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Original Article

Effect of temperature and light spectrum on the Lutein and Beta-carotenoids production in *Chlorella vulgaris* algae

Hiba Mahmood Al-Nassar, Sadiq Kadhum Lafta Alzurfi*

Department of Ecology and Pollution, Faculty of Science, University of Kufa, Kufa, Iraq.

Abstract: Algae contain carotenoids as granules that give them red, orange, or yellow hues. β-carotene acts as an antioxidant, protecting the body from free radicals, enhancing the immune system, and contributing to cell growth and differentiation. Lutein is a carotenoid found in plants and phototrophic microorganisms. Lutein is widely used as a nutraceutical for human health. This study aimed to investigate the impact of temperature (20, 25, and 30°C) and the light spectrum (red, blue, and white) on the production of lutein and β-carotene in Chlorella vulgaris. A special incubator cabin was designed for the study, containing three sections: the first part, corresponding to the red spectrum, had an illumination intensity of 152 µmol/m²/s; the blue spectrum had an illumination intensity of 1194 µmol/m²/s; and the white spectrum had an illumination intensity of 303 µmol/m²/s. Four replicates were prepared for each treatment, and two samples were drawn for lutein and β-carotene pigments on the 9th and 18th days. The results showed that the lutein concentrations ranged between <0.001-5.27 µg/ml at 25°C in the white and blue spectrums, respectively, while the highest concentrations of lutein were at 25°C in the blue spectrum on the 9th day. The results also indicated that the highest concentration of β-carotene at 20°C was 9.2 µg/ml in the blue spectrum on the 18th day. The study concluded that the best concentrations of lutein production were at 25°C in the blue spectrum on the 9th day, while the best concentrations of β-carotene production were at 20°C in the blue spectrum on the 18th day of the experiment.

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Introduction

β-carotene is a pigment responsible for many fruits and vegetables' red, orange, and yellow colors. It differs from alpha-carotene because it carries a β-ionone ring on both ends. Vitamin A is produced when β-carotene cleaves into two molecules (Shete and Quadrol, 2013). Consuming foods rich in β-carotene has various health benefits, including supporting vision, enhancing skin health, and boosting the immune system. β-carotene is also a fat-soluble provitamin; its active form is vitamin A (Valko et al., 2007). β-carotene is crucial as an antioxidant, shielding cells from damage caused by free radicals. Regular consumption of β-carotene in algae promotes skin health and contributes to a radiant complexion. Additionally, it is linked to various health benefits, including supporting heart health and strengthening the immune system. Furthermore, β-carotene protects the skin from UV damage, contributing to sun protection (Arct and Mieloch, 2016). Research suggests potential anti-inflammatory effects, promoting overall body health for β-carotene. It promotes cell growth and development, contributing to overall well-being and natural growth processes (Wu et al., 2023).

β-carotene is among the most important natural carotenoids produced by plants, algae, and microorganisms (Córdova et al., 2018). In recent years, there has been a significant increase in interest due to substantial evidence highlighting its benefits and importance for human health. Its significance extends to various uses, including pharmaceuticals, food coloring, and cosmetics (Johnson and Schroeder, 1995).

Plant Carotenoid production is influenced by environmental factors such as nutrients like

*Correspondence: Sadiq Kadhum Lafta Alzurfi
E-mail: sadiqk.alzurfi@uokufa.edu.iq
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phosphorus and nitrogen, high light intensity, and salinity. β-carotene accumulates in thylakoids in green plastids (Arora and Sahoo, 2015). The plant pigments are also affected by light, a crucial environmental factor influencing growth, chemical composition, and the increased efficiency of β-carotene. Studies have shown that an increase in light intensity and a decrease in nitrate concentration leads to enhanced β-carotene accumulation in green algae (Wu et al., 2016). Generally, the biomass system is light-dependent, as light easily absorbs and scatters through algal cells. Adaptation to light changes relies on alterations in both thylakoid numbers and pigment content (Khotimchenko and Yakoveleva, 2005; Jeon et al., 2006). The duration of daylight affects the circadian rhythm of biological processes such as photosynthetic activity, respiration, cell division, and growth rates (Bouterfas et al., 2006).

Lutein belongs to the carotenoid group of plant pigments and is yellow (Ozawa and Sasaki, 2014). It is a common carotenoid found in the macular area of the eye and helps prevent age-related macular degeneration (AMD) and damage caused by light (Mrowicka et al., 2022). Lutein, C40H56O2, has a reddish-yellow optical structure and is abundant in various plants and algae. It shields plant cells from UV light damage and assists in regulating the process of photosynthesis, absorbing and converting light into chemical energy. Additionally, lutein plays a role in safeguarding plants and algae from intense light and ultraviolet radiation, preserving biodiversity, and protecting them in diverse environments (Vishwanathan et al., 2016). It is exclusively produced by photosynthetic organisms such as wild plants and microalgae (Becerra et al., 2020).

Fariz-Salinas et al. (2024) evaluated the impact of sunlight intensity on intracellular lutein pigment generation and phosphorus removal from secondary effluents by autoflocculating the microalgae consortium BR-UANL-01 in a photobioreactor culture. Microalgae were grown in a secondary effluent from a wastewater treatment plant, using a combination of low and high light conditions, and they produced significantly more lutein under low light conditions (Fariz-Salinas et al., 2024). In another study, blue light was found to have a stimulating effect on the synthesis of lutein and other carotenoids (Diaz-MacAdoo et al., 2022). Based on the above-mentioned background, the current study aims to investigate the impact of temperature (20, 25, and 30°C) and the light spectrum of red, blue, and white on the production of lutein and β-carotene in the algae *Chlorella vulgaris*.

Materials and Methods

Chlorella vulgaris algae were added to 1000 ml flasks and placed in special incubators containing three sections of spectra: the 1st part, corresponding to the red spectrum, had an illumination intensity of 152 µmol/m²/s, the 2nd part, corresponding to the blue spectrum, had an illumination intensity of 1194 µmol/m²/s, and the 3rd part, representing the white spectrum, had an illumination intensity of 303 µmol/m²/s. The four replicates of the prepared samples were incubated for 20 days in each section of the incubator. The three incubators were each adjusted to temperatures of 20, 25, and 30°C as temperature treatments.

According to Morowvat and Ghasemi (2016), Beta-carotene and lutein pigments were extracted with some modifications. A glass fiber filter (Whatman GF/F filters, 47 mm in diameter, and pore size of 0.45 µm) was used for filtration in a vacuum filtration apparatus. Subsequently, slightly over 0.2 milliliters of 1% magnesium carbonate solution were added. Samples were collected on the ninth day of the experiment, and the extraction process was conducted as follows: 25 ml of the sample was centrifuged at 10,000 rpm for half an hour, and the supernatant was discarded. The remaining pellet was preserved at -20°C. Then, 20 ml of hexane was added to the pellet and mixed with vortexing for 30 seconds. Ultrasonication at 100 watts for 60 minutes was used to disrupt algal cells, releasing their contents, including lutein and β-carotene. Subsequently, samples were centrifuged for 7 minutes at 8,400 rpm to remove crushed materials, separating the liquid portion containing lutein. The supernatant, containing lutein and a small amount of β-carotene, was

Table 1. The conditions of Beta-carotene and lutein measurements.

Component	Model	Manufacture
Binary high-pressure gradient pump	P6.1L	Knauer, Germany
Diode array detector	DAD 2.1L	Knauer, Germany
Sample loop (20µl) and injector	D1357	Knauer, Germany
Analyses and system control software	Claritychrom V7.4.2.107	Dataapex, Czech Republic

evaporated, and 20 ml of ethanol was added to the residue. Samples were centrifuged for 7 minutes at 8,400 rpm to remove crushed materials, separating the liquid portion containing β-carotene. Finally, the organic solvent was removed from the lutein extract by evaporating it under low pressure using a rotary evaporator, leaving behind a concentrated lutein extract.

Lutein and β-carotene measurement: The extracted samples (lutein and β-carotene) were measured using high-performance liquid chromatography (HPLC; Knauer, Germany, with a C18 column: 250-4.6 mm i.d., 5 µm particle size, 80 Å pore size). The gradient elution profile, consisting of water as mobile phase B and acetone as mobile phase A (refer to Table 1), was detected at 450 nm. Each compound was identified by comparing each standard's retention time and absorbance spectrum. The concentration was determined by serially diluting the external standard of lutein (Sigma Company, USA) to create a calibration curve that linked the concentration to the corresponding peak area or height.

The detection of each compound was carried out by comparing the retention time and absorbance spectrum of each standard; the concentration was determined by serial dilutions of external standard materials to establish a calibration curve correlating the concentration with its corresponding peak area or height. All calculated concentrations are based on 1 gram of fresh algae as follows:

Time / min	A / %	B / %
0	75	25
5	75	25
10	95	5
17	95	5
22	100	0
27	75	25

IBM SPSS Statistics v26.0, univariate analysis of variance (ANOVA) was employed for statistical data testing. The significant difference between means at $P<0.05$ was determined using the Tukey test.

Results and Discussions

The standard curves for lutein and Beta-carotene are presented in Figures 1 and 2.

Lutein pigment: The results of lutein production at 20°C showed the highest concentration on the last day in the blue spectrum (3.19 µg/ml), and the lowest concentration was recorded on the same day in the white spectrum (0.029 µg/ml) (Fig. 3a). In Figure 3b, the results of lutein at 25°C are presented, with the highest concentration of lutein recorded on the ninth day in the blue spectrum at 5.27 µg/ml, while no concentration was recorded on the same day in the white spectrum and the last day in the red spectrum.

Figure 4 displays the results of lutein at 30°C, where the highest concentration of lutein was recorded on the last day in the white spectrum at 0.66 µg/ml, while the lowest concentration was recorded on the ninth day in the white spectrum at 0.13 µg/ml. The statistical analysis revealed significant differences at $P<0.05$ (Tables 2-4).

The results indicate that the highest concentrations of lutein were observed on the ninth day at 25°C in the blue spectrum. This temperature can be considered ideal for the primary productivity of Chlorella algae. The lowest concentrations were observed on the ninth day at 25°C in the red and white spectra. The white spectrum at 30°C exceeded the other spectra on the 18th day. This suggests that the optimum temperature for lutein production is 25°C, and the optimal spectrum for lutein production is blue. These findings are consistent with those of Zhao et al. (2019), who reported that the highest lutein productivity occurs at the blue light wavelength.

The peak production of lutein pigment in Chlorella algae occurs at temperatures of 25 and 30°C because these temperatures are suitable for the activity of lutein enzymes (Ma et al., 2020). The blue spectrum was found to have a stimulating effect on the synthesis of lutein and other carotenoids (Diaz-MacAdoo et al.,

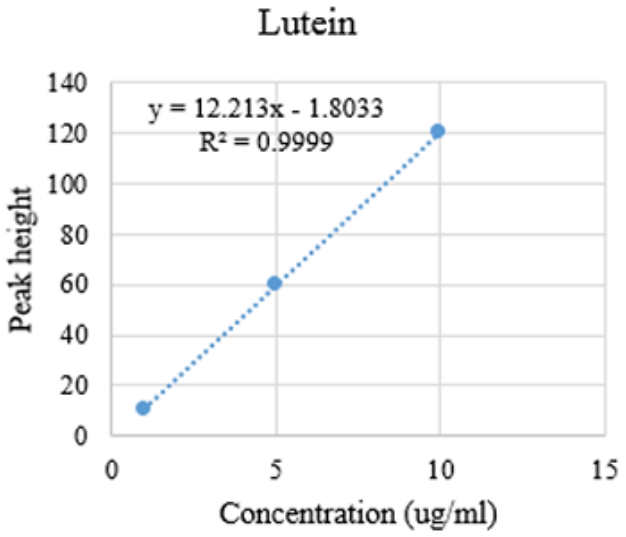

Figure 1. Standard curve of lutein.

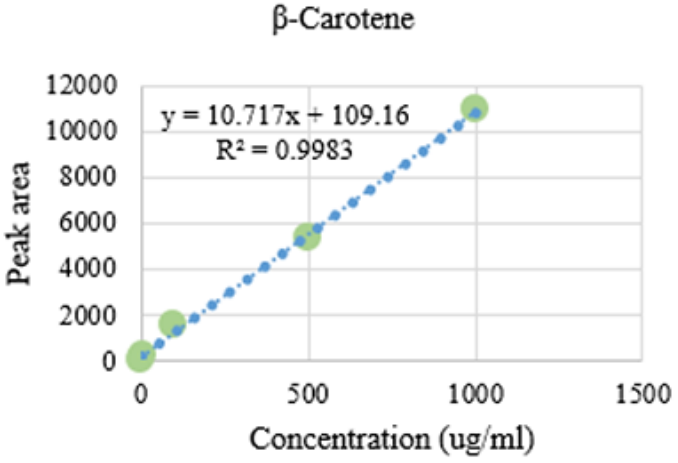

Figure 2. Standard curve of Beta-carotene.

2022). The white spectrum of Chlorella algae reflects sunlight and helps protect it from damage. The peak production of lutein pigment in Chlorella algae is achieved at a temperature of 30°C (Chini Zittelli et al., 2023) because this temperature is suitable for the activity of lutein enzymes responsible for lutein synthesis. High temperatures also help increase the metabolic rate of Chlorella algae, leading to increased lutein pigment production (Lou et al., 2021). The white color of Chlorella algae reflects sunlight and aids in protection. It also allows more light to penetrate the cell, enhancing lutein pigment production, which in turn protects the algae from sun damage (Diaz-MacAdoo et al., 2022). Since sunlight can cause cell damage by generating free radicals, lutein pigment acts as an antioxidant, neutralizing free

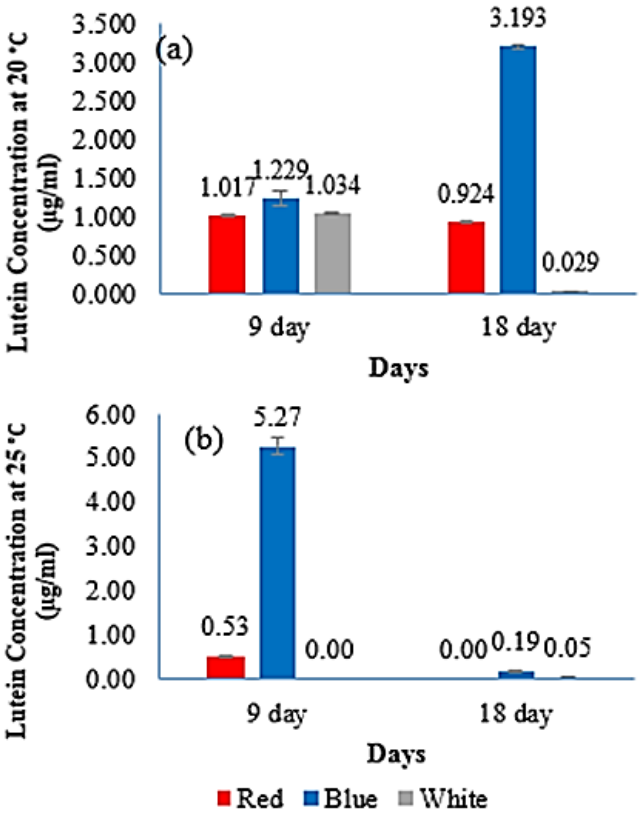

Figure 3. Lutein concentrations in *Chlorella vulgaris* (a) at 20°C, and (b) at 25°C.

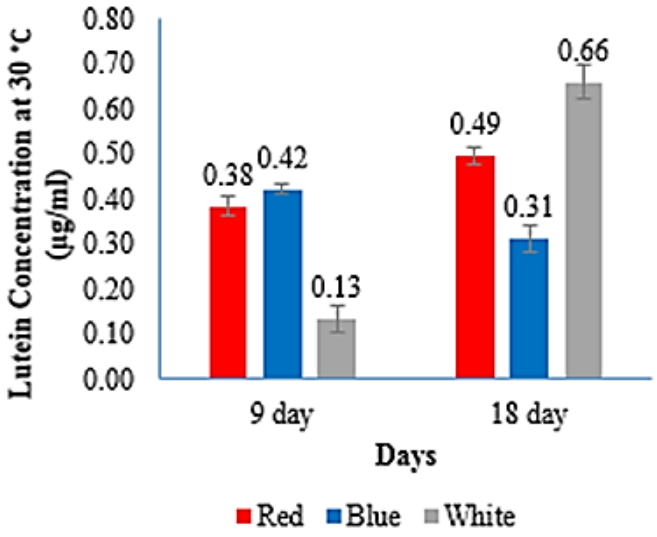

Figure 4. Lutein concentrations in *Chlorella vulgaris* at 30°C.

radicals and protecting cells from damage. In conclusion, the production of lutein pigment in Chlorella algae is a complex process influenced by various factors, including temperature, color, and antioxidants (Coulombier et al., 2021).

β-Carotene: Figure 5a displays the results of β-carotene at 20°C, where the highest value of β-

Table 2. ANOVA test results of lutein incubated at 20°C during the the experiment.

Tests of Between-Subjects Effects					
Dependent Variable: Lutein con. at 20°C					
Source	Type III SS	df	MS	F	P
Corrected Model	16.395[a]	5	3.279	1714.376	0.000
Intercept	27.571	1	27.571	14415.165	0.000
Days	0.373	1	0.373	195.065	0.000
Spectrum	9.096	2	4.548	2377.853	0.000
Days × Spectrum	6.926	2	3.463	1810.555	0.000
Error	0.023	12	0.002		
Total	43.990	18			
Corrected Total	16.418	17			

[a.] R Squared = 0.999 (Adjusted R Squared = 0.998)

Table 3. ANOVA test results of lutein incubated at 25°C during the the experiment.

Tests of Between-Subjects Effects					
Dependent Variable: Lutein con. at 25°C					
Source	Type III SS	df	MS	F	P
Corrected Model	66.539[a]	5	13.308	1960.245	0.000
Intercept	18.382	1	18.382	2707.660	0.000
Days	15.587	1	15.587	2295.929	0.000
Spectrum	27.094	2	13.547	1995.467	0.000
Days × Spectrum	23.859	2	11.929	1757.181	0.000
Error	0.081	12	0.007		
Total	85.003	18			
Corrected Total	66.621	17			

[a.] R Squared = 0.999 (Adjusted R Squared = 0.998)

Table 4. ANOVA test results of lutein incubated at 30°C during the experiment.

Tests of Between-Subjects Effects					
Dependent Variable: Lutein con. at 30°C					
Source	Type III SS Squares	df	MS	F	P
Corrected Model	0.472[a]	5	0.094	131.847	0.000
Intercept	2.856	1	2.856	3985.186	0.000
Days	0.140	1	0.140	195.977	0.000
Spectrum	0.015	2	0.007	10.326	0.002
Days × Spectrum	0.317	2	0.159	221.302	0.000
Error	0.009	12	0.001		
Total	3.337	18			
Corrected Total	0.481	17			

[a.] R Squared = 0.982 (Adjusted R Squared = 0.975)

carotene was recorded on the 18th day in the blue spectrum at 9.199 µg/ml, while the lowest value was recorded on the 18th day in the white spectrum at 2.13 µg/ml. In Figure 5b, presenting the results of β-carotene at 25°C, the highest value of β-carotene was recorded on the ninth day in the white spectrum at 4.0 µg/ml, while the lowest value was recorded on the 18th day in the white spectrum at 0.7 µg/ml. Figure 6 and Tables 5-7 illustrate the results of β-carotene at 30°C, where the highest value of β-carotene was

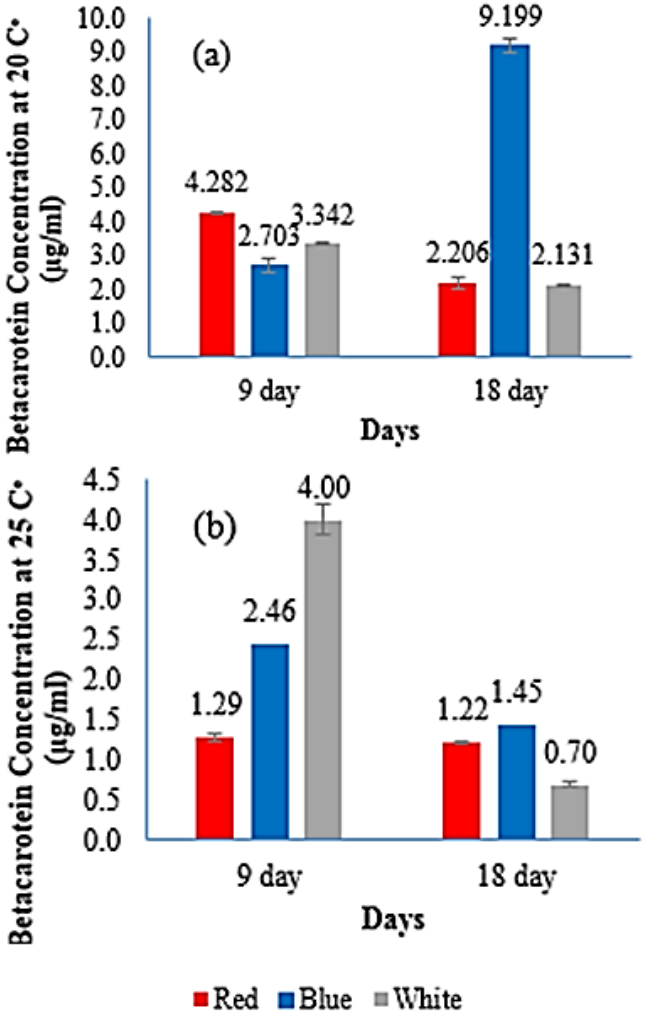

Figure 5. Beta-carotene concentrations in *Chlorella vulgaris*, (a) at 20°C, and (b) at 25°C.

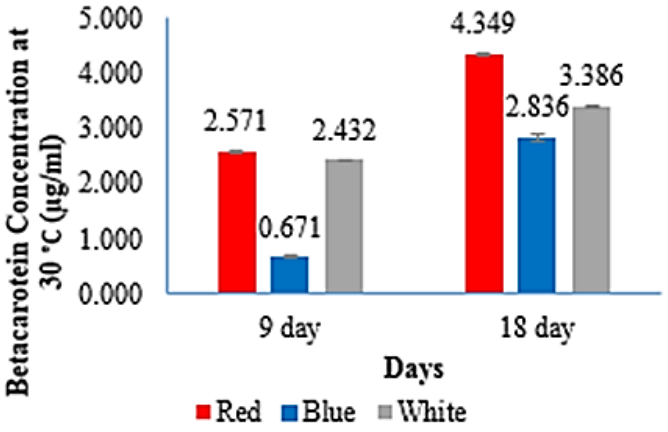

Figure 6. Beta-carotene concentrations in *Chlorella vulgaris* at 30°C.

recorded on the 18th day in the red spectrum at 4.35 µg/ml, while the lowest value was recorded on the

Table 5. ANOVA test results of beta-carotene that was incubated at 20°C during experiment.

Tests of Between-Subjects Effects					
Dependent Variable: Beta-carotene con. at 20°C					
Source	Type III SS	df	MS	F	P
Corrected Model	107.805[a]	5	21.561	1051.283	0.000
Intercept	284.725	1	284.725	13882.718	0.000
Days	5.149	1	5.149	251.058	0.000
Spectrum	35.847	2	17.923	873.915	0.000
Days × Spectrum	66.810	2	33.405	1628.763	0.000
Error	0.246	12	0.021		
Total	392.777	18			
Corrected Total	108.052	17			
[a.] R Squared = 0.998 (Adjusted R Squared = 0.997)					

Table 6. ANOVA test results of beta-carotene that was incubated at 25°C during the experiment.

Tests of Between-Subjects Effects					
Dependent Variable: Betacarotene con. at 25°C					
Source	Type III SS	df	MS	F	P
Corrected Model	21.577[a]	5	4.315	578.611	0.000
Intercept	61.845	1	61.845	8292.077	0.000
A	9.613	1	9.613	1288.831	0.000
B	3.710	2	1.855	248.729	0.000
A × B	8.255	2	4.127	553.382	0.000
Error	0.089	12	0.007		
Total	83.512	18			
Corrected Total	21.667	17			
[a.] R Squared = 0.996 (Adjusted R Squared = 0.994)					

Table 7. ANOVA test results of beta-carotene that was incubated at 30°C during the experiment.

Tests of Between-Subjects Effects					
Dependent Variable: Beta-carotene con. at 30°C					
Source	Type III SS	df	MS	F	P
Corrected Model	22.248[a]	5	4.450	3775.054	0.000
Intercept	131.958	1	131.958	111952.160	0.000
Days	11.999	1	11.999	10179.449	0.000
Spectrum	9.101	2	4.550	3860.571	0.000
Days × Spectrum	1.149	2	0.574	487.340	0.000
Error	0.014	12	0.001		
Total	154.220	18			
Corrected Total	22.262	17			
[a.] R Squared = 0.999 (Adjusted R Squared = 0.999)					

ninth day in the blue spectrum at 0.67 µg/ml. The results indicated significant differences at the $P<0.05$ level. The study results also indicate that the highest concentrations of beta-carotene were observed on the 18th day at a temperature of 20°C for all treatments. This temperature can be considered ideal for the primary productivity of Chlorella algae. The lowest concentrations were observed on the 18th day at a temperature of 25°C. The red color at a temperature of 30°C exceeded the other spectra on the 18th day. This suggests that the optimum temperature for beta-carotene production is 20°C, and the optimal spectrum for beta-carotene production is blue. These results are consistent with Baidya et al. (2021), where it was found that the highest beta-carotene productivity occurs at the blue light wavelength within a temperature range of 20-26℃.

The results showed that with increasing temperature, the beta-carotene content significantly increased. Additionally, the extraction rate of beta-carotene was enhanced by increasing exposure to red light spectra (Li et al., 2020). Notably, in all cases, more than 80% of the total carotenoid content was beta-carotene (Han et al., 2019). Han et al. (2019) assessed beta-carotene synthesis in D. salina cells under 50 µmol/(m^2.s) of red, blue, and white light. Moslemipetroudi et al. (2021) show that the highest beta-carotene content was recorded in a combination of four colored lights: white, blue, red, and yellow, each in equal periods of six consecutive days. From these studies, it can be concluded that for optimal beta-carotene synthesis, cells should be exposed to white and blue light in the early days of cultivation to promote a favorable growth pattern (lag phase and early stages of logarithmic phase) and in the later stages from the log phase to the stationary phase, red light followed by yellow light.

Conclusion

The study concluded that the highest concentrations of lutein were observed at 25°C in the blue spectrum on the 9th day, followed by 20°C in the blue spectrum on the last day, and 30°C in the white spectrum on the 18th day of the experiment. The highest concentrations of β-carotene were observed at 20°C in the blue spectrum on the 18th day of the experiment, followed by 30°C in the red spectrum on the 18th day, and 25°C in the white spectrum on the 9th day.

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