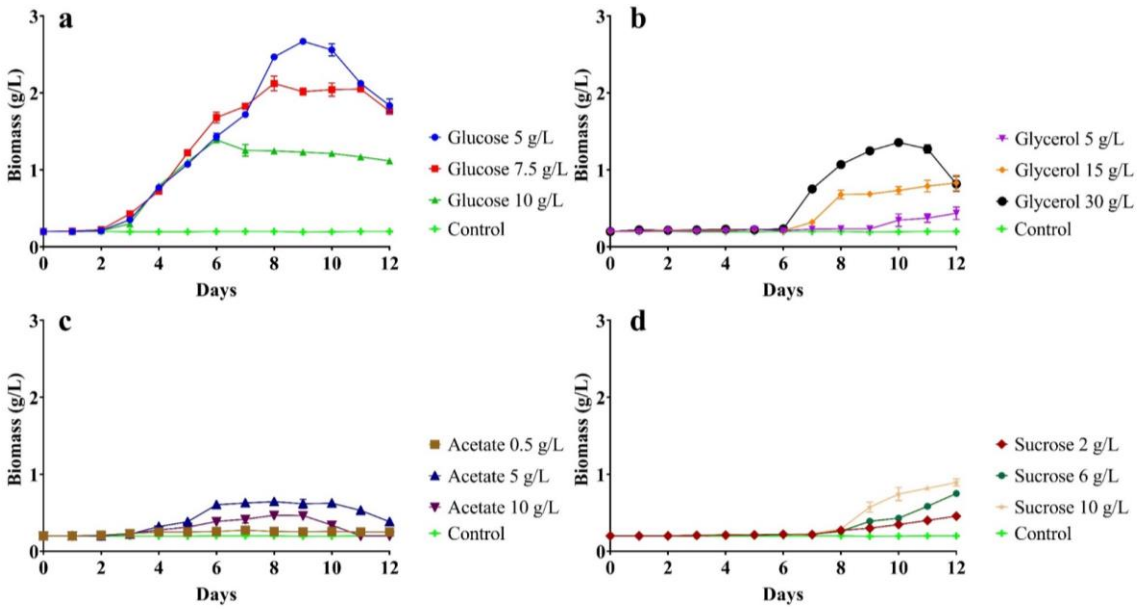

Figure 2: Effect of different concentrations of glucose (a), glycerol (b), acetate (c), and sucrose (d) on biomass production of *Chlorococcum* sp.

3.2 *Effect of different nitrogen sources in BG–11 enriched with glucose*

Since nitrogen is a key component in the formation of amino acids and nucleic acids, which are among the building blocks of DNA and RNA. Nitrogen is necessary for the metabolism of microalgae. Depending on the microalgae strain and the type of nitrogen utilized, nitrogen comprises 1–10% of the protoplasm [42]. Therefore, urea, ammonium chloride, and sodium nitrate at different concentrations were employed in the modified BG–11 with 5 g/L of glucose. The IVF and biomass production under different nitrogen sources and concentrations are depicted in Figures 3 and 4, respectively. Urea addition to the modified BG–11 increased the IVF of *Chlorococum* sp. cultures rather than the use of ammonium chloride and sodium nitrate. However, IVF values and biomass decreased significantly with the increase of urea concentrations from 0.1 to 0.5 and 1.0 g/L (Figures 3(a) and 4(a)). Nevertheless, the IVF from all urea concentrations was still higher than the negative control (BG–11 without modification). Urea is a relatively small organic molecule that can be readily transported across the cell membrane. Many microalgae possess the enzyme urease, which rapidly hydrolyzes urea into ammonium chloride and bicarbonate. The produced ammonium chloride can then be directly assimilated via the GS/GOGAT pathway, similar to the externally supplied ammonium chloride, but potentially at a controlled rate that minimizes toxicity. The bicarbonate produced during urea hydrolysis can also be a readily available inorganic carbon source for photosynthesis, potentially enhancing growth, especially under carbon-limiting conditions [43].

Using ammonium chloride as a nitrogen source with different concentrations (0.5, 1.5, and 3.0 g/L) in *Chlorococcum* sp. significantly decreased IVF from day 3 of cultivation, and the IVF (Figure 3(b)) was also lower than the negative and positive control (BG–11 with glucose 5 g/L). Although ammonium chloride is often a preferred nitrogen source for microalgae growth due to its direct incorporation into amino acids via the glutamine synthetase/glutamate synthase (GS/GOGAT) pathway, high concentrations of

ammonium chloride can be toxic to microalgae, inhibiting growth and photosynthesis. This toxicity arises from the disruption of internal pH gradients and energy metabolism [44], [45]. The ammonium chloride concentrations used in this study were extracted and referenced from similar prior research conducted on other microalgae [33]–[35]. It can be assumed that the employed ammonium chloride was considerably higher than the microalgae required and led to the toxicity on the microalgae growth (Figures 3(b) and 4(b)). Additionally, Figures 3(c) and 4(c) present a slow increase of IVF and biomass because microalgae must first reduce nitrate (NO_3^-) to nitrite (NO_2^-), and then finally to ammonium (NH_4^+) before it can be assimilated into biomass. This reduction process requires energy input (in the form of reduced ferredoxin or NADPH) and the enzymes nitrate reductase and nitrite reductase [46].

The effect of sodium nitrate in modified BG–11 with 5 g/L of glucose showed that all concentrations of sodium nitrate can significantly increase IVF better than the negative control from day 4, but not significantly different when compared with the positive control (Figure 3(c)).

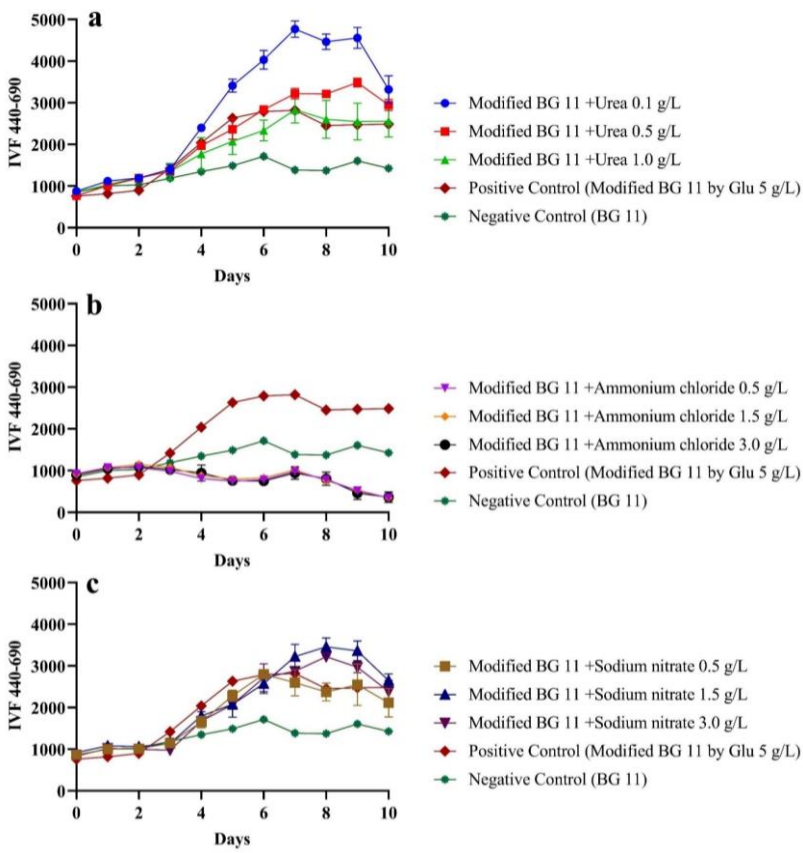

Figure 3: Comparison of different concentrations of urea (a), ammonium chloride (b), and sodium nitrate (c) in modified BG–11 with glucose (5 g/L) on IVF of *Chlorococcum* sp. cultures.

The modified BG–11 with glucose and urea at 0.1, 0.5, and 1.0 g/L (Figure 4a) resulted in the highest biomass production of 4.56 ± 0.28 g/L, 3.88 ± 0.30 g/L, and 3.19 ± 0.26 g/L, respectively, on day 7. Ammonium chloride in all concentrations did not contribute significantly to reaching the higher biomass production than the positive control (Figure 4(b)). For the cultures enriched with 0.5, and 1.5 g/L of sodium nitrate, the biomass production of *Chlorococcum* sp. slowly increased after day 3 and presented the highest concentration of 2.63 ± 0.95 g/L, and 3.26 ± 0.36 g/L, respectively, at day 9, while cultivation with 3.0 g/L of sodium nitrate produced the highest biomass at day 8 (Figure 4(c)).

Some research has shown similar response results using urea to cultivate green microalgae.

According to Erratt *et al*., [47], the authors tracked how three types of freshwater cyanobacteria (*Microcystis*, *Dolichospermum*, and *Synechococcus*) grew and performed photosynthesis when provided with only sodium nitrate, ammonium chloride, or urea as their nitrogen source. The authors found that urea specifically results in greater cell growth and pigment production compared to ammonium chloride or sodium nitrate because urea supplies twice the nitrogen and an extra carbon source, potentially making it more energy-efficient. All three cyanobacterial species grew similarly well on urea and sodium nitrate, but their growth was only half as much on ammonium chloride. However, the cyanobacterial cells contained higher amounts of pigments when grown on urea versus sodium nitrate and ammonium chloride. These results indicate that the extra building blocks from the breakdown of urea were not used only for active growth but instead accumulated, leading to increased production of nitrogen-rich substances like pigments.

Chandra *et al*., [48] found that among all nitrogen sources investigated, urea was the best for *Scenedesmus obtusus*, indicating a two-fold increase in biomass production when compared to the medium containing sodium nitrate as the nitrogen source. However, according to Nayak *et al*., [33], raising urea levels over the ideal threshold will cause an alkalization process because more ammonia will be produced. The stability of the ideal pH will be altered by this alkalization process, which will impede the growth of microalgae. Furthermore, when more than 40 ppm of urea was applied, the stationary phase of the *Clamidomonas* growth curve tended to diminish. The high ammonia levels could result in the microalgae's death [49]. Hence, the results corresponded to previous reports that using urea at optimal concentration would be an excellent choice to enhance the growth and biomass production in many microalgae species.

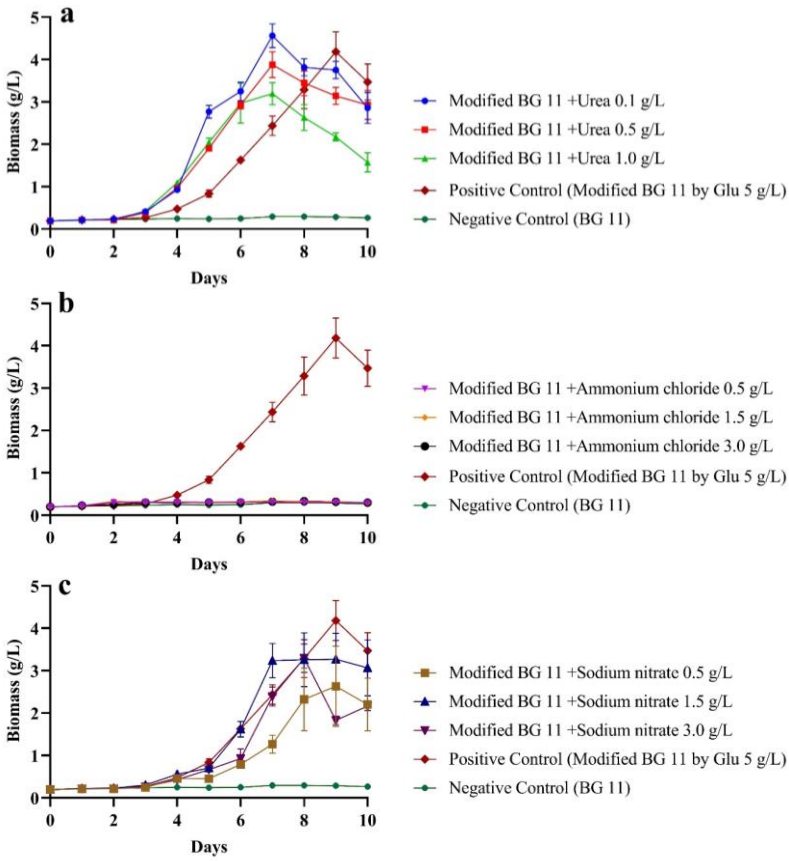

Figure 4: Comparison of different concentrations of urea (a), ammonium chloride (b), and sodium nitrate (c) in modified BG–11 with glucose (5 g/L) on biomass production of *Chlorococcum* sp.

3.3 *Optimization of urea and glucose concentrations via Central Composite Design (CCD)*

Optimization of glucose and urea concentrations for the cultivation of *Chlorococcum* sp. performed by CCD with the biomass production at the late log phase of the cultivation, is presented in Table 2. The experimental results of the CCD were fit to a second-order polynomial as shown in Equation (5):

$$Y = 3.803 + 0.062X_1 + 13.889X_2 - 0.903X_1X_2 - 0.033X_1^2 - 39.600X_2^2 \quad (5)$$

Based on the coefficient of determination, or R^2, model Y's fitness was assessed. It was 0.9413, meaning that the model could account for 94.13% of the response's variability (Table 3). At the 99% confidence level, this regression was statistically significant (p-value < 0.0001), according to an F-test ANOVA used to assess the model equation's statistical significance. The model's regression coefficient's significance is presented in Table 3, which reveals that urea (X_2; p-value = 0.0409) and glucose (X_1; p-value < 0.0001) significantly impacted biomass production. At the 99% confidence level, the impact of the glucose (X_1) and urea (X_2) interaction was significant (p-value = 0.0105). Figure 5 represents the response surface plot described by model Y. Table 2 shows the effect of modification of BG–11 media with glucose and urea on biomass production. The maximum biomass was not significantly different (p-value < 0.05) when comparing treatment 3 (4.73 ± 0.03 g/L) and treatment 5 (4.81 ± 0.04 g/L). However, the calculation using Design-Expert software showed the maximum biomass of *Chlorococcum* sp. was approximately 4.8 g/L using the optimal concentrations of glucose at 1.6 g/L, and urea at 0.16 g/L. Therefore, the optimal concentrations of glucose and urea were selected for the modified BG–11 medium for the next experiment, as choosing the most efficient and cost-effective organic carbon and nitrogen sources is crucial.

Table 2: Central composite experimental design and biomass production at the late log phase of cultivation.

Treatment	Coded Values		Real Values		Response
	X_1	X_2	X_1, Glucose (g/L)	X_2, Urea (g/L)	Y, Biomass Production (g/L)
1	–1	–1	1.59	0.08	4.6 ± 0.11^{ab}
2	+1	–1	4.41	0.08	3.94 ± 0.25^{cd}
3	–1	+1	1.59	0.22	4.73 ± 0.03^{a}
4	+1	+1	4.41	0.22	3.71 ± 0.05^{d}
5	–α	0	1.00	0.15	4.81 ± 0.04^{a}
6	+α	0	5.00	0.15	3.80 ± 0.19^{d}
7	0	–α	3.00	0.05	4.15 ± 0.06^{c}
8	0	+α	3.00	0.25	3.94 ± 0.12^{cd}
9	0	0	3.00	0.15	4.47 ± 0.22^{b}
10	Positive control (BG–11 with glucose 5 g/L)				2.46 ± 0.14^{e}
11	Negative control (Normal BG–11)				0.55 ± 0.01^{f}

Values are the average ± standard deviation of triplicates
Different superscript letters in the same column correspond to significant differences (p-value ≤ 0.05)

Table 3: Analysis of variance for the experimental results of the central composite design (CCD).

Source	Sum of Squares	DF	Mean Squares	F-value	p-value
X_1	3.63	1	3.63	292.46	< 0.0001
X_2	0.0589	1	0.0589	4.75	0.0409
X_1X_2	0.0979	1	0.0979	7.89	0.0105
X_1^2	0.0396	1	0.0396	3.19	0.0886
X_2^2	0.3421	1	0.3421	27.58	< 0.0001
Model	4.18	5	0.8356	67.35	< 0.0001*
Error	0.1901	18	0.0106		
Total	4.44	26			
R^2 = 0.9413			Adj-R^2 = 0.9273		

*Statistically significant at a probability level of 99%

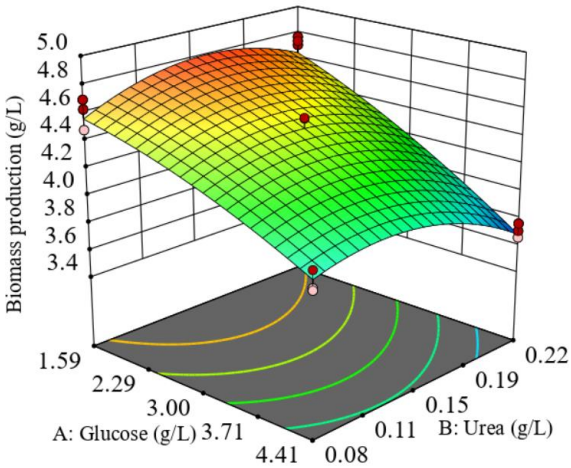

Figure 5: Response surface plot from the experimental results of the central composite design (CCD) represents the effect of glucose and urea on biomass production.

3.4 *Cultivation of Chlorococcum* sp. *in modified BG–11 under different light modes*

The optimal light mode for microalgae cultivation depends on various factors, including desired product (biomass, pigments, etc.), specific microalgae strain, and economic considerations [50], [51]. The effect of different light modes, including mixotrophic and heterotrophic cultivations under aeration and agitation, was investigated with the modified BG–11 medium. Figure 6(a) presents the IVF values from different light modes in modified BG–11 medium. The IVF under mixotrophic cultivation with aeration was not significantly different when compared with mixotrophic cultivation with agitation (approximately 5600). However, the IVF under mixotrophic cultivation with aeration and mixotrophic cultivation with agitation were significantly higher than both conditions under heterotrophic cultivation under aeration and agitation (p-value ≤ 0.05).

Figure 6(b) shows the absorbance at 680 nm, representing the microalgal cell accumulation. The absorbance at 680 nm from different light modes with modified BG–11 medium was consistently higher than the control at the same starting condition until 7 days of cultivation. The absorbance of both mixotrophic cultivation with aeration (2.34 ± 0.08) and agitation (2.35 ± 0.07) was higher than that of heterotrophic with agitation (2.10 ± 0.01), with aeration (1.68 ± 0.08), and control (0.73 ± 0.02). Figure 6(c) represents the biomass production of *Chlorococcum* sp. cultivated in modified BG–11 under different light modes. The biomass production of aerated and agitated cultures of *Chlorococcum* sp. under heterotrophic mode expeditiously increased 24 h after inoculation, compared to both conditions under the mixotrophic mode.

Along with the light, simultaneously adding an external nutrient source, e.g., carbon, nitrogen, or phosphorus, follows various patterns depending on each species' nutrient requirements and metabolism. For instance, nitrogen is a main factor in microalgae growth and the augmentation of metabolites [52]–[54]. Similar to the current study, Mohamadnia *et al.* [16], [17] also found that the production of biomass was considerably enhanced under optimized mixotrophy culture enriched with sodium nitrate when compared to the phototrophic culture. Besides, the optimized ratio of carbon to nitrogen in the microalga growth media also increased the biomass production and fucoxanthin in *Tisochrysis lutea*.

Another important nutrient was glucose, the organic carbon source, leading to enhanced growth and biomass production under mixotrophic cultivation than heterotrophic conditions. Glucose consumption in the modified BG–11 medium was analyzed by HPLC, and the results were presented in Figure 7. Under all conditions, the glucose concentrations decreased after 24 h and were less than 0.5 g/L after day 4. Therefore, glucose addition on day 4 or 5 of cultivation might be an approach for further boosting the microalgal growth in the fed-batch process. Moreover, the decrease of glucose in the medium means the whole carbon source has been converted to biomass (Figure 6(c)). Interestingly, *Chlorococcum* sp. can grow in heterotrophic conditions where photosynthesis is impossible, demonstrating that glucose as an energy source can replace photosynthesis and promote the growth of green microalgae [55].

Finally, the kinetic parameters of the *Chlorococcum* sp. growth were calculated and are demonstrated in Table 4. According to Table 4, the cells grew fastest at mixotrophic cultivation with aeration among all tested conditions. They reached the highest biomass production, specific growth rate (μ), and division per day of 0.90 ± 0.01 g/L/d, 0.65 ± 0.01 day^{-1}, and 0.94 ± 0.01, respectively. In addition, mixotrophic cultivation with aeration also showed the lowest doubling time of 1.07 ± 0.02 days.

In summary, applying modified BG–11 medium for the growth of the *Chlorococum* sp., under

mixotrophic conditions with glucose (1.6 g/L), and urea (0.16 g/L), resulted in the maximum biomass production of 4.90 ± 0.14 g/L and 4.85 ± 0.07 g/L with aeration and agitation, respectively. Interestingly, *Chlorococum* sp. could grow under heterotrophic conditions with aeration (4.30 ± 0.00 g/L) and agitation (4.25 ± 0.07 g/L). Moreover, the use of modified BG–11 medium showed higher biomass than the control (1.90 ± 0.00 g/L). Therefore, under optimized medium and conditions, the microalgae biomass and cellular metabolites can significantly increase.

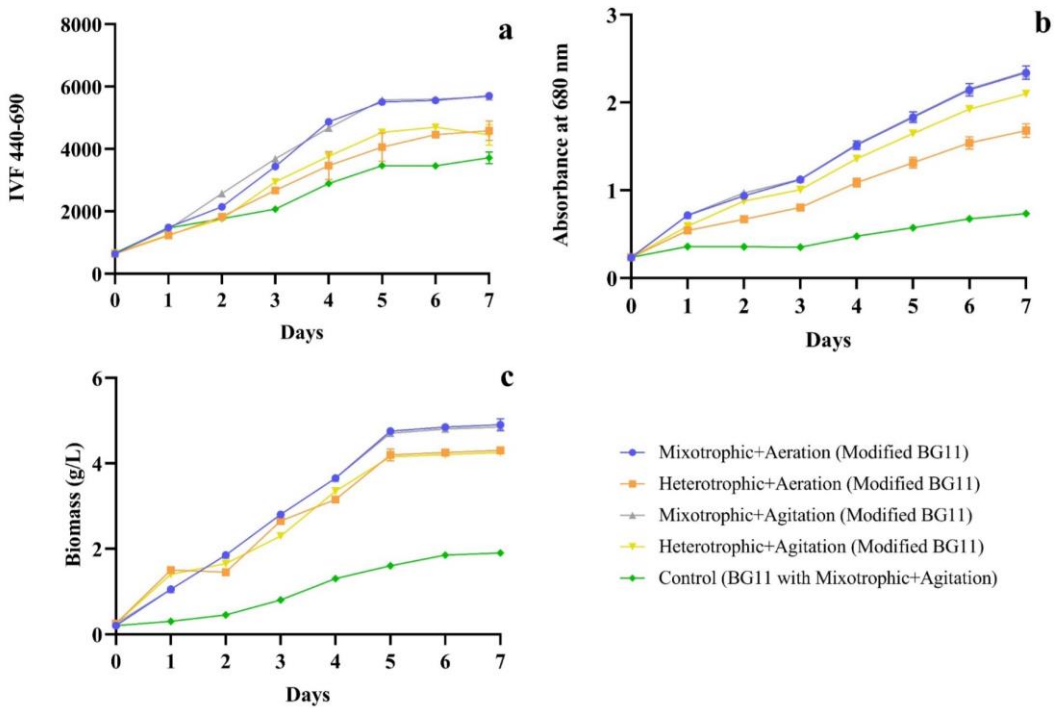

Figure 6: Effect of different light modes with modified BG–11 medium on IVF (a), absorbance at 680 nm (b), and biomass production (c) of *Chlorococcum* sp.

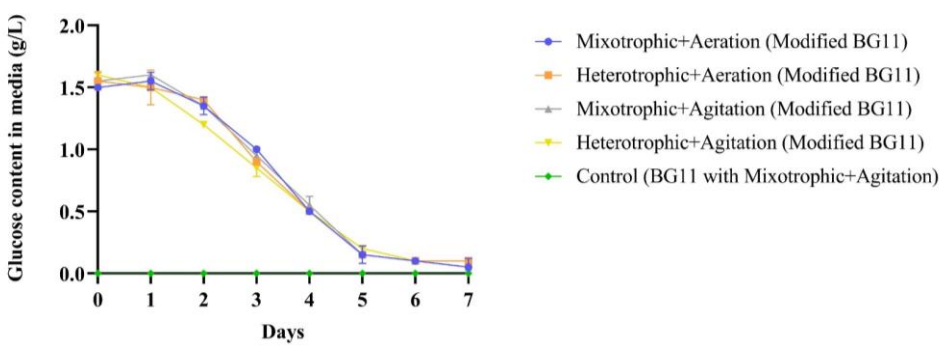

Figure 7: Glucose consumption profile of *Chlorococcum* sp. cultivated in the modified BG–11 under different light modes.

Table 4: Effect of different light modes with modified BG–11 on the microalgal growth rate.

Treatment	Biomass Productivity (g/L/day)	Specific Growth Rate (day^{-1})	Division per Day (Dd)	Doubling Time, td (day)
Control	0.28 ± 0.01^{c}	0.42 ± 0.03^{c}	0.60 ± 0.04^{c}	1.67 ± 0.01^{a}
Mixotrophic + Aeration	0.90 ± 0.01^{a}	0.65 ± 0.01^{a}	0.94 ± 0.01^{a}	1.07 ± 0.02^{c}
Heterotrophic + Aeration	0.79 ± 0.03^{b}	0.57 ± 0.03^{b}	0.82 ± 0.04^{b}	1.23 ± 0.00^{b}
Mixotrophic + Agitation	0.89 ± 0.01^{a}	0.59 ± 0.03^{ab}	0.85 ± 0.04^{ab}	1.09 ± 0.06^{c}
Heterotrophic + Agitation	0.79 ± 0.01^{b}	0.58 ± 0.02^{b}	0.83 ± 0.04^{b}	1.21 ± 0.03^{b}

Values are the average and standard deviation of triplicates
Different letters in the same column differ significantly by Duncan's test (p-value ≤ 0.05)

3.5 *Optimization of the β-cryptoxanthin production by Chlorococcum* sp.

The chlorophyll *a*, chlorophyll *b*, and total carotenoid content of *Chlorococcum* sp. under different light modes with a modified BG–11 medium are presented in Figure 8. The three studied conditions of mixotrophic, including mixotrophic with aeration, mixotrophic with agitation, and the control (mixotrophic with agitation), showed chlorophyll *a* of 106.42 ± 0.29, 110.98 ± 1.44, and 113.51 ± 1.70 mg/g DCW, respectively. Among the studied conditions, the control (BG–11 medium without modification) was found to produce higher chlorophyll *b* (68.86 ± 0.38 mg/g DCW) than the other tested light modes. Total carotenoid content under mixotrophic with aeration and mixotrophic with agitation was not significantly different, indicating 99.88 ± 0.75 and 99.12 ± 0.75 mg/g DCW, respectively. On the other hand, total carotenoid content under both mixotrophic conditions was significantly higher than the control (78.81 ± 0.65 mg/g DCW), heterotrophic with aeration (22.97 ± 0.22 mg/g DCW), and heterotrophic with agitation (24.27 ± 1.83 mg/g DCW).

β-cryptoxanthin, lutein, and β-carotene content of *Chlorococcum* sp. are presented in Figure 9. β-cryptoxanthin content in the microalga that grew under mixotrophic cultivation with aeration (3.44 ± 0.10 mg/g DW) was not significantly different from mixotrophic cultivation with agitation (4.02 ± 0.49 mg/g DW), and the control (3.09 ± 0.95 mg/g DW). However, those mixotrophic conditions showed a higher β-cryptoxanthin content than heterotrophic cultivation with aeration (1.53 ± 0.59 mg/g DW), and heterotrophic with agitation (1.44 ± 0.80 mg/g DW). Mixotrophic with aeration (12.91 ± 0.44 mg/g DW) and mixotrophic with agitation (12.76 ± 0.26 mg/g DW) presented a higher lutein content than the heterotrophic cultivation with aeration and heterotrophic cultivation with agitation. Additionally, the lutein content of microalgae from all light modes tested using the modified BG–11 medium was significantly higher than the control (5.73 ± 1.33 mg/g DW). Interestingly, *Chlorococcum* sp. was grown by using modified BG–11 medium with different light modes, reported a non-significant difference of β-carotene content (p-value ≥ 0.05).

In addition, Figures 8 and 9 show that microalgae can produce biomass and pigments in the mixotrophic cultures better than in heterotrophic cultures. The significantly higher biomass observed under mixotrophic conditions with aeration suggests a synergistic effect where the availability of both light energy and organic carbon, coupled with enhanced oxygen supply for respiration, provided the cells with abundant resources for growth and cell division [52], [56]. The result aligns with the studies by Licata *et. al.*, [57] that photosynthetic microorganisms, especially microalgae, are impressive because they can use sunlight and carbon dioxide to create various useful compounds. Microalgae are important for fighting climate change as they absorb carbon dioxide and produce valuable substances. Among different ways to grow them, mixotrophic growth is a special method that uses both light and inorganic and organic carbon, which can help increase their growth and their bioactive compounds. The lower pigments production in heterotrophic cultures could be attributed to the downregulation of photosynthetic pathways when light is absent, as pigments are often associated with light-harvesting complexes [58]. The positive impact of agitation on biomass in phototrophic cultures likely resulted from improved light penetration and nutrient distribution, ensuring a more homogenous environment for cell growth. However, excessive agitation in some studies has been reported to cause shear stress, which should be considered. The interaction between aeration and the carbon and nitrogen source was evident in our results, in which aeration had a more pronounced positive effect on biomass under heterotrophic conditions. This could be due to the increased reliance on oxidative phosphorylation for ATP production when light energy is not available [59], [60].

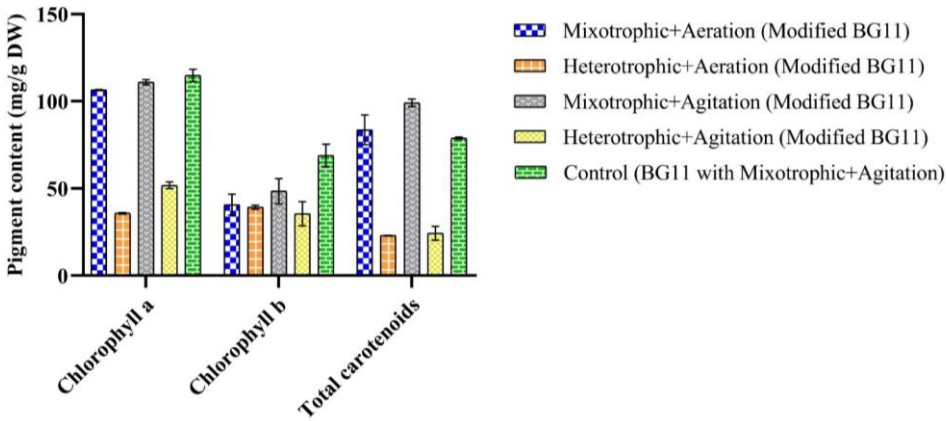

Figure 8: Effect of different light modes with modified BG–11 on pigment production.

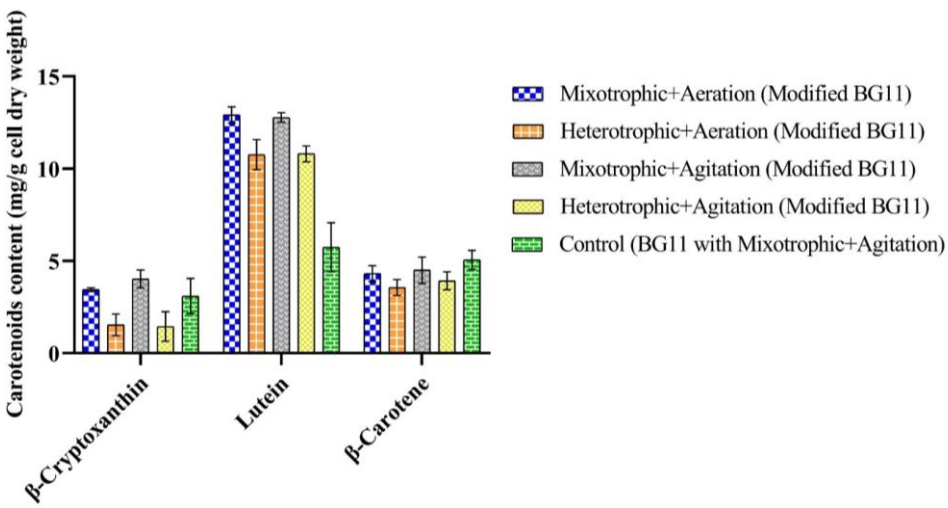

Figure 9: Effect of different light modes with modified BG–11 on β-cryptoxanthin, lutein, and β-carotene production.

4 Conclusions

In this study, the optimal conditions for biomass production and the content of β-cryptoxanthin, lutein, and β-carotene of *Chlorococcum* sp. were improved by microalgae cultivation using the modified BG–11 medium with 1.6 g/L of glucose and 0.16 g/L of urea and applying between mixotrophic and heterotrophic conditions. Employing optimum concentrations of glucose and urea plays an important role in biomass production. Moreover, mixotrophic with aeration (filtered air was provided at a rate of 2.5 L/min and 0.5% of CO_2), a growth offers promising strategies for enhancing pigment production in microalgae higher than non-modified BG–11 medium. However, consideration of the benefits, challenges, and specific needs of the microalgae strain and desired pigment is crucial for successful implementation.

These results demonstrated that the use of mixotrophic conditions in combination with BG–11 medium supplemented with glucose and urea certainly improved the production of biomass and pigment, especially β-cryptoxanthin. Our future research will employ microalgae as a novel carotenoid manufacturing source, specifically for β-cryptoxanthin. The sustainability issue will be resolved, and this methodology will support the objectives of developing environmentally responsible and commercially feasible procedures. Further research will be conducted on the other factors that impact carotenoids, specifically beta-cryptoxanthin, such as temperature and salinity. However,

considering that *Chlorococcum* sp. biomass can be used for β-cryptoxanthin production, the cost of the growth medium, which accounts for a significant part of the β-cryptoxanthin production costs, should be improved for large-scale production.

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Author Contributions

S.C.: conceptualization, investigation, reviewing and editing; V.R.: funding acquisition, methodology, data curation, writing an original draft, reviewing and editing; S.M.: research design, data analysis; I.A.: conceptualization, data curation, writing, reviewing and editing, funding acquisition, project administration. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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