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Research Article **Araştırma Makalesi**

Growth and Biochemical Responses of *Haematococcus pluvialis* to Different Arginine Concentrations under Red LED Light

Gökçe Kendirlioğlu Şimsek[1,*], Ahmet Kadri Çetin[1]

[1]Fırat University, Faculty of Science, Departmant of Biology, Elazığ-TÜRKİYE

*Corresponding author: gksimsek@firat.edu.tr

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Abstract: *Haematococcus pluvialis* is a microalgal species of considerable biotechnological interest due to its outstanding capacity for natural astaxanthin production. However, one of the major constraints limiting astaxanthin productivity in this organism is its relatively slow growth and the resulting low biomass yield. In this study, the time-dependent effects of different concentrations of exogenous L-arginine (0.1, 0.5, and 1.0 mM) on the growth performance and biochemical composition of *H. pluvialis* cultures were investigated under red LED illumination. Cultures were maintained for 10 days under a 16/8 h light–dark photoperiod at an irradiance of 3000 lux, and optical density (OD_{680}) as well as the contents of protein, lipid, astaxanthin, chlorophyll a, and chlorophyll b were determined. The results indicated that red light alone promoted cellular growth and metabolite accumulation only to a limited extent compared with the control. In contrast, supplementation with higher arginine concentrations markedly enhanced growth and substantially altered the biochemical profile of the cultures. The highest optical density and the greatest accumulation of protein, lipid, and astaxanthin were observed in cultures treated with 1.0 mM L-arginine under red light. Similarly, chlorophyll-a and b contents increased in an arginine concentration-dependent manner. Overall, these findings demonstrate that L-arginine supplementation in combination with red-wavelength illumination represents an effective and feasible strategy to improve both biomass formation and, in particular, astaxanthin production in *H. pluvialis*. This approach may contribute to the development of more sustainable and economically viable cultivation systems for natural astaxanthin production.

Keywords
- Microalgae
- Arginine
- Astaxanthin
- Red light

1. INTRODUCTION

Microalgae are among the most remarkable microorganisms due to their photosynthetic capacity to convert light and carbon dioxide into biomass, their rapid growth rates, and their broad ecological adaptability. Distributed across a wide range of habitats (including freshwater and marine ecosystems, soil surfaces and semi-terrestrial microenvironments) these algal groups possess a rich biochemical composition containing vitamins (vitamin A, B-complex vitamins and vitamin C), essential amino acids, antioxidant compounds, polyunsaturated fatty acids (PUFAs), minerals and valuable pigments such as chlorophyll and carotenoids (Bai et al., 2001; Azaman et al., 2017; Akter et al., 2021). This metabolic diversity enables microalgae to be utilized not only as nutritional ingredients and dietary supplements, but also as sustainable biomass sources for various sectors, including biofuels, cosmetics, pharmaceuticals and bioenergy production (Sandnes et al., 2005; Becker, 2007; Kar et al., 2008; Fathi et al., 2013). Although microalgal biomass productivity and biochemical profiles are strongly species-dependent, they are also shaped by multiple environmental parameters such as light, temperature, CO_2 availability, culture medium composition and pH (Li et al., 2020). Among these factors, light intensity, spectral quality and wavelength play central roles in regulating and activating key biochemical pathways throughout the growth cycle of microalgae (Pereira & Otero, 2020; Li et al., 2020). Recent studies have

consistently demonstrated that red and blue light wavelengths can markedly influence both growth and pigment accumulation in various algal species and strains (Li et al., 2023; Lemoine & Schoefs, 2010; Oslan et al., 2021). Das et al. (2011) reported that blue, white, green and red wavelengths exert distinct effects on the growth parameters of *Nannochloropsis* species. Similarly, under nutrient-replete conditions, high-intensity red light significantly enhanced carotenoid accumulation in *Dunaliella salina*, whereas high-intensity blue light supported growth rates comparable to those under red or white light but did not promote carotenoid accumulation (Xu & Harvey, 2019).

In contrast, another study indicated that red light is more favorable for the growth of *Haematococcus pluvialis*, while blue light preferentially supports astaxanthin accumulation (Xi et al., 2016). *H. pluvialis* is a unicellular freshwater green microalga with a broad distribution worldwide and is particularly distinguished by its ability to synthesize and store high levels of astaxanthin under environmental stress (Li et al., 2023). Astaxanthin is a lipophilic carotenoid characterized by a polyene chain structure and is widely recognized for its strong antioxidant activity (Sung & Sim, 2022). Unlike other common antioxidants such as β-carotene, vitamin C, and vitamin E, astaxanthin can localize across the lipid bilayer of cell membranes, thereby providing protection against oxidative processes both inside and outside the cell (Yu et al., 2021). This unique biophysical property allows astaxanthin to effectively suppress cellular damage induced by reactive oxygen species. In line with its high antioxidant capacity, astaxanthin has been reported to exhibit anticancer potential and to exert beneficial effects on liver function, skin health and the cardiovascular system (Hwang et al., 2019). These multifunctional biological properties have led to a rapidly growing demand for astaxanthin in the food, pharmaceutical, cosmetic and feed industries (Hong et al., 2018). Accordingly, *H. pluvialis* is regarded as one of the most important biological systems for the production of naturally derived astaxanthin (Ambati et al., 2014). This microalgal species is characterized by two distinct developmental stages that differ in both metabolic and morphological traits: a green (autotrophic) stage associated with rapid cell proliferation and a red cyst stage in which astaxanthin accumulation becomes pronounced. Under adequate nutrient supply and appropriate light regimes, temperature and pH, biomass production increases substantially during the green stage; however, upon exposure to environmental stressors, cells transition into the cyst stage and shift toward dominant astaxanthin synthesis (Mularczyk et al., 2020).

In this context, given the decisive influence of environmental conditions on both cell growth and pigment biosynthesis, the development of suitable cultivation strategies is essential to achieve the desired biomass or astaxanthin yield (Hagen et al., 2001).

Arginine (L-arginine) is an essential amino acid with a high nitrogen-to-carbon ratio and plays multifaceted roles in plant and microalgal metabolism. It serves as a major precursor for the biosynthesis of stress-related compounds such as polyamines (PAs), proline and notably nitric oxide (NO) (Liu et al., 2006; Zeid, 2009). Arginine can enhance intracellular NO production by activating nitric oxide synthase (NOS) and NO has been shown to upregulate the expression of genes associated with astaxanthin biosynthesis in *H. pluvialis* (Ding et al., 2018). Moreover, arginine supplementation is known to strengthen antioxidant enzyme activities, including catalase (CAT) and superoxide dismutase (SOD), thereby alleviating oxidative stress and supporting cellular survival (Karpets et al., 2018). Considering the strong antioxidant capacity of astaxanthin, the ability of arginine to reinforce antioxidant defense mechanisms may indirectly promote pigment production. Despite being regarded as a “trifunctional” metabolite due to its simultaneous roles in proline, polyamine and NO synthesis, the regulatory effects of arginine on biomass accumulation and astaxanthin biosynthesis in *H. pluvialis* remain insufficiently explored.

The primary aim of this study is to optimize the metabolic responses of *H. pluvialis* to enhance the biotechnological production of high value-added bioactive compounds. Today, naturally derived astaxanthin has gained strategic importance in the health, pharmaceutical and cosmetic industries owing to its potent antioxidant properties. In addition, microalgae-derived lipids offer considerable potential for sustainable energy production through biofuel technologies, while the high protein content of microalgae provides an alternative and environmentally sustainable protein source for the food and feed sectors.

Within this framework, the present study investigates the combined effects of red LED light and different arginine concentrations on cellular growth, pigment production, energy-storing lipid accumulation, and protein synthesis that contributes to nutritional value in *H. pluvialis* cultures. The findings are expected to clarify how the red light and arginine interaction directs cellular metabolic pathways. Ultimately, this approach may not only contribute to improving astaxanthin production efficiency but also offer a strategic perspective for advancing microalgae-based applications in food, health and energy biotechnology.

2. MATERIAL AND METHODS

2.1. Strain and Culture Conditions

The microalgae *H. pluvialis* was obtained from the stock cultures of the Algal Biotechnology Laboratory at Fırat University. Cultivation was carried out in BG11 medium at 23 ± 1 °C under a 16:8 h light/dark photoperiod with an illumination intensity of 3000 lux (≈ 45–55 µmol photons m^{-2} s^{-1}) using an incubator (Nüve ES 120/252). The BG11 medium was prepared according to the standard formulation by Allen and Stanier (1968). The prepared medium was sterilized by autoclaving at 121 °C and 15 min. After sterilization, the medium was allowed to cool to room temperature Experimental cultures were prepared in 500 mL volumes by inoculating with the stock vegetative phase of *H. pluvialis* cultures at a 1:1 (v/v) ratio.

2.2. Preparation of Arginine Stock Solution and Supplementation to The Culture Medium

L-arginine (Merck, Germany) was used for the preparation of the stock solution. A total of 1.742 g of arginine was dissolved in 100 mL of sterile distilled water to obtain the stock solution.

Since arginine cannot be autoclaved and to avoid compromising the sterility of the culture medium, the stock solution was sterilized using a 0.22 µm membrane filter under aseptic conditions. Following the sterilization of the culture medium, arginine was added aseptically to obtain final concentrations of 0.1 mM, 0.5 mM and 1.0 mM in the experimental flasks.

2.3. Red LED Illumination Set Up

Red light was selected based on previous studies demonstrating its significant effects on microalgal growth, pigment accumulation and protein content (Kendirlioglu & Cetin, 2017). A dedicated LED illumination system was established to provide controlled red light conditions for the experiment in incubator. The setup consisted of red LED light emitting within the 660–680 nm wavelength range and the light intensity was calibrated to 3000 lux (≈ 45–55 µmol photons m^{-2} s^{-1}) using a digital internal lux meter. Timer programmed to maintain a 16 h light / 8 h dark photoperiod, ensuring a consistent and automatic illumination cycle throughout the experiment in incubator **(**Nüve ES 120/252). The ambient temperature was maintained at 23 ± 1 °C. The Erlenmeyer flasks were positioned at equal distances from the light source by surrounding them with the LED strips and each flask was gently rotated daily to maintain uniform light exposure. A total of five 500 mL Erlenmeyer flasks were used in the experimental design. The first flasks served as the control group, containing *H. pluvialis* cultured in BG11 medium without any treatment and was incubated under 3000 lux white light with a 16/8 h photoperiod. The second flasks was also prepared with BG11 medium but incubated under 3000 lux red LED light with the same photoperiod, allowing evaluation of the independent effect of red light on *H. pluvialis*. In the third, fourth, and fifth flasks, arginine was aseptically added to the BG11 medium to obtain final concentrations of 0.1 mM, 0.5 mM and 1.0 mM, respectively. These cultures were incubated under the same conditions as the second flask 3000 lux red LED illumination and 16/8 h light/dark photoperiod.

2.4. Biochemical Analysis

2.4.1. Optical Density Measurements

For optical density readings of *H. pulivialis* cultures were mixed homogeneously and 2 ml of each sample was transferred to quartz cuvette using a micropipette. UV/VIS spectrophotometer (Isolab uv/vıs spectrophotometer) readings at 680 nm wavelength were taken for 10 days. Measurements were performed in triplicate and the average absorbance values were recorded. The OD_{680} value was not converted into biomass.

2.4.2. Total Protein Assay

Total protein content was determined using a Total Protein Assay Kit (Sigma Aldrich TP0300). For this purpose, 1 mL of sample was treated with 100 µL of deoxycholate solution and 100 µL of trichloroacetic acid solution, followed by centrifugation. Subsequently, 1 mL of Lowry reagent was added, and the mixture was incubated for 20 minutes. After this step, 1 mL of Folin–Ciocalteu reagent was added, and the samples were further incubated for 30 minutes. Finally, absorbance measurements were performed at 750 nm using a UV/VIS

spectrophotometer (ISOLAB) to determine total protein content (Lowry et al., 1951).

2.4.3 Determination of Chlorophyll-a and b

Chlorophyll content was determined using methanol extraction method. 2 mL sample was centrifuged at 2000 rpm for 10 minutes. The resulting pellet was treated with 2 mL of 99.9% methanol (Merck) and incubated in the dark at 45°C for 24 hours. Following incubation, samples were centrifuged again at +4°C (Nüve NF 800R), and the supernatant was collected for absorbance measurements at 665 nm and 652 nm (Lichtenthaler, 1987).

Pigment quantity calculations were performed using the following formulas (1-2):

Chlorophyll-a: Chl-a (mg L−1) = 16.72 $OD_{665.2}$ *− 9.16* $OD_{652.4}$ *(1)*

Chlorophyll-b: Chl-b (mg L−1) = 34.09 $OD_{652.4}$ *− 15.28* $OD_{665.2}$ *(2)*

2.4.4. Total Lipid Content

5 ml of sample was mixed with Methanol-Chloroform, and then 2 ml of 0.4% CaCl2 was added to the mixture. After filtration through filter paper (Scleicher & Schuell, 125 mm), the filtered samples were kept in an incubator at 105°C for 2 hours in a dark environment for 24 hours. Afterward, the samples were placed in a hot water bath (Isolab) at 80°C and kept in the incubator for 1 hour at 90°C to evaporate all the chloroform (Bligh and Dyer,1959). The flask was pre-weighed. Once cooled, the tubes were weighed using a sensitive balance and lipid quantities were calculated using the equation (3);

Lipid content (%) = [Weight of Flask + Lipid (g) - Weight of Flask (g)] × 100 / Sample Weight (g) (3)

2.4.5. Astaxanthin Analysis

5 ml of sample was centrifuged for 10 minutes and then 5 ml of 5% KOH and 30% methanol (v/v) were added to the pellet and incubated at 65°C for 15 minutes. Afterward, 5 ml of Dimethyl sulfoxide (DMSO) was added, and sonication was carried out for 10 minutes at 3.0 kHz using an Ultrasonic Homogenizer (Bandelin HD2070) until coloration was observed. This process was repeated until coloration was visible (Boussiba and Vonshak, 1991). Samples were then read at 490 nm wavelength using spectrophotometer and the concentration (c) was calculated using the formula(4):

c (mg L-1) = $4.5 \times OD_{490} \times Va/Vb$ *(4)*

Va = The volume of extracts

Vb=The volume of culture simple (Davies,1976).

2.5. Statistical Analysis

All experiments were performed in triplicate and data are presented as mean ± SD. Pearson correlation heatmap is an analytical approach that visualizes the direction and strength of the linear relationship between two continuous variables based on the Pearson correlation coefficient (r). The Pearson correlation coefficient ranges from −1 to +1, where positive values indicate that the variables tend to increase together, whereas negative values reflect an inverse relationship. This method enables a comprehensive and comparative evaluation of associations among quantitative datasets, including optical density and biochemical parameters. All correlation heatmaps were generated using Matplotlib (version 3.7.4).

3. RESULTS

3.1. Growth Characteristics of *H. pluvialis* Under Red Light and Arginine Supplementation

The optical density (OD_{680}) values measured in *H. pluvialis* cultures demonstrated that both the applied light conditions and arginine supplementation played a decisive role in cellular growth. In the control group, the OD_{680} value increased steadily from an initial 0.409 to 0.975 by day 10 of incubation. Under red light, OD_{680} rose from 0.407 to 0.996, indicating a slightly enhanced growth profile compared to the control. Notably, the growth response under red light became markedly stronger with arginine supplementation.

In particular, the group exposed to red light and supplemented with 0.5 mM arginine reached an OD_{680} of 1.772 at the end of day 10, clearly exceeding the value obtained under red light alone. This finding highlights the growth-promoting effect of arginine on cell proliferation. The highest growth performance was observed in cultures cultivated under red light with 1.0 mM arginine supplementation.

In this group, which started at an OD_{680} of 0.405, a rapid and continuous increase was recorded throughout the incubation period, reaching 2.177 on day 10, the highest value among all experimental groups (Figure 1). Overall, these results indicate that the combination of red light and 1.0 mM arginine most effectively enhanced *H. pluvialis* cell proliferation.

In general, OD_{680} values increased significantly in parallel with rising arginine

concentration, with the lowest growth detected in the control group and the highest growth in the red light treatment supplemented with 1.0 mM arginine. Collectively, these findings suggest that arginine, particularly at higher doses, acts as a strong metabolic regulator that promotes *H. pluvialis* growth.

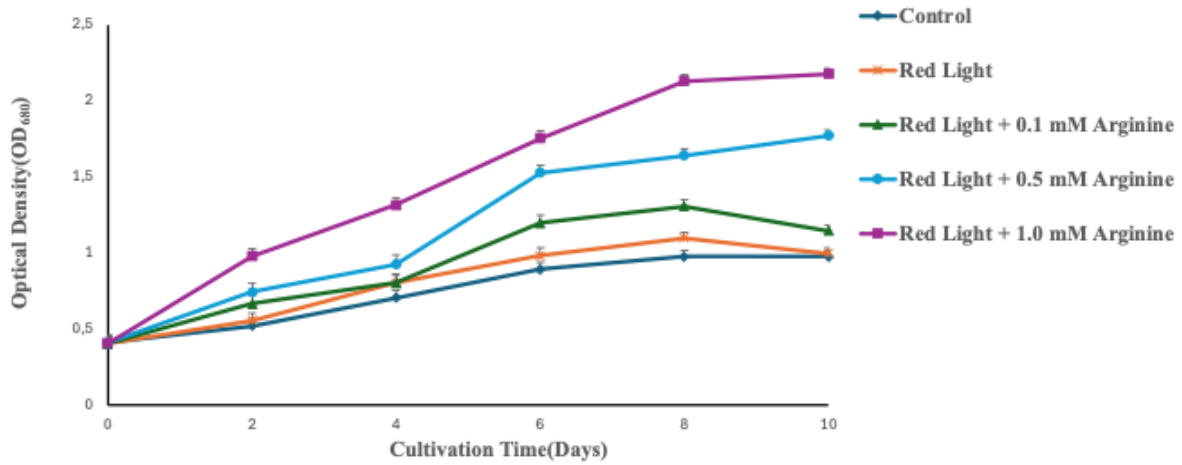

Figure 1. Optical density (OD_{680}) response of *H. pluvialis* under red light and arginine supplementation over 10 day cultivation period.

Correlation heatmap analysis was employed to quantitatively evaluate the relationships among growth trends induced by different light and arginine treatments in *H. pluvialis* cultures. The Pearson correlation heatmap revealed that the associations between optical density (OD_{680}) values across cultures grown under different light and arginine conditions were predominantly strong and positive.

In particular, a very strong positive correlation was observed between the control group and the red light–treated cultures ($r \approx 0.97–0.99$), indicating that red light maintained a growth pattern over time that was largely comparable to the control conditions. Similarly, high correlation coefficients were recorded among the arginine-supplemented groups ($r > 0.95$), suggesting that the time-dependent increase in growth followed a shared dynamic across these cultures. However, the culture incubated under red light with 1.0 mM arginine exhibited relatively lower correlation coefficients with the other groups ($r \approx 0.90–0.93$), indicating that its growth response diverged more clearly and followed a distinct trajectory. This pattern suggests that high-dose arginine exerts a stronger and more specific effect on cellular proliferation (Figure 2).

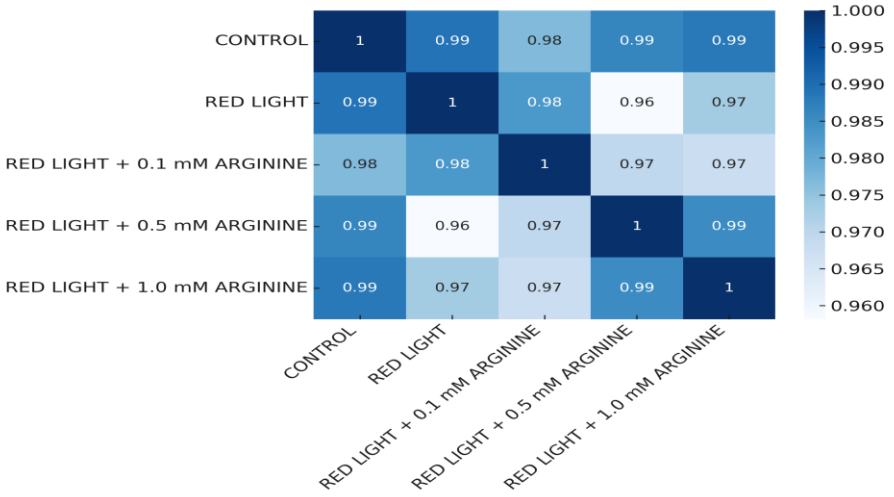

Figure 2. Pearson Correlation Heatmap of OD_{680} amounts Under Different Light and Arginine Treatments.

The high positive correlation values indicate that growth increased consistently over time across all experimental groups and that the light–arginine treatments influenced growth kinetics in the same direction, albeit with different magnitudes.

Notably, the partially divergent correlation profile observed for the 1.0 mM arginine treatment further supports, from a statistical perspective, that this condition most strongly promoted *H. pluvialis* growth.

3.2 Effect of Red Light and Arginine on Protein Accumulation of *H. Pluvialis*

Changes in protein content in *H. pluvialis* cultures subjected to stress conditions generated by different arginine concentrations and red-wavelength light were evaluated over a 10-day cultivation period. Red light was applied as a stress factor to impose metabolic pressure and elicit physiological responses in the cells. In the control group, the protein concentration was initially 0.507 µg/mL, increased to 1.219 µg/mL on day 6, and reached 1.009 µg/mL at the end of incubation. In the culture exposed to red light without arginine supplementation, protein levels were higher than those of the control group; protein content increased to 1.376 µg/mL on day 6 and was recorded as 1.438 µg/mL on day 10. This result indicates that red-wavelength light alone triggered a stress response that enhanced protein synthesis in *H. pluvialis* cells. Under red light, arginine supplementation further promoted protein accumulation in a concentration-dependent manner. In the 0.1 mM arginine group, protein content reached 1.647 µg/mL on day 6 and was measured as 1.650 µg/mL on day 10. Supplementation with 0.5 mM arginine stimulated protein synthesis more strongly under stress conditions, with protein levels rising to 1.885 µg/mL on day 6 and reaching 1.797 µg/mL on day 10.

The highest protein accumulation was observed in cultures supplemented with 1.0 mM arginine. In this group, protein content increased rapidly under red light–induced stress, rising from 0.504 µg/mL at the start to 2.206 µg/mL on day 6 and remaining at 2.204 µg/mL by day 10, representing the highest level among all treatments (Figure 3).

These findings demonstrate that protein accumulation increased in response to both red light exposure and increasing arginine concentration, suggesting that arginine acts as an effective metabolic modulator supporting protein biosynthesis in *H. pluvialis* under stress conditions.

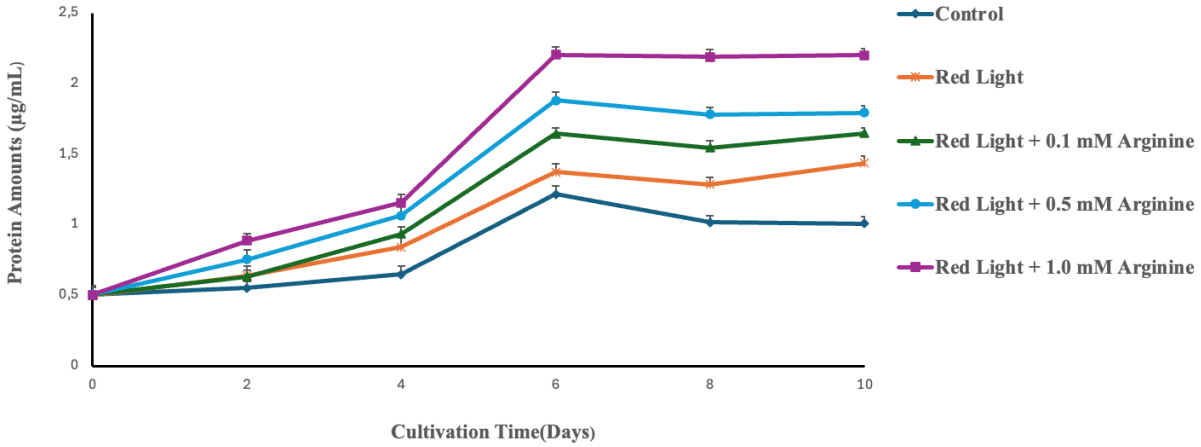

Figure 3. Changes in protein content of *H. pluvialis* cultures exposed to red light and arginine supplementation.

The obtained correlation coefficients indicated strong positive relationships among the arginine-treated groups incubated under red-wavelength light ($r > 0.95$). This finding suggests that, under red light–induced stress conditions, the temporal pattern of protein synthesis is governed by a similar metabolic regulation, largely independent of arginine concentration. Although absolute protein levels increased markedly with increasing arginine concentration, the consistently high correlation coefficients among the groups demonstrate that these increases occurred proportionally and in a synchronized manner over time. The very strong correlation observed between the 0.5 mM and 1.0 mM arginine treatments further indicates that, despite affecting

protein biosynthesis at different quantitative levels, these conditions produced largely overlapping time-dependent response patterns. In contrast, the relatively lower correlation coefficients between the control group (white light) and the red light and arginine treatments imply that protein metabolism is sensitive to changes in light spectrum and that red light directs protein accumulation through a distinct physiological framework.

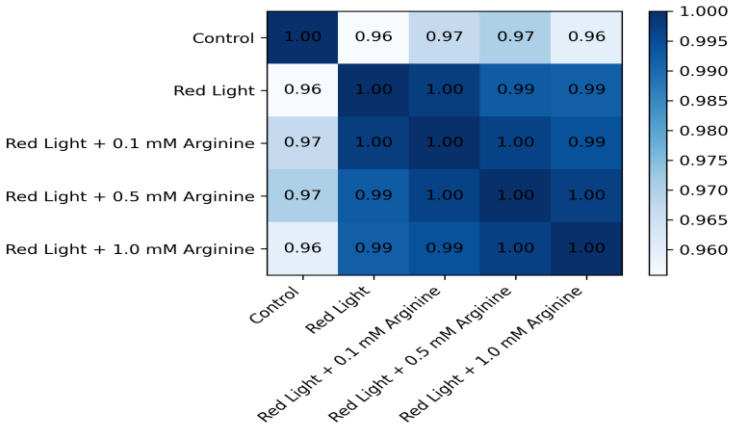

Figure 4. Correlation heatmap of protein contents in *H. pluvialis* cultures exposed to red light and arginine-ınduced stress.

Group-wise correlation results indicate that arginine supplementation under red light influenced protein biosynthesis in a consistent, reproducible, and temporally coordinated manner, while the arginine dose primarily modulated this process at a quantitative level. These findings demonstrate that the interaction between red light and arginine exerts a combined regulatory effect on protein metabolism in *H. pluvialis*.

3.3 Astaxanthin Accumulation Response to Increasing Arginine Levels Under Red LED Light

Changes in astaxanthin content were evaluated over a 10-day cultivation period in *H. pluvialis* cultures incubated under red-wavelength LED light and supplemented with different arginine concentrations. The results showed that astaxanthin production increased with cultivation time; however, the magnitude of this increase was strongly dependent on both arginine concentration and exposure to red light. In the control group (white light, without arginine supplementation), astaxanthin content increased from 0.302 µg mL^{-1} on the day of inoculation to 0.637 µg mL^{-1} by day 10. In cultures exposed to red light without arginine, astaxanthin reached 0.793 µg mL^{-1} on day 10, indicating that red light alone exerted a limited stimulatory effect.

In contrast, astaxanthin accumulation increased markedly in the arginine-supplemented groups. Under red light, supplementation with 0.1 mM arginine raised astaxanthin content to 1.165 µg mL^{-1} by day 10. Increasing the arginine concentration to 0.5 mM further enhanced pigment accumulation, resulting in an astaxanthin level of 1.794 µg mL^{-1}. The highest astaxanthin content was observed in the 1.0 mM arginine group. In this treatment, astaxanthin increased from 0.302 µg mL^{-1} at inoculation to 2.115 µg mL^{-1} by day 10, representing the maximum value among all experimental groups (Figure 5). Compared with lower arginine concentrations, this increase was substantial, indicating that 1.0 mM arginine most effectively stimulated astaxanthin biosynthesis under red-wavelength light conditions.

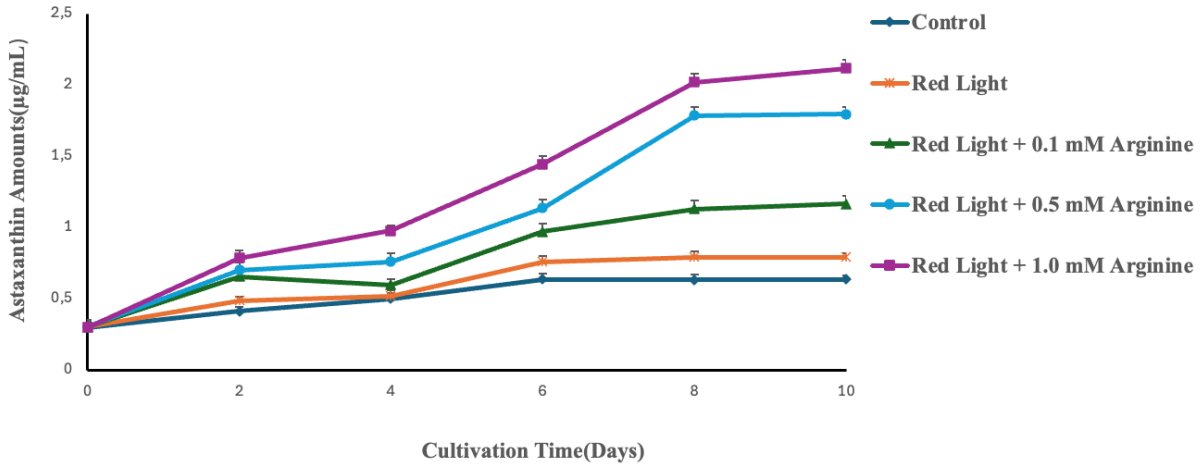

Figure 5. Time dependent astaxanthin accumulation under red LED light and arginine supplementation.

The results indicate that astaxanthin production increased in a dose-dependent manner with arginine supplementation, and that the 1.0 mM arginine treatment, in combination with red-wavelength LED light, represented the most effective condition for maximizing astaxanthin accumulation in *H. pluvialis*. This outcome suggests that the metabolic pressure induced by high arginine concentration strongly promotes astaxanthin biosynthesis.

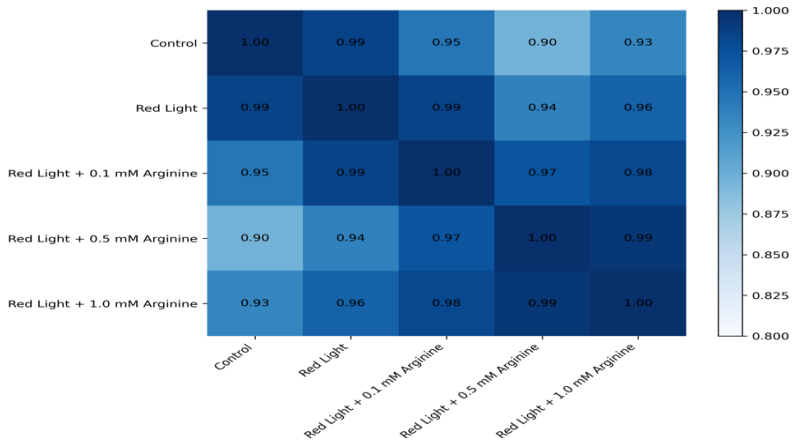

Figure 6. Correlation heatmap of astaxanthin accumulation under red light and arginine supplementation.

The resulting correlation heatmap indicated that astaxanthin accumulation exhibited a strong and positive time-dependent association across all experimental groups. Correlation coefficients (r) were predominantly above 0.90, demonstrating that pigment accumulation followed a consistent increasing trend throughout the cultivation period. Notably, the group treated with 1.0 mM arginine under red-wavelength light showed the highest and most stable correlation coefficients when compared with all other treatments ($r \approx 0.98–0.99$). This finding indicates that, under this condition, astaxanthin production not only reached higher levels but also increased in a more regular and predictable manner over time (Figure 6). Although positive correlations were also observed at lower arginine concentrations (0.1 and 0.5 mM), these relationships were relatively weaker than those recorded for the 1.0 mM arginine group. Similarly, while the control and red light–only groups maintained an overall upward trend with time, their lower correlation coefficients suggest that astaxanthin accumulation was more limited under these conditions.

3.4 Changes in Lipid Accumulation Under Red Light and Different Arginine Concentration

In this study, time-dependent changes in lipid accumulation were investigated in *H. pluvialis* cultures throughout the cultivation process conducted under red-wavelength LED light and different arginine concentrations. The results demonstrated that lipid content increased over the

cultivation period in all groups; however, the magnitude of this increase varied substantially depending on the applied light condition and arginine level.

In the control group, lipid content was measured as 12.19% on the day of inoculation and increased to 30.13% by day 10. In the red light–only group, lipid accumulation reached higher levels than the control, with lipid content rising to 35.34% at the end of the cultivation period. This outcome indicates that red-wavelength light alone exerted a stimulatory effect on lipid accumulation. Lipid accumulation became more pronounced in the arginine-supplemented groups. Under red light, supplementation with 0.1 mM arginine increased lipid content to 44.67% by day 10, representing a notable increase compared to the red light–only treatment. Increasing arginine concentration to 0.5 mM further enhanced lipid accumulation, with the final lipid content reaching 52.13%.

The highest lipid accumulation was observed in cultures supplemented with 1.0 mM arginine under red-wavelength light. In this group, lipid content increased from 12.15% at inoculation to 57.72% by day 10, representing the maximum value among all experimental groups (Figure 7). This pronounced increase suggests that high-dose arginine, when combined with red light, strongly promotes lipid biosynthesis.

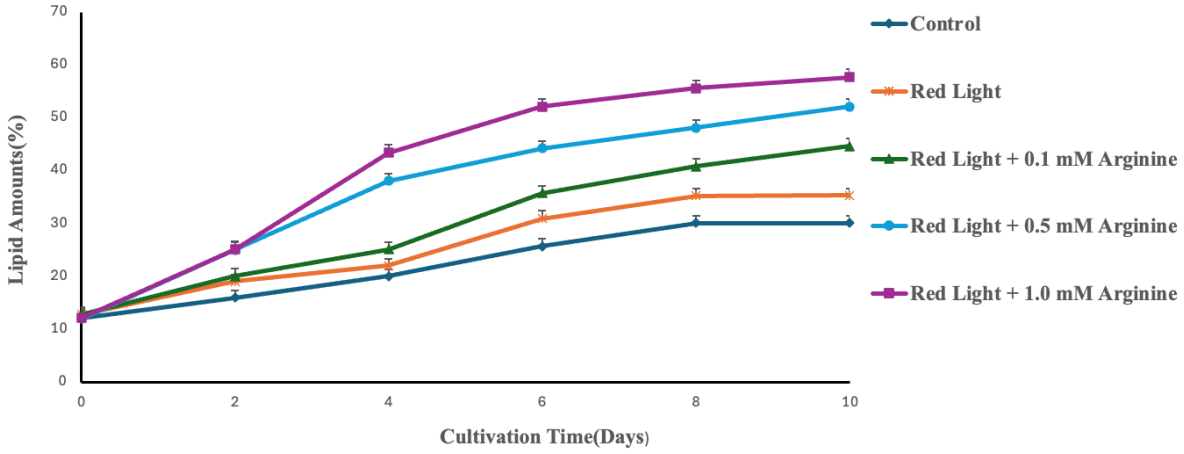

Figure 7. Effect of red light and different concentration of arginine on lipid content (%) in *H. pluvialis.*

The findings of this study indicate that lipid accumulation increased in an arginine concentration–dependent manner and that high concentration arginine supplementation particularly enhanced lipid production under red light conditions.

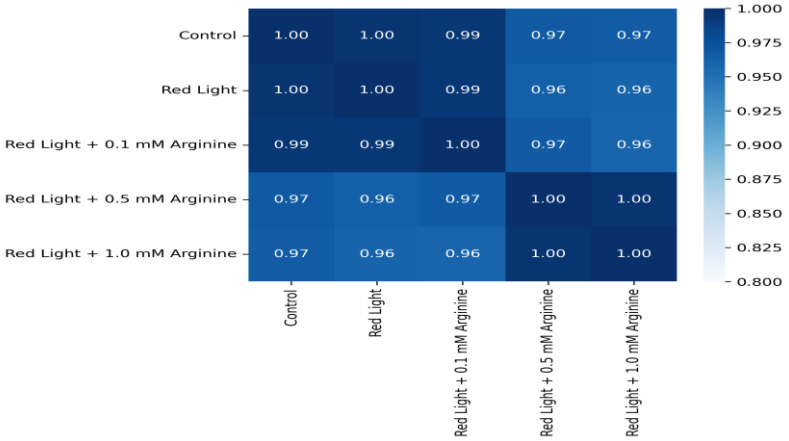

Figure 8. Heatmap of lipid content in *H. pluvialis* exposed to red light and arginine.

The correlation heatmap for lipid content revealed predominantly strong and positive relationships among the different light and arginine treatments. High correlation coefficients were observed between the control group and the red light–only cultures ($r \approx 0.99$), indicating that

lipid accumulation followed a similar time-dependent pattern in these two conditions.

Notable differences in the correlation structure emerged in the groups incubated under red-wavelength light with arginine supplementation. In particular, very high correlation coefficients were obtained between the 0.5 mM and 1.0 mM arginine treatments under red light ($r \geq 0.98$). These strong associations suggest that high arginine concentrations regulate lipid accumulation through comparable metabolic routes. The highest correlation values were recorded in cultures treated with 1.0 mM arginine under red light, which exhibited strong positive correlations with all other treatments (Figure 8). This pattern indicates that high-dose arginine promotes lipid biosynthesis in a more stable and predictable manner under red light–induced stress conditions. Overall, the correlation analysis demonstrates that lipid accumulation is sensitive to both light spectrum and arginine concentration, and that higher arginine levels markedly shape lipid production patterns.

3.5. Impact of Red Light and Different Concentration Arginine on Chlorophyll-a and b Content in *H. pluvialis.*

In this study, changes in chlorophyll-a content (µg/mL) were monitored over a 10-day cultivation period in *H. pluvialis* cultures incubated under red light and different arginine concentrations. Under white light (control), chlorophyll-a content increased from 0.145 µg/mL on the day of inoculation to 0.446 µg/mL by day 10, with a limited increase observed after day 6. In cultures exposed to red-wavelength light without arginine supplementation, chlorophyll-a accumulation was slightly higher than in the control group, reaching 0.478 µg/mL on day 10. Under red light, cultures supplemented with arginine exhibited higher chlorophyll-a levels compared with both the control and red light–only groups. In the 0.1 mM arginine treatment, chlorophyll-a increased steadily throughout the cultivation period and reached 0.555 µg/mL on day 10. Supplementation with 0.5 mM arginine further enhanced chlorophyll-a accumulation, resulting in a value of 0.672 µg/mL at the end of the cultivation period.

The highest chlorophyll-a content was observed in cultures treated with 1.0 mM arginine under red light (Figure 9). In this group, chlorophyll-a increased from 0.148 µg/mL at inoculation to a maximum of 0.754 µg/mL on day 6 and remained high at 0.722 µg/mL by day 10. These findings indicate that high arginine concentration strongly stimulates chlorophyll-a synthesis under red light conditions.

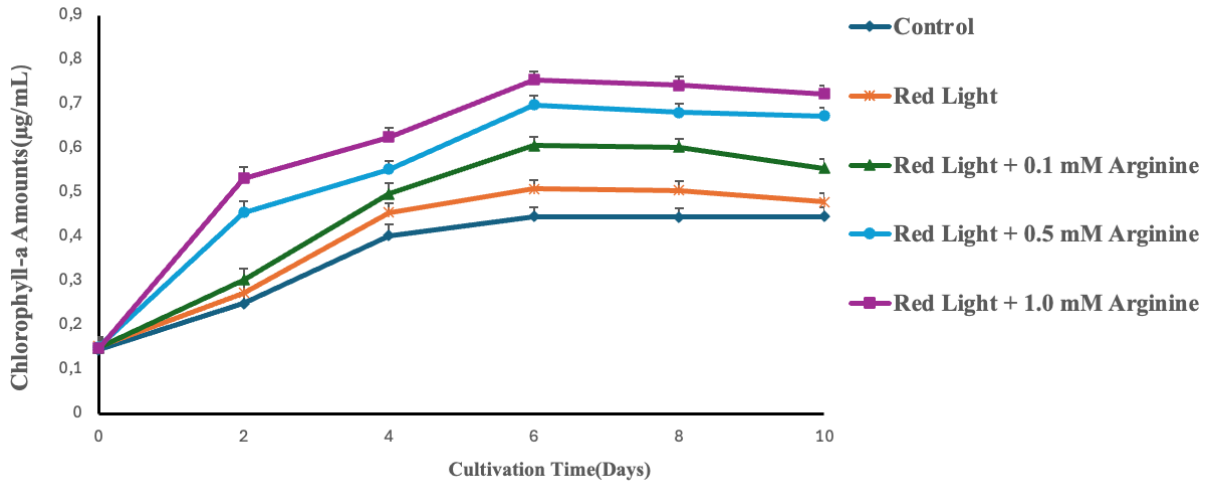

Figure 9. Changes in chlorophyll-a content of *H. pluvialis* under red light conditions in response to increasing arginine concentrations.

The correlation heatmap based on chlorophyll-a content revealed strong linear relationships among the datasets obtained under different light and arginine treatments. The fact that all calculated correlation coefficients were positive and within a high range ($r > 0.90$) indicates that chlorophyll-a accumulation exhibited similar time-dependent increasing trends across all experimental groups (Figure 10). In particular, the very high correlation coefficients observed among the arginine-supplemented cultures under red-wavelength

light suggest that pigment accumulation increased consistently with arginine concentration and followed comparable patterns across different doses.

The highest correlation values recorded between the 0.5 mM and 1.0 mM arginine treatments further indicate that chlorophyll-a metabolism produced comparable physiological responses within this concentration range.

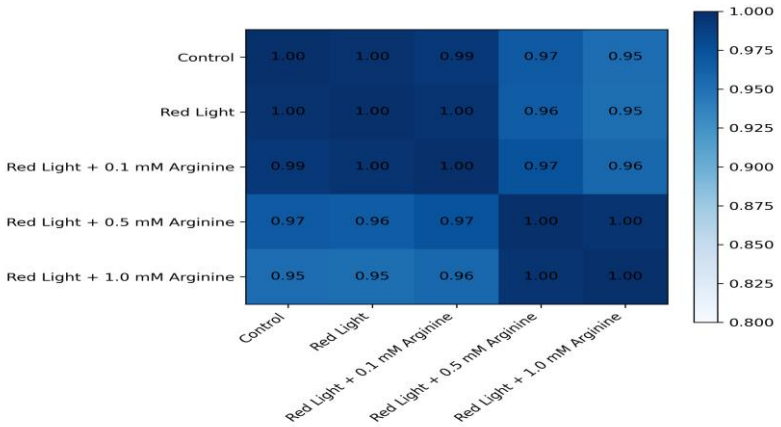

Figure 10. Heatmap representation of correlations among chlorophyll-a contents under different cultivation conditions.

In parallel with the changes observed in chlorophyll-a levels, the effects of different light wavelength conditions and arginine concentrations on chlorophyll-b content were also evaluated. Data obtained over the 10-day cultivation period showed that chlorophyll-b exhibited an increasing trend in all groups; however, the magnitude of this increase varied depending on the applied light condition and arginine concentration. In the control group, chlorophyll-b content was initially 0.098 µg/mL, and the rate of increase decreased after day 6, reaching a stable level of 0.356 µg/mL by day 10. In cultures exposed to red-wavelength light alone, chlorophyll-b accumulation was higher than in the control group, reaching 0.404 µg/mL by day 10, indicating that red light supported chlorophyll-b synthesis. Under red light stress, supplementation with 0.1 mM arginine increased chlorophyll-b content to 0.503 µg/mL on day 6, while the final value was measured as 0.490 µg/mL at the end of the experiment.

This result suggests that low-dose arginine positively influenced photosynthetic pigment composition. In the 0.5 mM arginine treatment, chlorophyll-b reached 0.625 µg/mL on day 6 and remained at 0.612 µg/mL by day 10. The highest chlorophyll-b content was recorded in cultures supplemented with 1.0 mM arginine, where chlorophyll-b increased to 0.682 µg/mL on day 6 and was measured as 0.652 µg/mL at the end of cultivation.

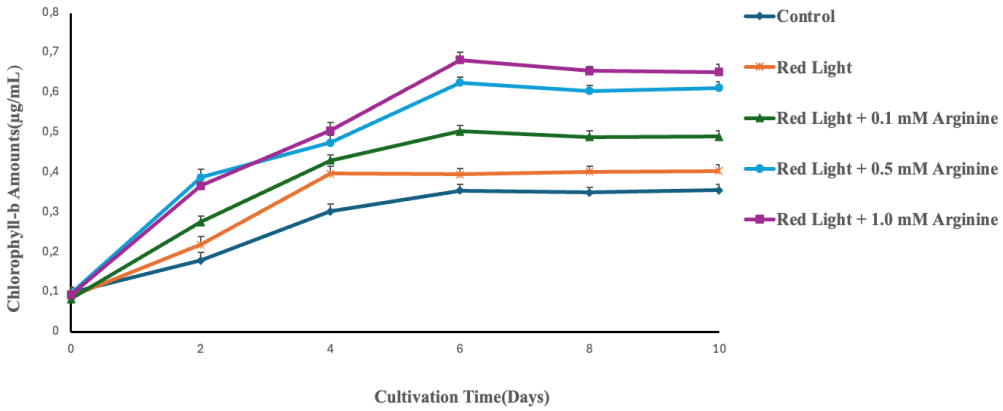

Figure 11. Effects of red light and different arginine concentrations on chlorophyll-b amounts in *H. pluvialis*

According to the data obtained in this study, chlorophyll-b content increased in an arginine concentration manner under red-wavelength light conditions, with the highest pigment accumulation observed at 1.0 mM arginine (Figure 11).

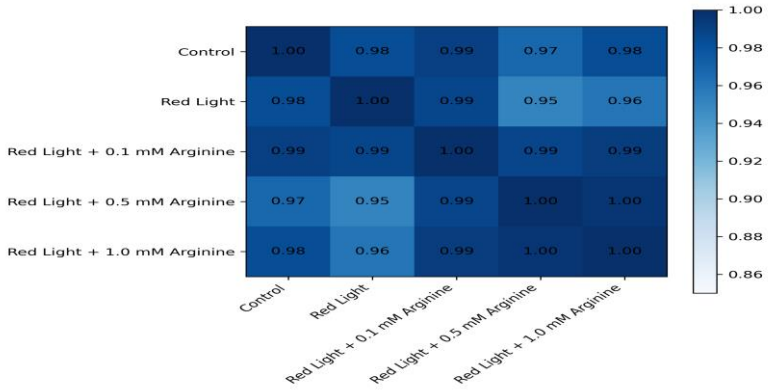

Figure 12. Correlation heatmap of chlorophyll-b contents in *H. pluvialis* cultures exposed to red light and different arginine concentrations over a ten-day cultivation period.

The correlation heatmap for chlorophyll-b content revealed that the time-dependent pigment accumulation profiles exhibited predominantly positive relationships across the treatment groups. In particular, the high correlation coefficients observed between the 0.5 mM and 1.0 mM arginine-supplemented cultures under red light indicate that these two treatments regulated chlorophyll-b accumulation in a comparable manner, both in terms of the direction of increase and its temporal pattern. In contrast, chlorophyll-b accumulation followed a more limited trend in the control and red light–only cultures; therefore, correlations between these groups and the arginine-supplemented treatments remained relatively lower. Overall, the correlation heatmap suggests that chlorophyll-b accumulation reflects not only a time-dependent increase but also a response that is modulated by arginine concentration under red light conditions, with higher arginine levels being associated with enhanced pigment synthesis.

4. DISCUSSION

Previous studies have reported that arginine supplementation at different concentrations improves carbon utilization in *H. pluvialis*, activates arginine-related metabolic pathways and promotes a synergistic increase in biomass, astaxanthin and lipid production by regulating the expression of genes associated with carotenoid and lipid biosynthesis (Acheampong et al., 2024). These findings are consistent with the growth enhancement and metabolite accumulation observed in the present study, particularly at higher arginine concentrations. Similarly, in *Microcystis aeruginosa* cultures, arginine addition at different doses has been shown to induce distinct growth patterns (Dai et al., 2019), supporting the view that arginine can species and dose-dependent physiological responses. Given its central role in nitrogen metabolism, arginine provides an important framework for interpreting its effects on pigment and protein synthesis.

In a multi-omics study conducted on *Phaeodactylum tricornutum* under mixotrophic conditions, arginine-based nitrogen metabolism was found to accelerate intracellular nitrogen utilization, thereby simultaneously supporting photosynthetic activity as well as chlorophyll *a* and carotenoid synthesis, and enhancing photosystem efficiency during early growth stages (Li et al., 2025).

This mechanism may help explain the increased chlorophyll *a/b* contents recorded in the arginine-supplemented groups in our study. In *H. pluvialis*, astaxanthin is primarily stored in lipid droplets, highlighting the close association between lipid metabolism and pigment production (Zhang et al., 2019). Previous reports have shown that stress-related compounds such as melatonin, proline and GABA can indirectly elevate astaxanthin levels by enhancing lipid accumulation under unfavorable environmental conditions (Xing et al., 2023; Ding et al., 2018; Li et al., 2021). In this context, the pronounced increase in lipid content induced by arginine supplementation in the present study appears to be a key factor supporting astaxanthin accumulation, particularly at higher concentrations. In *Gracilariopsis lemaneiformis* exposed to high-temperature stress, arginine supplementation at 0.5 and 1 mM was reported to

preserve phycobiliprotein content, whereas lower doses showed limited effects (Zhang et al., 2021).

Likewise, in *Dunaliella salina*, arginine application under high-salinity conditions increased both cell number and protein content (Bamary & Einali, 2022). These observations suggest that the physiological impact of arginine may become more pronounced as stress intensity increases.

Light quality is another major environmental determinant shaping the physiological and biochemical responses of microalgae. Several studies have demonstrated that red light can enhance growth and biochemical composition by supporting photosynthetic carbon fixation.

In *Chlorella sorokiniana*, red light stimulated carbohydrate and protein production during the early cultivation phase (He et al., 2022), while in *Chlorella vulgaris*, it increased lipid levels compared to white light (Chang et al., 2022).

Studies on *H. pluvialis* have further shown that red light promotes CO_2 fixation and maintains pH stability in the medium, thereby supporting algal growth (Wongsnansilp & Khamcharoen, 2024). In addition, red light applied at different intensities has been reported to increase biomass and protein content (Sui & Harvey, 2021).

Taken together with the mechanisms proposed in the literature, the findings of the present study indicate that high-concentration arginine supplementation under red-wavelength light represents an effective strategy to simultaneously promote growth, pigment synthesis and the production of energy-storing lipids in *H. pluvialis*. This approach may therefore provide a sustainable and feasible route for the biotechnological optimization of natural astaxanthin production.

5. CONCLUSION

In this study, the effects of red-wavelength light and different L-arginine concentrations on growth and biochemical composition (protein, astaxanthin, lipids, and chlorophyll-a,b) in *H. pluvialis* cultures were evaluated over a 10-day period. The results demonstrated that red light alone provided only limited support for cellular development and metabolite accumulation compared with the control conditions (white light, arginine-free culture). In contrast, arginine supplementation, particularly at 1.0 mM, markedly enhanced growth and metabolic responses under red light. High concentration arginine treatment resulted in pronounced increases in protein, lipid, chlorophyll-a,b and especially astaxanthin contents, suggesting a regulatory role of arginine linked to nitrogen metabolism, photosynthetic activity, and stress-related responses. The parallel increase in astaxanthin accumulation and lipid content was interpreted as a physiologically consistent response, in agreement with the storage strategy of this pigment. Likewise, the elevation of chlorophyll-a,b levels indicates that photosynthetic capacity can be maintained more effectively under arginine-supported conditions.

In conclusion, arginine supplementation in combination with red-wavelength light was identified as an effective cultivation strategy for promoting both growth and the production of high value-added metabolites in *H. pluvialis*. This approach represents a promising alternative for improving the efficiency of natural astaxanthin production in biotechnological applications and may provide a basis for the development of sustainable microalgae-based production systems.

Future studies focusing on the molecular regulatory effects of arginine and its performance under long-term cultivation conditions will be essential for translating this strategy to industrial-scale applications.

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CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

AUTHOR CONTRIBUTIONS

G.K.Ş. designed the study, conducted the experiments, analyzed the data and wrote the original draft of the manuscript. A.K.Ç. contributed to data interpretation and revised the manuscript for intellectual content. All authors read and approved the final version of the manuscript.

ETHICAL STATEMENTS

Local Ethics Committee Approval was not obtained because experimental animals were not used in this study.

DATA AVAILABILITY STATEMENT

Data supporting the findings of the present study are available from the corresponding author upon reasonable request.

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