Thesis for the Degree of Master of Science

Effects of light-emitting diode on the growth and biochemical composition of *Chlorella vulgaris*

by

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Division of Earth Environmental System Science

The Graduate School

Pukyong National University

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발광다이오드 광량 및 파장에 따른 *Chlorella vulgaris*의 생장 및 생화학적 조성 변화 연구

Advisor: Prof. Seok Jin Oh

by

Ji Seung Han

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Abstract

Microalgae play a crucial role in marine ecosystems by synthesizing organic matter using light, water, and carbon dioxide, thus providing primary production. Unlike terrestrial photosynthetic plants, microalgae contain a balanced composition of carbohydrates, proteins, and lipids, and produce a variety of useful substances. As photosynthetic organisms, they fix carbon dioxide and utilize various nutrients for cell growth, aiding in environmental solutions like water purification and heavy metal removal.

Efficient light sources are vital for effective microalgae cultivation. The recently developed LED light source, with its small size, excellent energy efficiency, and the ability to irradiate specific wavelengths. In particular, recent studies have shown that by irradiating specific light intensity appropriately, wavelength, and pulse of LED according to the cultivation stage, the synthesis of useful physiologically active substances such as photosynthetic pigments, antioxidants, and lipids can be promoted. This study aims to identify the optimal wavelength for the growth of *Chlorella*

vulgaris and the effective wavelength for promoting useful substances using LED.

In this study, the *C. vulgaris* strains used were wild type PKVL7422 and a genetically modified strain, PKVL7422, engineered to insert the flounder growth hormone gene for enhanced growth hormone production. Under a single wavelength, the maximum growth rate μ_{max} of the wild type was high in both blue and red wavelengths, with a threefold lower K_s in the red wavelength compared to other wavelengths. The growth rate of the genetically modified strain was highest in red and blue wavelengths, similar to the wild type. Under mixed wavelengths, the growth rate of *C. vulgaris* showed no significant difference in wavelength ratio for both strains. The highest growth rate under light-dark cycles was observed with an optimal adjustment of 12 hours light (L) and 12 hours dark (D).

The carbohydrate content of the wild type under a single wavelength was highest in the green wavelength at a normal light intensity of 10 μmol/m^2/s, correlating with a lower growth rate. The transformed strain showed a carbohydrate content approximately 3.6 times lower than that of the wild type and did not show a significant difference by wavelength. Also, proteins and lipids appeared highest in the blue wavelength of normal light intensity 100 μmol/m^2/s for both wild type and transformed strains, and in particular, the lipid content of the transformed strain showed a content about 1.4 times higher than that of the wild type. The pigment content also peaked in the blue wavelength at 100 μmol/m^2/s for chlorophyll-*a*, chlorophyll-*b*, lutein, zeaxanthin, violaxanthin, and neoxanthin in both strains.

In a two-stage cultivation approach, cells were irradiated with the red wavelength until the late exponential phase to promote cell density, was irradiated until the late

exponential phase, and the blue wavelength was irradiated from the normal phase to promote useful substances, the carbohydrate content of the wild type did not show a statistically significant difference ($P<0.05$). In the case of proteins and lipids, they showed a content 1.5 times higher than that of the red single wavelength, but lower than that of the blue single wavelength. The pigment content was 4.4 times higher in violaxanthin compared to fluorescent lamps, but lower for other pigments than under the blue single wavelength.

The results of changes in biochemical composition according to the control of the light-dark cycle showed that most of them reached their peak when the light-dark cycle was set to 12L:12D, and the differences were not significant. The pigment content also appeared highest in chl-*a*, chl-*b*, and lutein, zeaxanthin, violaxanthin, and neoxanthin when the light-dark cycle was 12L:12D.

In mixed wavelength conditions, where red and blue wavelengths were combined at specific ratios, the carbohydrate content of the wild type showed no statistically significant difference ($P<0.05$), while the transformed strain's content was about 1.1 times lower than that of the wild type. The protein content was highest at a 7:3 ratio favoring the blue wavelength for both strains. In the case of the wild type, this ratio resulted in a protein content 2.1 times higher than under the blue single wavelength, and the transformed strain showed a content 2.4 times higher. Lipids also reached their highest content at the 7:3 ratio, with the lipid content of the transformed strain being 3 times higher than under the blue single wavelength. Additionally, the pigment content of both strains was highest at the 7:3 ratio for chl-*a*, chl-*b*, lutein, zeaxanthin, violaxanthin, and neoxanthin, with chl-*a*, chl-*b*, and lutein content in the transformed

strain being 1.3, 1.5 and 1.2 times higher, respectively.

The experimental results demonstrate that the red wavelength serves as an economical light source to promote cell density in *C. vulgaris*, while the blue wavelength is effective in enhancing valuable biochemical substances such as proteins, lipids, and pigments. Notably, experiments with mixed wavelengths, combining the growth-promoting red and the substance-enhancing blue wavelengths, revealed that a higher ratio of the blue wavelength in this mix leads to more effective accumulation of these substances. Therefore, employing a mixed wavelength with a predominant blue wavelength ratio in the construction of a photobioreactor (PBR) could yield economic benefits and increased productivity.

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1. Introduction

In recent times, the field of biotechnology (BT) has been categorized into various sectors. One of these sectors is White Biotechnology (WBT), which specializes in the production of bioenergy. Due to the depletion of petroleum energy and rising oil prices, the biofuel industry, particularly one that uses microalgae, is gaining significant attention as a promising future resource (Bentahar et al., 2023). Microalgae-based bioenergy production utilizes solar energy, thereby avoiding depletion issues. Moreover, the CO_2 generated during production is reabsorbed through plant photosynthesis, making the process renewable and capable of reducing carbon dioxide emissions (Kim and Choi, 2014). Additionally, producing bioenergy with microalgae does not compete with terrestrial edible crops and does not require deforestation to secure farmland. This makes it a particularly relevant field for maritime countries like ours, which have larger sea areas than land territories (Jo and Cha, 2010). The second sector is Red Biotechnology (RBT), focused on extracting intracellular nutrients for use as raw materials in pharmaceuticals and food (Bentahar et al., 2023). While microalgae are primarily composed of polysaccharides, there are also varieties rich in vitamins, proteins, and unsaturated fatty acids (Kim and Choi, 2014). For example, the blue-green algae *Spirulina* is used as a protein supplement health food because it has very high intracellular protein per dry weight (Kim et al., 1996), and the green algae *Chlorella* contains a variety of vitamins such as vitamin C, linolenic acid, linoleic acid, and 16 other vitamins, as well as chlorophyll, carotenoids, and mineral components (Kim et

al., 2014), so it is used as a comprehensive nutritional biological resource (Safi et al., 2014). The third sector is Green Biotechnology (GBT), which addresses the problem of food resources by purifying domestic and industrial wastewater and sludge (Bentahar et al., 2023). Green Biotechnology (GBT) utilizing microalgae doesn't demand advanced technology for carbon fixation conditions, nor does it necessitate a separate treatment device. It offers numerous additional advantages. In addition, it can not only purify eutrophicated water quality, but also, green algae such as *Chlorella* and *Scenedesmus* have a high ability to absorb heavy metals, so they can be used to remove heavy metals from factory wastewater (Kim et al., 2013; Yan et al., 2013). The biomass produced can substitute for feed resources like corn and enhance functional substances when mixed with commercial feed. It is also used as a feed organism in the aquaculture industry, for organisms such as rotifers and fingerlings (Bentahar et al., 2023).

For commercial use of microalgae, mass cultivation is essential. Mass cultivation methods can be broadly categorized into open and closed systems (Chisti, 2007). Open cultivation devices are economically advantageous over other production methods, such as chemical synthesis, due to their low cost and simple maintenance and operation. However, they present challenges in controlling the cultivation environment, have a high risk of contamination, and struggle with uniform light penetration, which is critical for microalgae growth, especially when cell density increases (Javanmardian et al., 1991; Chio and Lee, 2012).

In contrast, closed cultivation methods, particularly Photobioreactors (PBs),

are considered highly effective, especially in countries with seasonal variations and limited land space (Gim et al., 2012). These systems are actively used for cultivating microalgae that produce high-value physiologically active substances. Moreover, large-scale photobioreactors are crucial for producing substantial quantities of microalgae for commercialization (Kim et al., 2011). However, an increase in culture solution volume in large-scale systems can reduce light transmittance. Fluorescent lamps, commonly used as light sources in these systems, have the drawbacks of large size, low transmittance due to external irradiation, and high operating and initial investment costs (Choi et al., 2012; Yeo 2016). Consequently, research has been directed toward more environmentally friendly, efficient, and economical light sources, leading to the growing use of Light Emitting Diodes (LEDs), which are known to be more economically efficient and effective than traditional light sources (Chen et al., 2008; Mata et al., 2010).

The Light Emitting Diode (LED) is attracting attention as a light source to maximize the cultivation efficiency of microalgae. The LED is a semiconductor that emits light by sending a current to compounds such as gallium arsenide (GaAs) or gallium phosphide (GaP), and unlike fluorescent lamps that convert only 5-10% of the consumed power into light, it converts 90% into light, emitting brightness twice that of fluorescent lamps and ten times that of incandescent lamps, giving it a great advantage in energy efficiency (Park et al., 2013). Additionally, LEDs have a smaller form factor than traditional light sources, allowing them to be placed directly inside photobioreactors, resulting in high light transmission efficiency (Carvalho et al., 2011). They also emit

wavelengths favorable for photosynthesis, unlike fluorescent lamps that irradiate a broader spectrum, many parts of which are unnecessary for photosynthesis. This specificity can enhance the nutrition and growth of microalgae, enabling the cultivation of commercially advantageous strains (Lee et al., 1994; Katsuda et al., 2004; Wang et al., 2007).

Moreover, numerous studies have reported that the synthesis of useful physiologically active substances, such as photosynthetic pigments, antioxidants, and lipids inside microalgae is promoted by irradiating specific luminance, wavelength, and pulse of LED according to the cultivation stage (Katsuda et al., 2004; Menon et al., 2013; Xu et al., 2013; Oh et al., 2015). According to the research by Katsuda et al. (2004), the green algae *Haematococcus plucialis* increased the production efficiency of astaxanthin when using the blue wavelength (wavelength 460 nm), and Wang et al. (2007) reported that the blue-green algae *Spirulina platensis* had a higher cultivation efficiency in the red wavelength (wavelength 660 nm) (Wang et al., 2007). Also, *Botryococcus braunii*, a green algae with a high intracellular lipid content for use in biodiesel, was confirmed to be able to change the content of intracellular carbohydrates and lipids depending on the green, red, and blue wavelengths (Baba et al., 2012). Fu et al. (2013) reported that when the red and blue wavelengths were mixed and the mixed wavelength was performed, the content of β-Carotene and lutein in *Dunaliella salina* increased, and the low-tide diatom *Nitzschia* sp. also reported that the content of chlorophyll *a* (chl-*a*) was higher in the blue wavelength than in the red and yellow wavelengths (Kwon et al., 2013). In addition, Abiusi et al. (2014) reported that the green algae

Tetraselmis suecica exhibited rapid growth under red wavelength irradiation, but higher carbohydrate content under green wavelength irradiation.

On the other hand, *Chlorella vulgaris*, the experimental species of this study, is being used as an important material for RBT, GBT, and WBT (Chisti, 2007; Kumar et al., 2010; Harun et al., 2010; Han and Oh, 2023). *C. vulgaris* accumulates useful substances such as intracellular carbohydrates, proteins, lipids, and pigments (Kim et al., 2014), and is rich in various nutrients such as minerals, vitamins, minerals, and amino acids (Safi et al., 2014), and is evaluated as a strong candidate for future food resources (Kang et al., 2004; Lee, 2007). In addition, the *Chlorella* Growth Factor (CGF) contained in *C. vulgaris* has been reported to have medical effects such as improvement and prevention of stroke, anticancer effect (Noda et al., 1996; Justo, 2001), growth promoting factor of animals and plants, and prevention of adult diseases (Hasegawa et al., 1995). In the fisheries industry, *C. vulgaris* is effective in improving pigment deposition, immunity, and antioxidant activity of cultured fish and crustaceans (Sergejevová and Masojídek, 2012; Xu et al., 2014), and is also used as a feed additive for aquaculture feed because it is rich in essential amino acids and polyunsaturated fatty acids (Enyidi 2017).

Therefore, this study initially investigates the effect of LED light intensity and wavelength on the growth of *C. vulgaris* to understand its potential as an effective biotechnological material. We identified the optimal wavelength for the growth of *C. vulgari*s. Additionally, by measuring changes in the content of carbohydrates, proteins, and lipids according to LED light intensity and wavelength, we determined the most effective wavelengths for promoting the

synthesis of useful substances. These results are anticipated to provide foundational data for the development of high-efficiency and functional photobioreactor systems and their future industrial applications.

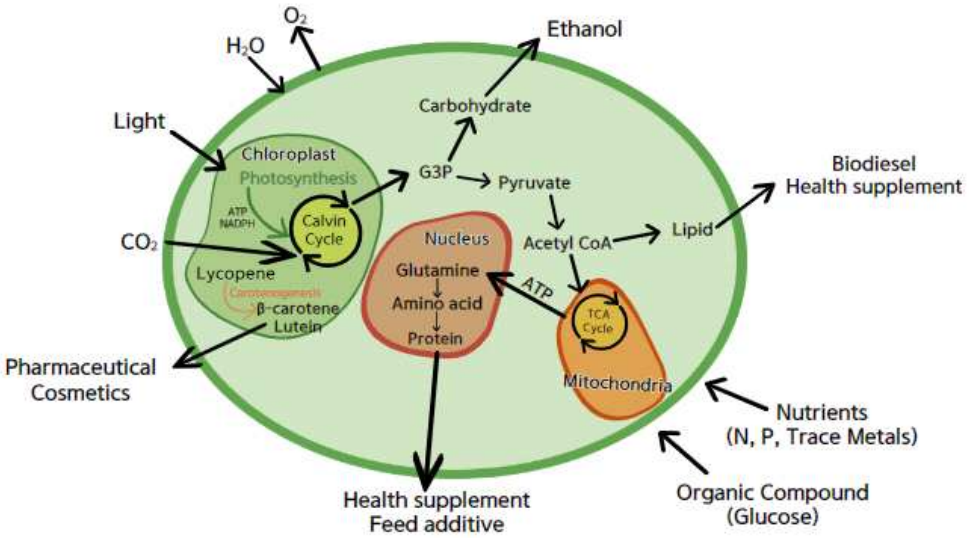

Figure 1. Commercially important metabolic pathways in microalgae.

2. Materials and Methods

2.1 Culture Conditions

The experiment utilized the green algae *Chlorella vulgaris,* including both the wild type PKVL7422 and a genetically modified strain of PKVL7422. This modified strain was engineered to produce a significant amount of growth hormone by incorporating a flounder growth hormone gene. The medium for the wild type was BG11 (Stanier et al., 1971; Anderson, 2005), prepared with ultra-pure ion water. In contrast, the genetically modified PKVL7422 strain, unable to utilize $NaNO_3$ as a nitrogen source due to the knockout of the nitrogen reductase gene, was cultured in BGP medium with $(NH_4)_2HPO_4$ as the nitrogen source. Cultures were maintained at a water temperature of 20℃ and a salinity of 0 psu, under lighting conditions of approximately 100 μ mol/m^2/s (12L:12D cycle) using cool-white fluorescent lamps. Although cultures were not sterilized, to prevent secondary biological contamination, experimental equipment was subjected to either high-pressure treatment (202 kPa for 30 min) or dry sterilization (185℃ for 1 hr). All experiments were conducted within a clean bench.

2.2 Characterization of Growth Specificity of *C. vulgaris* Based on Wavelengths

2.2.1 Monochromatic LED

The light source for the growth experiments was a fluorescent lamp (three-wavelength lamp, Namyoung Bulb Co., Ltd., Seoul, Korea) with multiple wavelengths. Single wavelengths of blue (λmax=450 nm), green (λmax=520 nm), and red (λmax=660 nm) were provided by LUMILEDS (Mansfield, TX, USA). The light intensity was adjusted to 5 levels (10, 50, 75, 100, and 200 μmol/m^2/s) using a visible light blocking film and a QSL-2100 instrument (Biospherical Instrument Inc., San Diego, CA, USA), with a light/dark cycle set to 12L:12D. Temperature and salinity conditions were consistent with those of the maintenance culture. After growing *C. vulgaris* to the exponential phase, it was inoculated into 1 L culture flasks to achieve a cell density of 1.2x10^5 cells/mL. Cell density was determined by measuring dry weight, 10 mL of cell culture was filtered (25 mm, GF/F), and the filter paper was dried in a dry oven at 75℃ for 2 hours before weighing. Dry weight was measured every two days.

The growth rate was calculated using the following formula, with experiments conducted in triplicate. The average value was reported, excluding any clearly erroneous values during the triplication:

$$\mu = \frac{1}{\Delta t} \ln \frac{N_t}{N_0} \cdots\cdots\cdots\cdots\cdots\cdots (1)$$

μ: growth rate (specific growth rate; /day)

N_0, N_t: Fluorescence value at the beginning and t hours (day) after the exponential phase

△t: Period of exponential phase (day)

The relationship between growth rate and light intensity was calculated using the following formula (2), which improved the model of Lederman and Tett(1981).

$$\mu = \mu_{\max} \frac{I - I_0}{(K_S - I_0) + (I - I_0)} \cdots\cdots\cdots\cdots (2)$$

μ: growth rate (specific growth rate; /day)

μ_{max}: maximum growth rate (maximum specific growth rate; /day)

I: light intensity (irradiance; μmol/m^2/s)

I_0: compensation light intensity (compensation photon flux density; μmol/m^2/s)

K_s: half-saturation light intensity (half-saturation light intensity; μmol/m^2/s)

2.2.2 Two-phased Culture

The first step in the two-stage culture involved irradiating with red LED to increase cell density until the late exponential phase. After reaching the stationary phase, blue LED was used to stimulate the accumulation of useful substances. The light intensity was set to 100 μmol/m^2/s (L:D=12L:12D) for all wavelengths. Temperature and salinity conditions were maintained as per the maintenance culture. Upon reaching the exponential phase, *C. vulgaris* was inoculated into 1 L culture flasks to achieve a cell density of $1.2x10^5$ cells/mL. The cell density was calculated by dry weight as mentioned in "2.2.1 Monochromatic LED", and the growth rate was calculated according to

formula (1).

2.2.3 LED Photoperiod

For the growth experiment based on light cycle, blue and red LEDs were tested with three light cycles of 24L:00D. 16L:8D, 12L:12D, which are extreme light cycle treatments without dark phase from 24L:00D to short light phase of 16L:08D. The temperature and salinity were the same as the maintenance culture conditions, and after growing *C. vulgaris* to the exponential phase, it was inoculated into a 1 L culture flask so that the cell density was $1.2x10^5$ cells/mL. The cell density was calculated by dry weight as mentioned in "2.2.1 Monochromatic LED", and the growth rate was calculated according to formula (1).

2.2.4 Mixed Wavelengths

For the growth experiment with mixed LED wavelengths, blue and red LEDs were combined in ratios of 5:5, 7:3, and 3:7, respectively. The light intensity was adjusted to approximately 100 μmol/m^2/s (12L:12D). Temperature and salinity conditions were as per the maintenance culture. *C. vulgaris,* grown to the exponential phase, was inoculated into 1 L culture flasks to achieve a cell density of $1.2x10^5$ cells/mL. Cell density and growth rate were calculated as in "2.2.1 Monochromatic LED", using formula (1).

2.3 Biochemical Composition Analysis of *C. vulgaris*

2.3.1 Experimental Condition

To measure the biochemical composition relative to light intensity and wavelength, experiment 2.2.1 was conducted at light intensities of 10 and 100 μmol/m^2/s (L:D=12L:12D) using blue, green, red, and fluorescent lamps(multiple wavelengths). Experiment 2.2.2 used red wavelengths, which are effective light sources for increasing cell density until the late exponential phase, and blue wavelengths were irradiated from the stationary phase to enhance useful substances (L:D=12L:12D). In experiment 2.2.3, only the L:D cycle was adjusted under the red single wavelength and blue single wavelength, respectively, and was adjusted to 12L:12D, 16L:08D, 24L:00D. Finally, in experiment 2.2.4, the blue wavelength and red wavelength were adjusted to a constant ratio of 7:3, 5:5, 3:7 (L:D=12L:12D). The temperature and salinity were the same as the maintenance culture conditions, and after growing *C. vulgaris* to the exponential phase, it was inoculated into 1 L culture flask so that the cell density was 1.2x10^5 cells/mL. For analysis, the cell culture solution during the mid-exponential phase and the stationary phase was filtered (47 mm, GF/F), and the filter paper was dried in a dry oven (75℃, 2 hours) and stored in a cold dark place (-70℃) until analysis.

2.3.2 Carbohydrate

Carbohydrates were analyzed using the Phenol-sulphuric acid method according to Dubios et al. (1956). A standard solution was prepared using a

glucose standard, and 1 mL of 5% phenol was added and left at room temperature for 40 minutes to extract intracellular carbohydrates. Then, 5 mL of H_2SO_4 was added and left for 10 minutes, and then mixed evenly and centrifuged at 2000 rpm for 15 minutes. After that, the absorbance at 490 nm was measured using a UV/Vis spectrophotometer (X-ma, 3000PC).

2.3.3 Protein

Proteins were analyzed using the method of Lowry et al. (1951). A protein standard solution was prepared, and 5 mL of alkaline copper solution was added and mixed evenly to extract the protein. Then, 0.5 mL of folin ciocalteu was added and left for 1 hour and 30 minutes, and then mixed evenly. After centrifuging at 3000 rpm for 10 minutes, the absorbance at 750 nm was measured using a UV/Vis spectrophotometer (X-ma, 3000PC).

2.3.4 Lipid

Lipids were analyzed by the method of Marsh and Weinstein (1966). Chloroform (1 mL) and methanol (2 mL) were added to the filter paper, which was then finely crushed and mixed. The mixture was refrigerated for 1 hour to prevent solvent evaporation, followed by centrifugation at 2000 rpm for 10 minutes. Distilled water was added to the extracted lipids to separate the lipid and chloroform layers from the water and methanol layers. The upper layers were removed, and the residue was dried in a dry oven at 40℃ for 48 hours. Then, 2 mL of H_2SO_4 was added and heated in a 200℃ block heater for 15 minutes. After adding 3 mL of ultra-pure water, the absorbance at 360

nm was measured using a UV/Vis spectrophotometer (Xma-3000PC, Human Cop., Seoul, Korea).

2.3.5 Pigment

The process of measuring pigment concentrations was described by Hyun et al. (2022), and we followed their detailed procedure. To maximize extraction efficiency, the filter papers were freeze-dried prior to extraction, soaked in 4 ml of an aqueous acetone solution (5:95 v:v), wrapped with aluminum foil to prevent exposure to light, and stored in a refrigerator at 4°C for 24 hours (Hyun et al., 2022; Hyun et al., 2023). The extracts were filtered again using 0.2-µm polytetrafluoroethylene syringe filters (Hyundai Micro, Seoul, Korea) to remove particles that may damage the HPLC system. After filtration, 1 ml of each filtrate was transferred into brown amber vials and 400 µl of HPLC-grade water was added. The pigments were separated and measured using an HPLC system (LC-2030c 3D; Shimadzu Corporation, Kyoto, Japan) following a modified version of the protocol described by Zapata et al. (2000). Specifically, the separation of pigments was performed through reverse-phase chromatography using a C8 column (150 × 4.6 mm, 3.5 µm particle size, 100 Å pore size; Waters Corporation, Milford, MA, USA). The concentrations of the separated pigments were measured using the 440-nm chromatogram detected with a photodiode-array detector. Additionally, the purity of each peak was confirmed through measurement of wavelengths from 370 to 800 nm. The factors used to convert peak area to pigment concentrations were determined on an annual basis prior to analysis when the

C8 column was replaced for maintenance. This process involved analysis of standard pigments (DHI LAB, Hørsholm, Denmark) and the generation of a calibration curve. Additionally, to facilitate peak identification, a mixture of standard pigments was evaluated (first and last samples daily) during HPLC operation.

3. Results and Discussion

3.1 Growth of *Chlorella vulgaris* as Influenced by Wavelength and Light Intensity

3.1.1 Monochromatic LED

Examining the relationship between light intensity, time, and dry weight of *C. vulgaris* across different wavelengths, we observed an increase in dry weight (indicating increased cell density) with rising light intensity and duration (Fig. 1). The maximum growth rate (μ_{max}) was high in blue wavelength (1.03 /day) and red wavelength (0.95 /day), followed by green wavelength (0.89 /day), and fluorescent lamp (0.83 /day) (Table. 1). Also, the minimum light intensity, i.e., the compensation light intensity(I_0), where cells can grow, meaning the same amount of respiration and photosynthesis, was the lowest in blue (15.90 μmol/m^2/s) and red wavelengths (11.26 μmol/m^2/s), followed by green wavelength (21.23 μmol/m^2/s) and fluorescent lamp (21.92 μmol/m^2/s). However, the affinity indicator (K_s) for light was about three times lower in red wavelength (47.08 μmol/m^2/s) compared to blue wavelength (137.60 μmol/m^2/s) (Fig. 2; Table 1).

Similarly, for the genetically transformed strain, an increase in dry weight was observed with increasing light intensity and duration, regardless of wavelength, without photoinhibition at certain intensities (Fig. 3). In terms of dry weight, it was highest in red wavelength at 2.2 g/L, followed by fluorescent lamp (1.7 g/L), blue wavelength (1.7 g/L), and green wavelength

(1.5 g/L). The growth rate was highest under the red wavelength (0.76 /day) and the blue wavelength (0.75 /day), while it was lower under both the fluorescent lamp (0.71 /day) and the green wavelength (0.71 /day) (Fig. 3; Table 2). Both the wild type and the genetically transformed strain showed high growth rates in red and blue wavelengths.

High growth rates in blue wavelengths have been reported in green algae *Nannochloropsis* sp. (Das et al., 2011) and the chrysophyceae *Isochrysis galbana* (Yoshioka et al., 2012), while high growth rates in red wavelengths have been reported in *S. platensis* (Wang et al., 2007) and green algae *C. pyrenoidosa* (Chu et al., 2021). Lee and Plasson (1994) and Yan et al. (2013) reported that *C. vulgaris* showed rapid growth in red wavelengths. Kim et al. (2014) reported that the cell density of *C. vulgaris* grown in red wavelengths was 1.5 times higher than that in blue wavelengths. Khalili et al. (2015) also conducted studies supporting the idea that *C. vulgaris* exhibits rapid growth in red wavelengths. Additionally, Habibi and Sibi (2019) reported that blue wavelengths (420-450 nm) and red wavelengths (660-700 nm) are effective for the growth of *C. vulgaris*.

Blue wavelengths are involved in photosynthesis and energy activation, help regulate gene transcription and enzyme activation (Ruyters et al., 1984), and phytochrome A (phy A gene; Lariguet and Fankhauser, 2004) that regulate phototropism and activate blue light receptors such as phototropin (plot 1 and plot 2; gene Takemiya et al., 2005), so they can show high growth rates. In addition, blue wavelengths act as catalysts to stimulate the photosynthetic pigments of microalgae (Atta et al., 2013), enhancing pigment accumulation

such as cryptochrome, a flavoprotein that detects blue light (Ma et al., 2012), and ultimately increasing chl-*a* and β-carotene, the main pigments of *C. vulgaris* (Baidya et al., 2021). Thus, the high growth rate in blue wavelengths is thought to be the result of accelerated cell division due to complex factors such as gene transcription and enzyme activity.

The high growth rate and low K_s in red wavelengths seem to be due to auxiliary pigments such as chl-*b* (chl-*b*) contained in green algae in addition to chl-*a*. chl-*b* absorbs blue wavelengths and red wavelengths in the range of 455 nm and 642 nm and transfers light energy to chl-*a*, so it is thought to have shown a high growth rate even in red wavelengths. Cunningham et al. (1990) reported that red wavelengths increase the activity of photosystem II in the red algae *Porphyridium cruentum* by more than three times compared to green wavelengths. In fact, *Chlorella* sp. is reported to contain high amounts of major photosynthetic pigments such as chl-*a* and chl-*b* (Richmond, 2003; Nurachman et al., 2015). Moreover, Oh et al. (2015) suggested that red wavelengths can be efficiently used for growth depending on the possession of auxiliary pigments such as chl-*b* that can efficiently absorb red wavelengths.

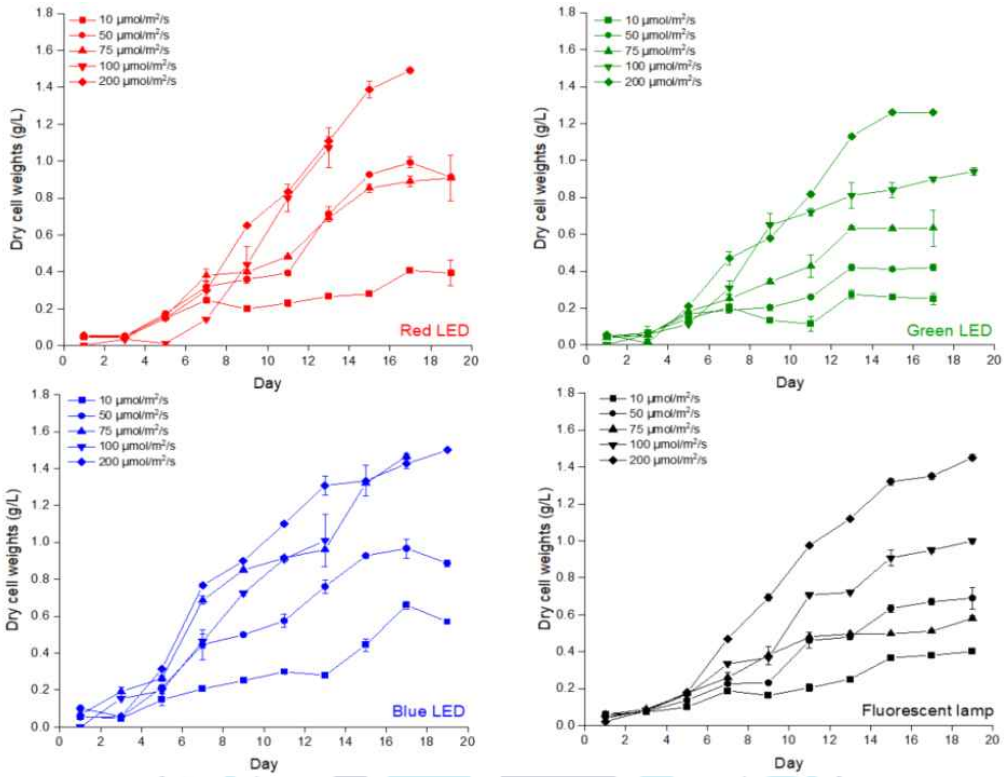

Figure 2. Growth curves of *Chlorella vulgaris* (PKVL7422) cultured at various irradiance under red LED, green LED, blue LED and fluorescent lamp.

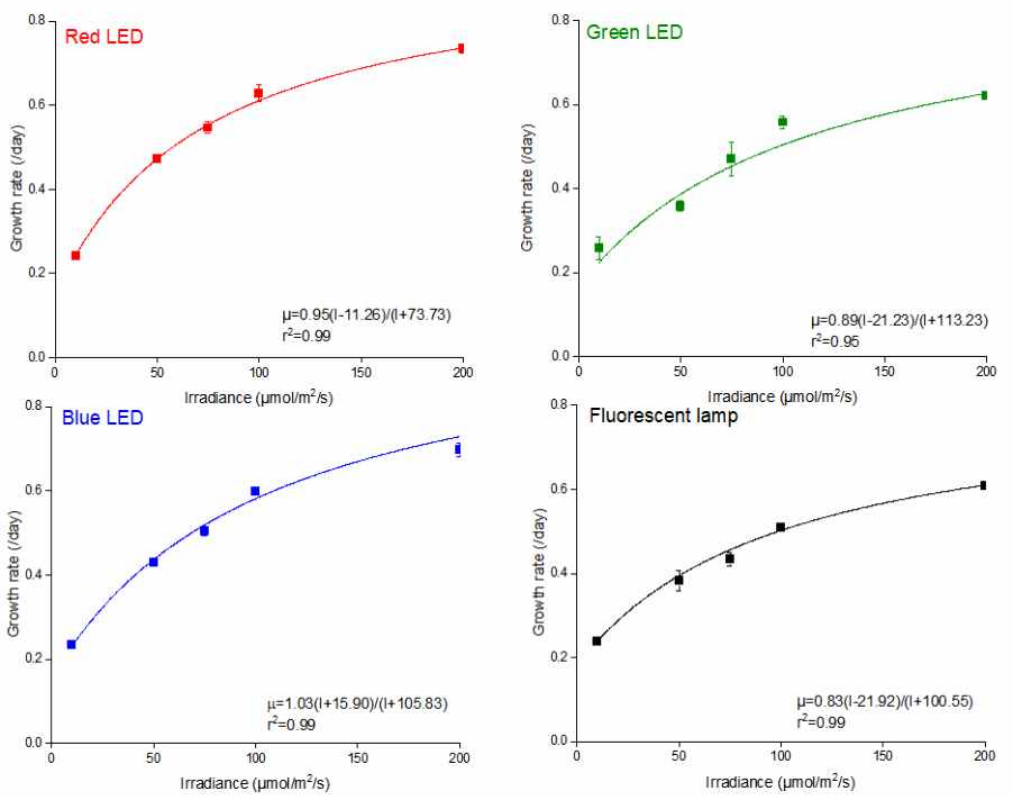

Figure 3. Specific growth rates of *Chlorella vulgaris* (PKVL7422) cultured under red LED, green LED, blue LED and fluorescent lamp.

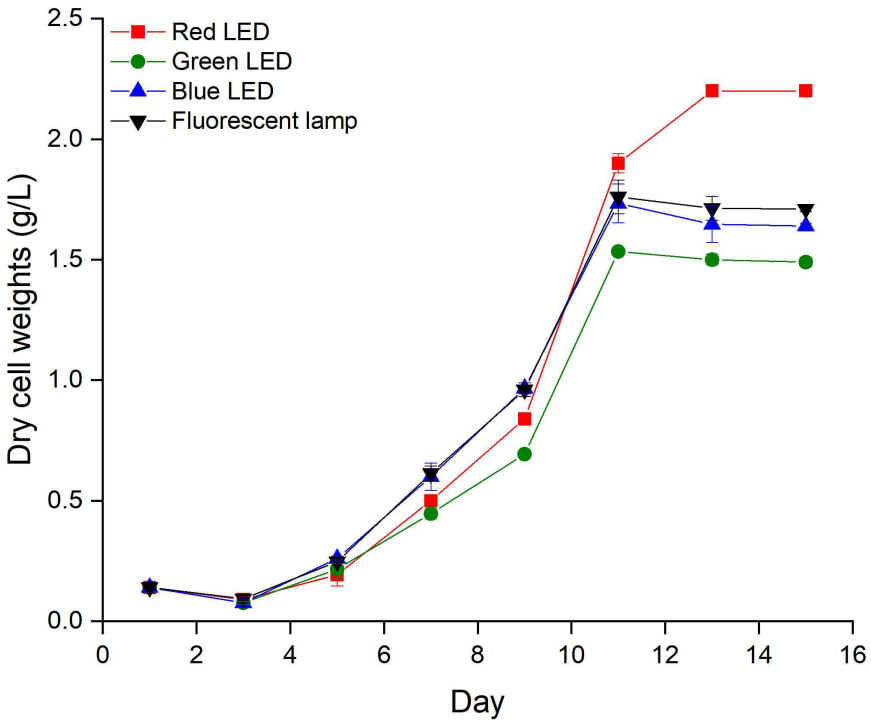

Figure 4. Growth curves of *Chlorella vulgaris* (transformed PKVL7422) cultured under red LED, green LED, blue LED and fluorescent lamp.

Table 1. Parameters of *Chlorella vulgaris* (PKVL7422) as a function of light intensity under red LED, green LED, blue LED and fluorescent lamp.

Wavelengths	Hyperbolic equation	μ_{max} (/day)	I_0 ($\mu mol/m^2/s$)	Ks ($\mu mol/m^2/s$)
Fluorescent lamp	μ=0.83(I-21.92)/(I+100.55)	0.83	21.92	144.39
Blue LED	μ=1.03(I-15.90)/(I+105.83)	1.03	15.90	137.63
Green LED	μ=0.89(I-21.23)/(I+113.23)	0.89	21.23	155.69
Red LED	μ=0.95(I-11.26)/(I+73.73)	0.95	11.26	47.08

Table 2. Growth rates of *Chlorella vulgaris* (transformed PKVL7422) cultured under red LED, green LED, blue LED and fluorescent lamp.

Wavelengths	Growth rate (/day)
Red LED	0.76
Blue LED	0.75
Green LED	0.71
Fluorescent lamp	0.72

3.1.2 Two-phased Light Culture

In the first stage, which showed high growth rates and dry weight until the late exponential phase, red wavelength light, characterized by the lowest light affinity index (K_s) and effective in increasing cell density, was used. From the late exponential to the stationary phase, blue wavelengths were irradiated to enhance the accumulation of useful substances (Fig. 4). Red wavelength was used for 15 days until the late exponential phase and the stationary phase, and the wavelength was changed from the stationary phase, and the blue wavelength was irradiated for 4 days. The growth rate was 0.56 /day, similar to the red wavelength (0.55 /day) with a light intensity of 100 μ mol/m^2/s.

Ra et al. (2016) reported results similar to our study, stating that the microalgae *N. salina*, *N. Oceanica*, and *N. oculata* exhibited effective biomass enhancement with blue wavelength injection until the late exponential growth phase. In multi-stage cultivation with green wavelength injection from the exponential growth phase, the cell growth rate was reported to be similar to that observed when exposed to a single blue wavelength. Additionally, Kim et al. (2019) found that *C. vulgaris*, injected with red wavelength for substantial microalgae biomass accumulation until the late exponential growth phase, displayed a growth rate comparable to that observed with single red wavelength injection, even when injected with green wavelength from the exponential growth phase for enhanced metabolite production. This corresponds with our study, where growth rates were measured using cells from the exponential growth phase for batch cultivation. Multi-stage cultivation involved

altering wavelengths during the stationary phase when cell proliferation slowed down and cell count increase ceased, making the growth rates comparable to the results of the single-wavelength LED experiments. Therefore, growth rates in multi-stage cultivation are considered similar to those obtained in single-wavelength experiments due to differences in microalgae pigment composition.

Richmond (2003) noted that all chlorophylls have major absorption bands at blue (400 – 500 nm) and red (600 – 700 nm) wavelengths, aligning with the chlorophyll absorption maxima. Korbee et al. (2005) pointed out that microalgae growth can be enhanced using blue or red light. This observation is attributed to the fact that the absorption of energy by photosynthetic organisms depends on the chemical composition of their pigments (Carvalho et al., 2011). Furthermore, the *Chlorella* strain used in our study, being a green algae, contains chlorophyll *a*, *b*, and the accessory pigment carotenoid for photosynthesis.

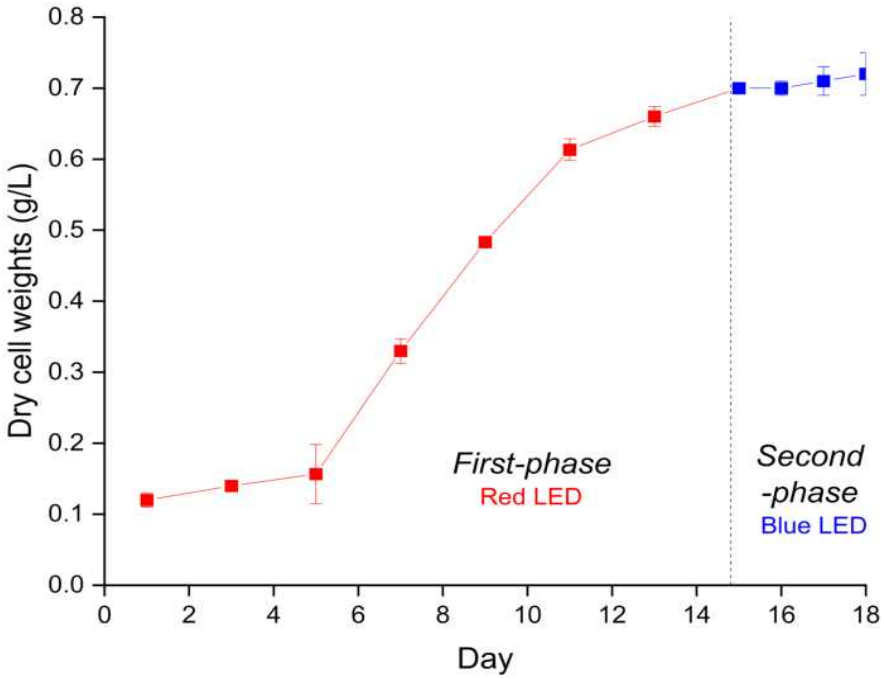

Figure 5. Two-phase cultures of *Chlorella vulgaris* (PKVL7422) involving biomass production in the first phase and lipid, protein production in the second phase.

3.1.3 LED Photoperiod

Both red and blue wavelengths demonstrated rapid growth rates when the light and dark cycle was set to 12L:12D, showing the lowest growth rates at 24L:00D (Fig. 6). This suggests that microalgae can grow sufficiently under high-intensity light, rendering a prolonged light cycle unnecessary (Wahidin et al., 2013). The fastest growth rate under red wavelength at 12L:12D (0.63 /day) was about 1.31 times faster than the slowest growth rate at 24L:00D (0.48 /day) (Table 3). Similarly, the highest growth rate under blue wavelength at 12L:12D (0.59 /day) was approximately 1.25 times faster than the lowest at 24L:00D (0.47 /day) (Table 3). Hence, adjusting the light and dark cycle to 12L:12D is an efficient culture condition that can optimize the growth of *C. vulgaris*.

Similar to our study findings, Meseck et al. (2005) reported that *Tetraselmis chui* achieved maximum cell biomass production under a 24L:00D photoperiod (among 12:12, 18:06, and 24:00 h dark/light cycles) at a light intensity of 110 μmol/m^2/s. Additionally, in Wahidin et al. (2013) research, *Nannoclopsis* sp. exhibited the highest cell density when subjected to a 24L:00D light/dark cycle (among 12:12, 18:06, and 24:00 h dark/light cycles) at a light intensity of 50 μmol/m^2/s.

Optimal photosynthetic efficiencies are achieved when the light/dark cycle period approaches the photosynthetic unit turnover time. The conversion process is traditionally divided into two stages: light reactions and dark reactions. In light reactions, occurring on the photosynthetic membranes of chloroplasts, light energy is converted into chemical energy, providing

$NADPH_2$ and the high-energy compound ATP (Wahidin et al., 2013). Dark reactions, or enzymatic reactions, take place in the chloroplast stroma (Wahidin et al., 2013). According to Anderson (2005), optimal light/dark regimes have been observed to range from 12:12 to 16:08 for most cultures. Exceeding a saturation point in light can lead to light inhibition, which can be mitigated by subjecting microalgal cells to very short cyclic periods of light and darkness (Pulz, 2001).

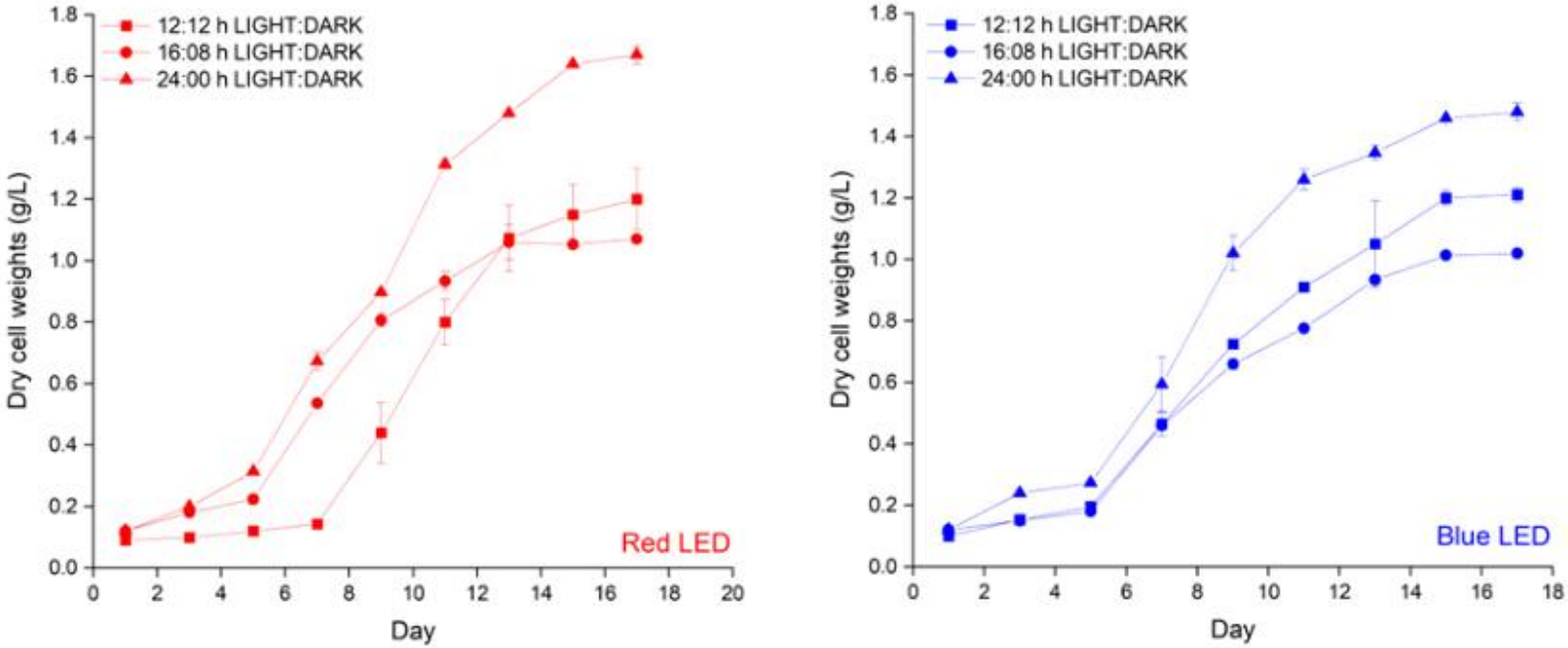

Figure 6. Growth curves of *Chlorella vulgaris* (PKVL7422) were observed under blue LED, red LED, and photoperiods of 12:12, 16:08, 24:00 h L:D.

Table 3. Growth rate of *Chlorella vulgaris* (PKVL7422) were observed under blue LED, red LED, and photoperiods of 12:12, 16:08, 24:00 h L:D.

Wavelengths	Light/Dark(L/D) cycle (h)	Growth rate (/day)
Red LED	12:12	0.63
	16:08	0.54
	24:00	0.48
Blue LED	12:12	0.59
	16:08	0.54
	24:00	0.47

3.1.3 Mixed Wavelengths

The dry weights in the 7:3 and 3:7 wavelength ratios showed similar values, while the lowest dry weight was observed in the 5:5 ratio (Fig. 6). The growth rate was highest at 0.49 /day in the 7:3 ratio, which has a higher proportion of red wavelengths, and lowest at 0.41 /day in the 3:7 ratio, with the lowest proportion of red wavelengths (Table 4). Observing the genetically transformed strain, similar to the wild type, the dry weights of the 7:3 and 5:5 ratios were similar, with the lowest in the 3:7 ratio, which has a high proportion of blue wavelengths (Fig. 7). The growth rate was highest at 0.50 /day in the 7:3 ratio and lowest at 0.47 /day in the 3:7 ratio (Fig. 7; Table 4). This is comparable to the wild type's 7:3 ratio (0.49 /day), and the slowest growing 3:7 ratio was about 1.1 times faster than the wild type (0.41 /day). Also, the growth rate of blue and red single wavelengths were 0.60 /day and 0.63 /day, respectively, slower than the mixed wavelengths.

Ruyters (1984) mentioned that blue and red wavelengths could induce photosystems I and II, respectively, and an appropriate ratio of red to blue wavelengths could lead to high biomass in microalgae. According to Kim et al. (2013), *Scenedesmus* sp. exhibited the highest biomass productivity in mixed wavelengths combining blue and red light. Moreover, Yan and Zheng (2014) conducted a study indicating that the dry weight was highest when the ratio of red to blue wavelengths was 5:5, rather than with single red or blue wavelengths.

The reason for the lower dry weight and growth rate observed in mixed wavelengths compared to single wavelengths is believed to be due to the stress-inducing role of blue light. According to the results of Kim et al.'s (2014) study, the application of blue light was reported to act as a stress factor, leading to an increase in cell size. Therefore, it is inferred that both the dry weight and useful substances within the cell increased. Consequently,

to investigate whether blue light acts as a factor enhancing photosynthetic efficiency in *Chlorella vulgaris*, as suggested in the results of Section 3-1-1, or conversely acts as a stress factor, additional research involving the measurement of reactive oxygen species (ROS) inside the cells and wavelength-specific cell size measurements would be necessary.

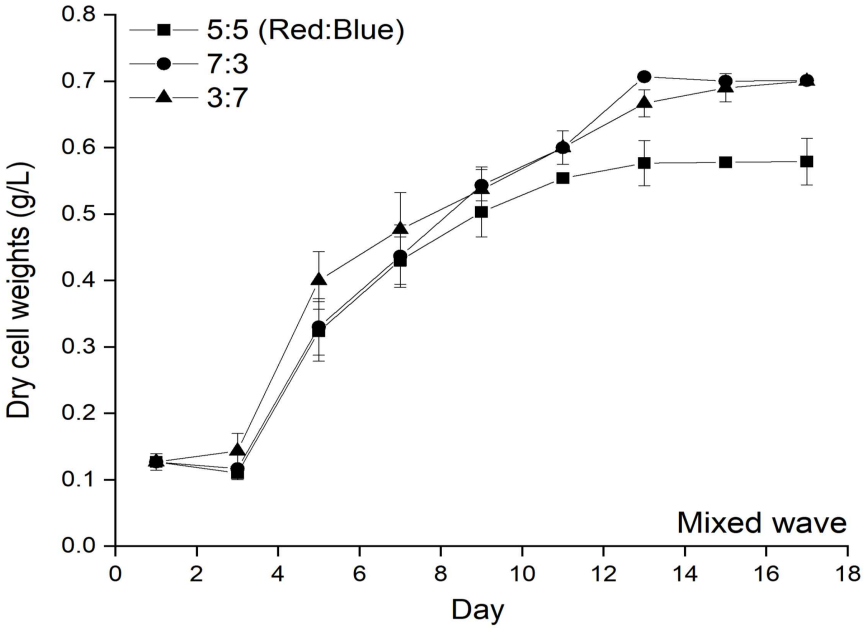

Figure 7. Growth curve of *Chlorella vulgaris* (PKVL7422) under mixed wavelengths.

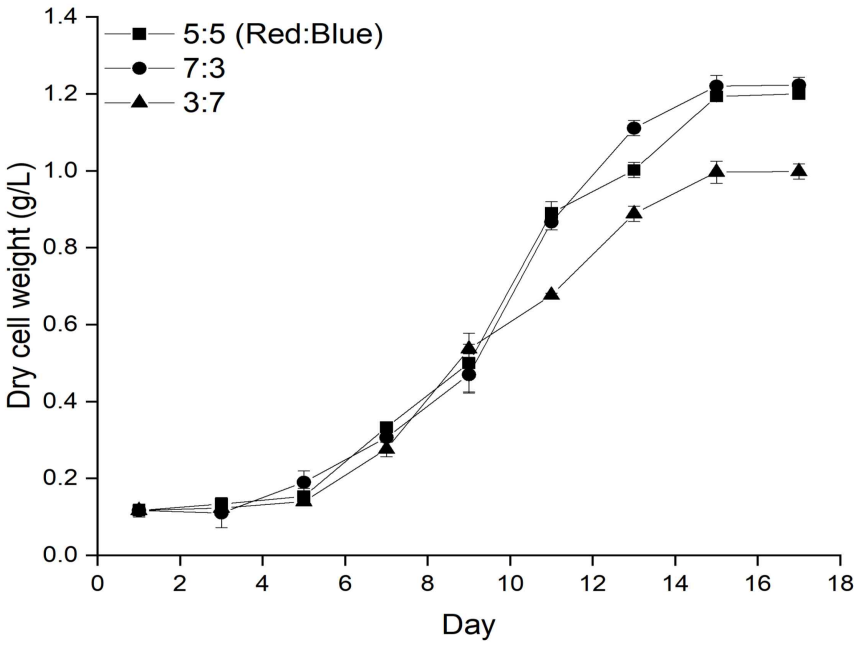

Figure 8. Growth curves of *Chlorella vulgaris* (transformed PKVL7422) under mixed wavelengths.

Table 4. Growth rate of *Chlorella vulgaris* (PKVL7422 and transformed PKVL7422) under mixed wavelengths.

	Wavelengths	Growth rate (/day)
PKVL7422	5:5 (Red:Blue)	0.48
	7:3	0.49
	3:7	0.41
transformed PKVL7422	5:5 (Red:Blue)	0.49
	7:3	0.50
	3:7	0.47

3.2 Biochemical Composition Analysis of *C. vulgaris*

3.2.1 Monochromatic LED

The carbohydrate content of the wild type did not show a clear difference by wavelength, but showed a large difference depending on the light intensity (Fig. 8). The green wavelength (0.26 /day), which showed a low growth rate in the stationary phase, showed the highest content (320 μg/g), while the blue wavelength (0.60 /day) in the stationary phase with a light intensity of 100 μmol/m^2/s showed a low content (133 μg/g). Also, the green wavelength (0.89 /day), which showed a low μ_{max}, showed about 1.29 times higher carbohydrate content than the fluorescent lamp and about 1.33 times higher than the blue wavelength. The carbohydrate ratio of the wild type, when viewed as a total biochemical ratio (carbohydrates, proteins, lipids), accounted for 24~32% of the carbohydrate content in the exponential phase at a light intensity of 10 μ mol/m^2/s, and 26~39% in the stationary phase. At a light intensity of 100 μ mol/m^2/s, the carbohydrate content in the exponential phase was 11~19%, and the carbohydrate content in the stationary phase was 11~13%, showing a relatively high ratio at low light intensity. The average carbohydrate content of the transformed strain was about 38.7 μg/g, which was about 3.58 times lower than the wild type (138 μg/g) (Fig. 9). Also, the transformed strain showed about 1.42 times higher carbohydrate content in the stationary phase than in the exponential phase, and there was no significant difference in carbohydrate content by wavelength in the stationary phase ($P>0.05$) (Fig. 14). Also, the carbohydrate ratio of the transformed strain, when viewed as a total

biochemical ratio, accounted for 2~4% of the carbohydrate content in the exponential phase and 3~4% in the stationary phase, which was relatively low compared to other biochemical constituents.

Suyono et al. (2014), *Chromochloris zofingiensis* showed a high dry weight in high light intensity of blue wavelength (9,000 lux; about 924 μmol/m^2/s), but the carbohydrate content was low, and it showed a high content at low light intensity (3,000 lux; about 346 μmol/m^2/s). He et al. (2023) also reported that *C. sorokiniana* showed the highest dry weight in white wavelength, but the carbohydrate content did not show a large difference by wavelength, which was similar to the results of this study. Also, according to the results of Korozi et al. (2023) reported that *C. vulgaris* exhibited a high growth rate under red and blue wavelengths, with the carbohydrate content showing minimal variation by wavelength. The carbohydrates produced at high light intensity are used as glucose, an energy source for cell division, rather than starch, a storage material in cells, reducing carbohydrate content (Chen et al., 2013; Suyono et al., 2014). Conversely, the increase in carbohydrate content at low light intensity means a decrease in cell division speed changes the enzyme activity and chemical composition of cells, increasing carbohydrate content (Markou, 2014), and a decrease in enzyme activity because enzyme activity is inversely proportional to carbohydrate degradation speed (Loganathan et al., 2020). Therefore, it is thought that *C. vulgaris* showed a high carbohydrate content in low light intensity, which acts as an environmental factor that stresses growth.

The protein content of the wild type was highest under blue wavelength

(509 μg/g) in the stationary phase light intensity 100 μmol/m^2/s, and appeared in the order of fluorescent lamp (461 μg/g), green wavelength (453 μg/g), red wavelength (355 μg/g) (Fig. 10). The blue wavelength of the stationary phase light intensity 100 μmol/m^2/s showed about 1.10 times higher protein content than the fluorescent lamp. Depending on the light intensity, it showed a higher content at 100 μmol/m^2/s than at 10 μmol/m^2/s, and the blue wavelength of the stationary phase light intensity 100 μmol/m^2/s showed about 1.53 times higher content than at 10 μmol/m^2/s. Also, depending on the growth stage, it showed a higher content in the stationary phase than in the exponential phase. The average protein content of the transformed strain was about 340 μg/g, which was about 0.88 times higher than the wild type (385 μg/g), but there was no big difference in protein content between the two strains. Also, the protein ratio of the transformed strain, when viewed as a total biochemical ratio, accounted for 30~41% of the protein content in the exponential phase and 30~39% in the stationary phase (Fig. 15).

Asuthkar (2016) reported that *C. pyrenoidosa* displayed the highest protein content under blue wavelengths, where it also showed the highest growth rate. Habibi and Sibi (2019) noted that the green algae *S. platensis, Scenedesmus obliquus*, and *C. vulgaris* showed high growth rates under red and blue wavelengths, with the protein content reaching maximum levels in blue and green wavelengths, varying according to the wavelength. Similarly, Baidya et al. (2021) observed that *C. ellipsoidea* had high dry weight and density under blue wavelength, with the highest protein content also in this wavelength range, aligning with the findings of our study. Blue wavelengths increase

amino acids such as aspartic acid, which constitute proteins, and glutamic acids used for protein synthesis, promoting gene transcription in photosynthesis and contributing to enzyme activation (Teo et al., 2014). In the case of *Chlamydomonas reinhardtii*, while only photosystem I is active at 705 nm, both photosystem I and II are active below 650 nm, allowing efficient photosynthesis in the blue wavelength range (450-500 nm) (Mulo et al., 2012). Kendirlioglu and Cetin (2017) reported a high growth rate and protein content in *C. vulgaris* under red wavelengths. Taufikurahman and Shafira (2019) observed that the protein content of *C. vulgaris* and *C. pyrenoidosa* did not significantly vary by wavelength. However, Guo and Fang (2020) reported that *C. pyrenoidosa* showed high cell density under blue wavelength, but the lowest protein content in this range. Therefore, it appears that *Chlorella* species and strains may exhibit varied biochemical composition responses to wavelength, but their basic photosynthetic pigment composition and enzyme activity seem similar. It is thus important to examine the physiological light responses across various strains.

The lipid content of the wild type was highest in the blue wavelength (715μg/g) of the stationary phase light intensity 100 μmol/m^2/s, and appeared in the order of red wavelength (576 μg/g), green wavelength (538 μg/g), and fluorescent lamp (526 μg/g) (Fig. 11). The blue wavelength of the stationary phase light intensity 100 μmol/m^2/s showed about 1.41 times higher lipid content than the fluorescent lamp. Depending on the light intensity, it showed a higher content at 100 μmol/m^2/s than at 10 μmol/m^2/s, and the blue wavelength of the stationary phase light intensity 100 μmol/m^2/s showed about

1.60 times higher content than at 10 μmol/m^2/s. Depending on the growth stage, the lipid content was 1.1~1.7 times higher in the stationary phase than in the exponential phase at a light intensity of 10 μmol/m^2/s, and 1.2~1.4 times higher at a light intensity of 100 μmol/m^2/s, showing a higher content in the stationary phase in all wavelengths. Also, the lipid ratio of the wild type, when viewed as a total biochemical ratio (carbohydrates, proteins, lipids), accounted for 37~41% of the lipid content in the exponential phase at a light intensity of 10 μmol/m^2/s, and 37~40% in the stationary phase. At a light intensity of 100 μmol/m^2/s, the lipid content in the exponential phase was 41~52%, and the lipid content in the stationary phase was 47~54%. Also, the transformed strain showed a high lipid content in the stationary phase, and showed the highest lipid content (985 μg/g) in the blue wavelength (Fig. 16). The average lipid content of the transformed strain was 809 μg/g, which was about 1.38 times higher than the wild type (588 μg/g). The lipid ratio of the transformed strain, when viewed as a total biochemical ratio, accounted for 55~66% of the lipid content in the exponential phase and 57~67% in the stationary phase. Both the wild type and the transformed strain showed a relatively high lipid content compared to other biochemical constituents, which was consistent with the reports of Chisti (2007) and Hu et al. (2008) that microalgae can synthesize lipids of 20~50% or more of their dry weight.

Teo et al. (2014) reported that *Nannochloropsis* sp. and *Tetraselmis* sp. showed the maximum lipid content in the blue wavelength where they exhibited a high growth rate. Baidya et al. (2021) found that *C. ellipsoidea* showed the highest lipid content in the blue wavelength, where it also

exhibited high dry weight and density. Similarly, Habibi and Sibi (2019) reported that *C. vulgaris* showed the highest lipid content in the blue wavelength, correlating with high dry weight. This is because the blue wavelength promotes the transcription of photosynthesis genes and activates enzyme activity, increasing the content (Ruyters et al., 2014). However, Hultberg et al. (2014) reported that *C. vulgaris* showed the highest biomass in the yellow wavelength, but the lipid content was highest in the purple wavelength where the biomass content was the lowest. Therefore, as mentioned in the protein, it can be seen that the specificity varies depending on the species or strain in green algae. These results suggest the need to confirm the light specificity of green algae isolated in Korea in the future.

The pigment content of wild type was higher at a light intensity of 100 μmol/m^2/s than at 10 μmol/m^2/s, and it was higher in the stationary phase than in the exponential growth phase (Fig. 12; Fig. 13). Also, most of the pigments were higher in the blue wavelength. In the case of the main pigments chl-*a* and chl-*b*, compared to the conventional mass culture light source, the fluorescent lamp, the blue wavelength was 2.64 and 2.14 times higher in the exponential growth phase and stationary phase at a light intensity of 10 μmol/m^2/s (Fig. 12), 1.33 times higher in the exponential growth phase and stationary phase at a light intensity of 100 μmol/m^2/s (Fig. 13). As for the content of auxiliary pigments by wavelength, violazanthin and lutein were highest in the blue wavelength regardless of light intensity, and compared to the fluorescent lamp, they were 2.42, 2.09 times higher in the stationary phase at a light intensity of 10 μmol/m^2/s, and 4.82, 1.15 times higher in the

stationary phase at a light intensity of 100 μmol/m^2/s. The pigment content of the transformed strain also showed high levels of the main pigments chl-*a* and chl-*b* in the blue wavelength (Fig. 17). In the transformed strain, the main pigments chl-*a* and chl-*b* were highly expressed in the blue wavelength. Also, when compared with conventional light source, fluorescent lamps, the main pigments chl-*a* and chl-b were 1.41 and 1.28 times higher in the exponential growth phase, and 1.40 and 4.56 times higher in the stationary phase, respectively. The results of the wavelength-specific content of auxiliary pigments showed that lutein, α-carotene, and β-carotene were highest in the blue wavelength regardless of the growth stage, and among them, the content of neozanthin, violazanthin, zeazanthin, lutein, α-carotene, and β-carotene was highest in the blue wavelength in the stationary phase. In this study, it was determined that both strains generally accumulate pigments effectively under light stress in the blue wavelength.

Katsuda et al. (2004) found that the green alga *Haematococcus pluvialis* increased astaxanthin production efficiency under blue wavelength (460 nm), and Fu et al. (2013) reported that *Dunaliella salina* increased β-carotene and lutein content with a mixed wavelength combining blue and red wavelengths. The benthic diatom *Nitzschia* sp. showed higher chl-*a* content under blue than under red and yellow wavelengths (Kwon et al., 2013). Therefore, it is believed that the change in pigment content and composition in microalgae according to environmental conditions appears to be species-specific. The higher protein, lipid and pigment content in the transformed strain compared to the wild type is attributed to the characteristics of the transformed strain. The

genetically modified strain has been engineered to enhance fish growth by knocking out the nitrogen-reducing enzyme gene present in the wild type and introducing a flounder growth hormone. Consequently, the genetically modified strain has the potential to efficiently produce energy and nutrients internally, aiming to enhance fish growth.

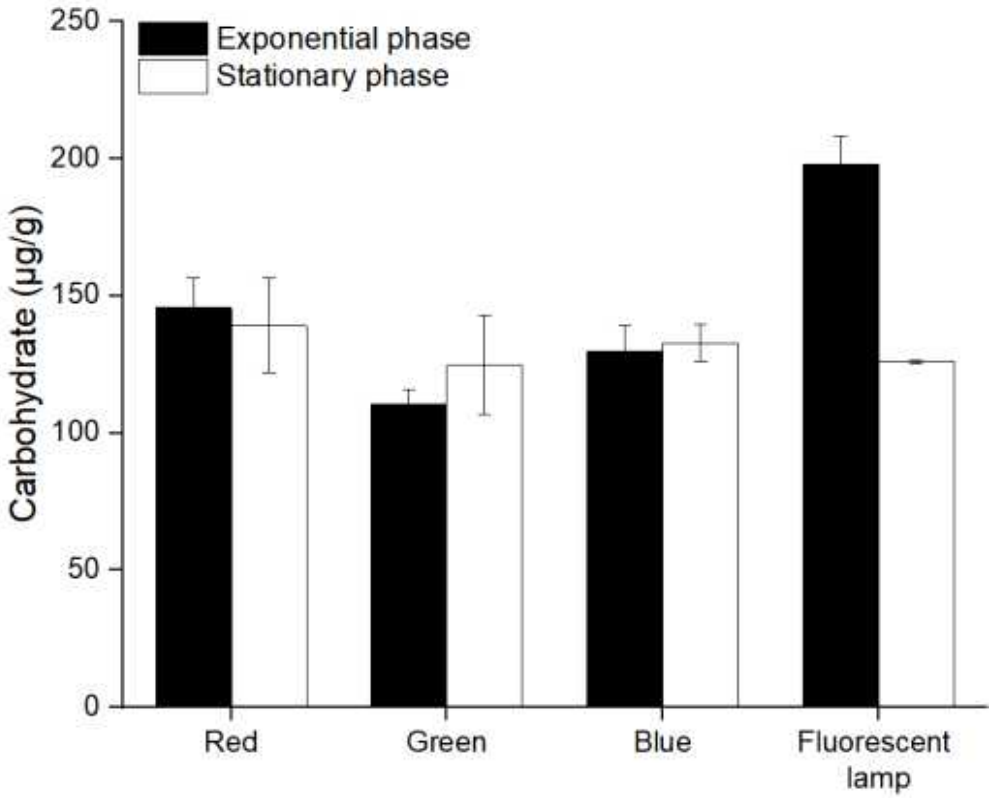

Figure 9. Carbohydrate changes in *Chlorella vulgaris* (PKVL7422) cultured under red LED, green LED, blue LED and fluorescent lamp.

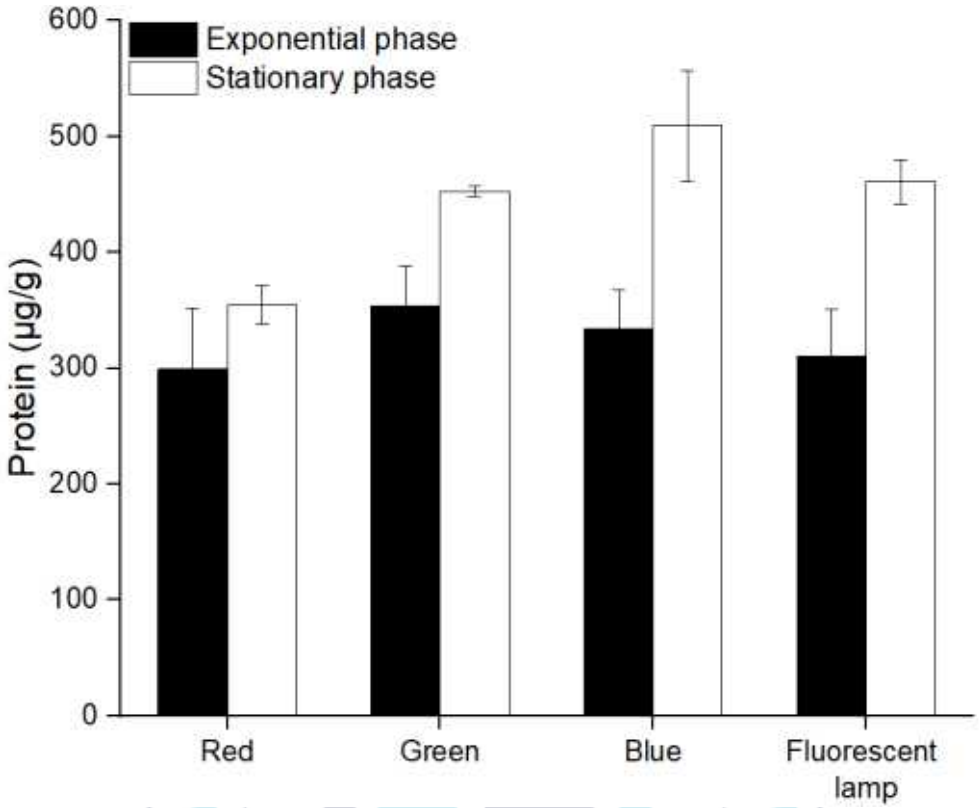

Figure 10. Protein changes in *Chlorella vulgaris* (PKVL7422) cultured under red LED, green LED, blue LED and fluorescent lamp.

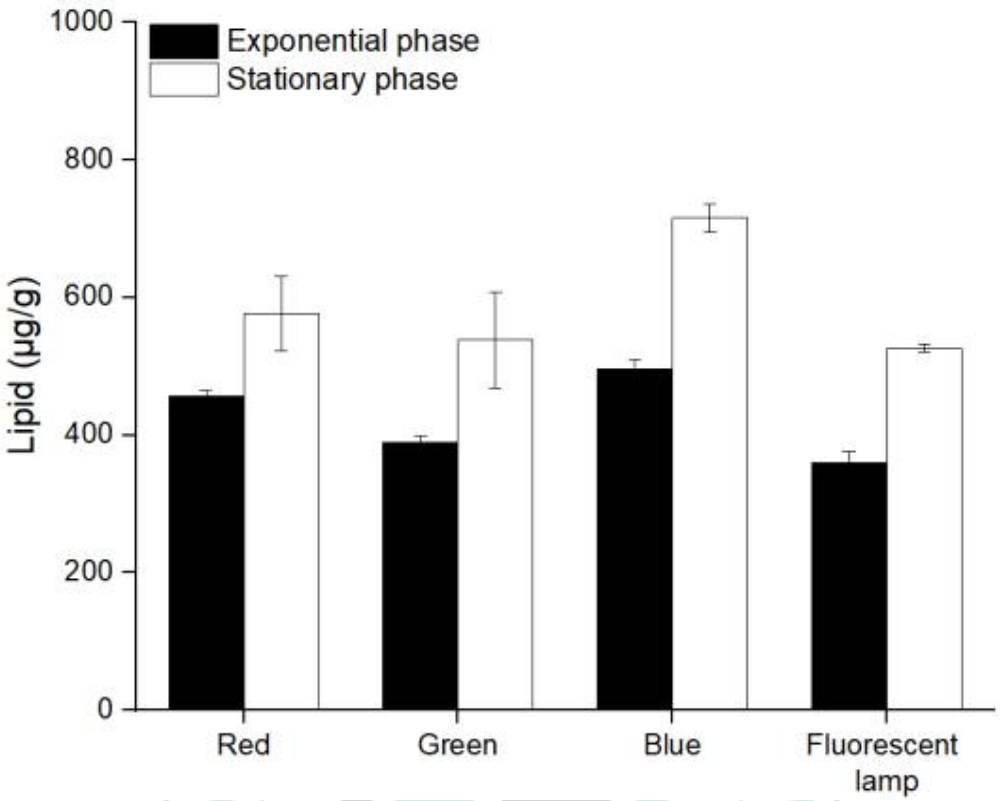

Figure 11. Lipid changes in *Chlorella vulgaris* (PKVL7422) cultured under red LED, green LED, blue LED and fluorescent lamp.

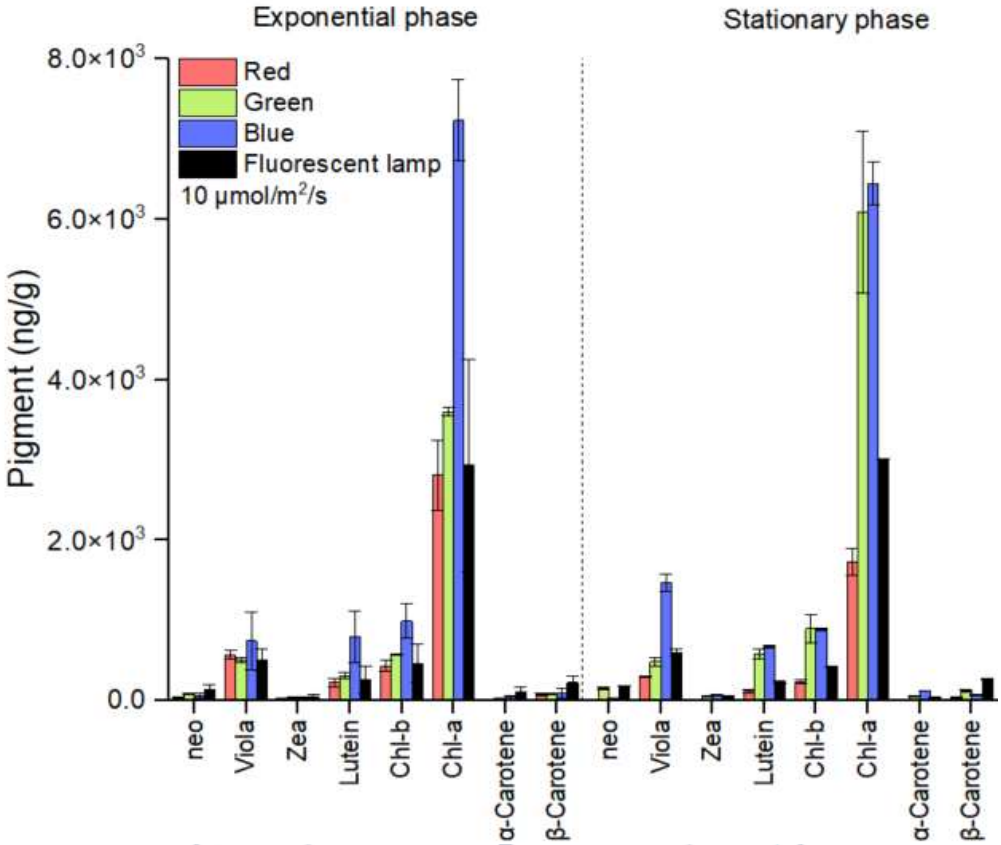

Figure 12. Pigment contents of *Chlorella vulgaris* (PKVL7422) under red LED, green LED, blue LED and fluorescent lamp at 10 µmol/m^2/s.

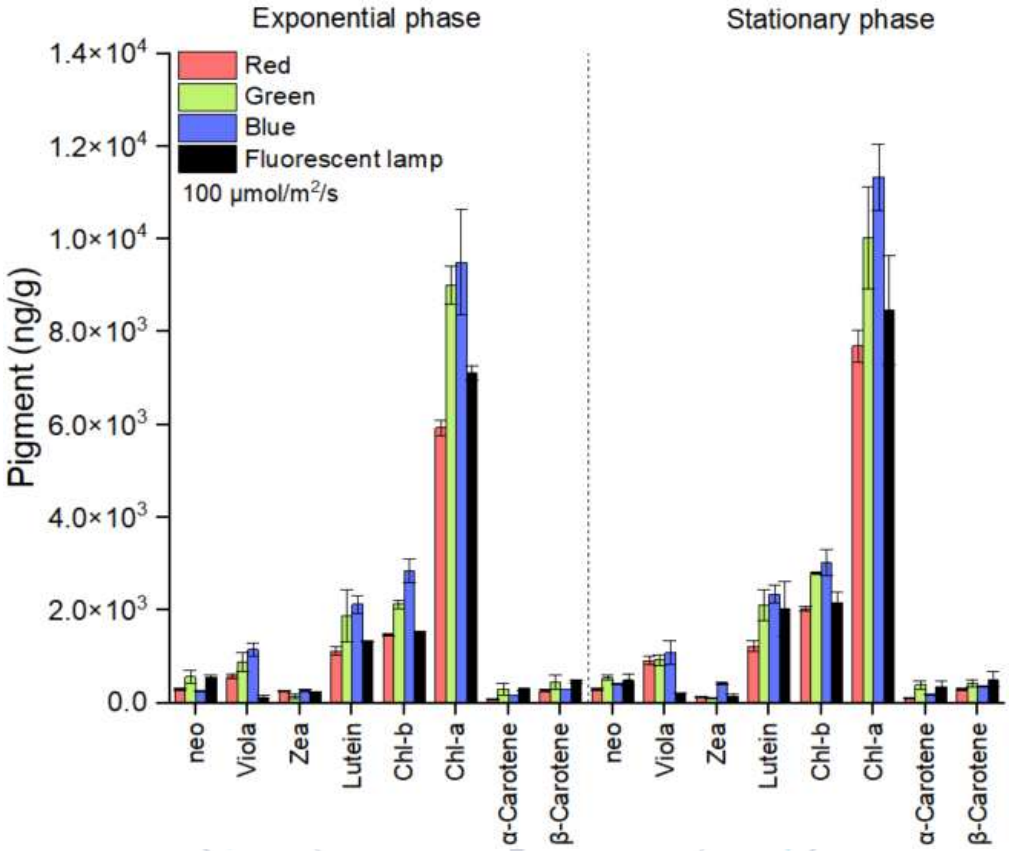

Figure 13. Pigment contents of *Chlorella vulgaris* (PKVL7422) under red LED, green LED, blue LED and fluorescent lamp at 100 µmol/m^2/s.

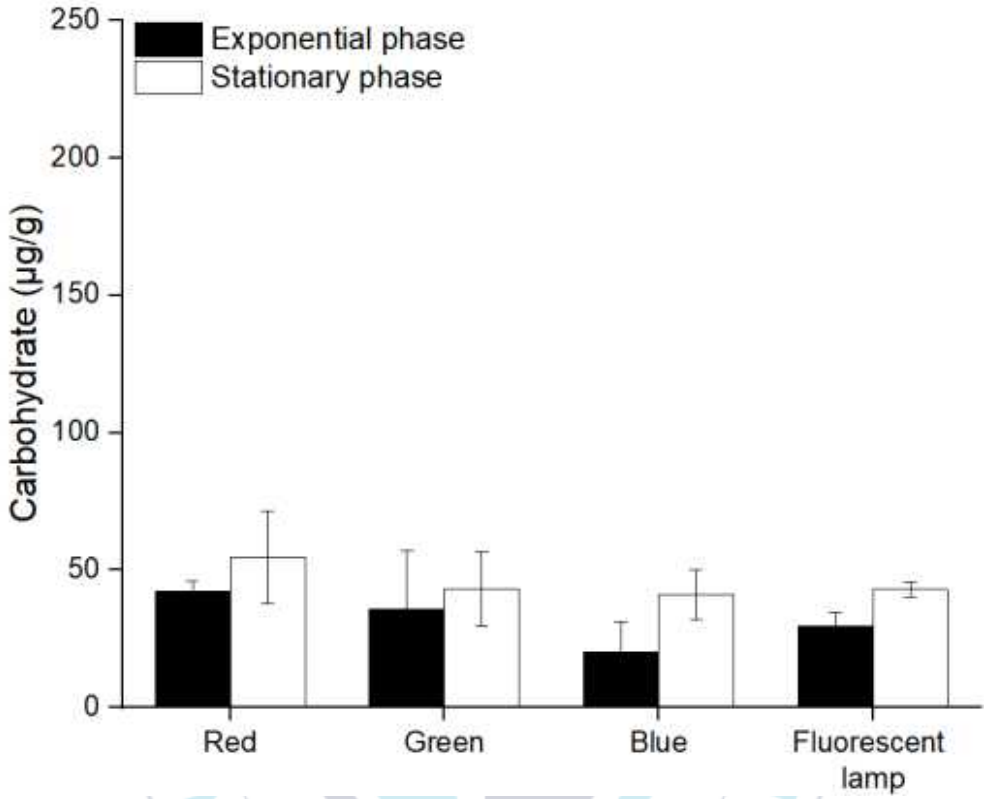

Figure 14. Carbohydrate changes in *Chlorella vulgaris* (transformed PKVL7422) cultured under red LED, green LED, blue LED and fluorescent lamp.

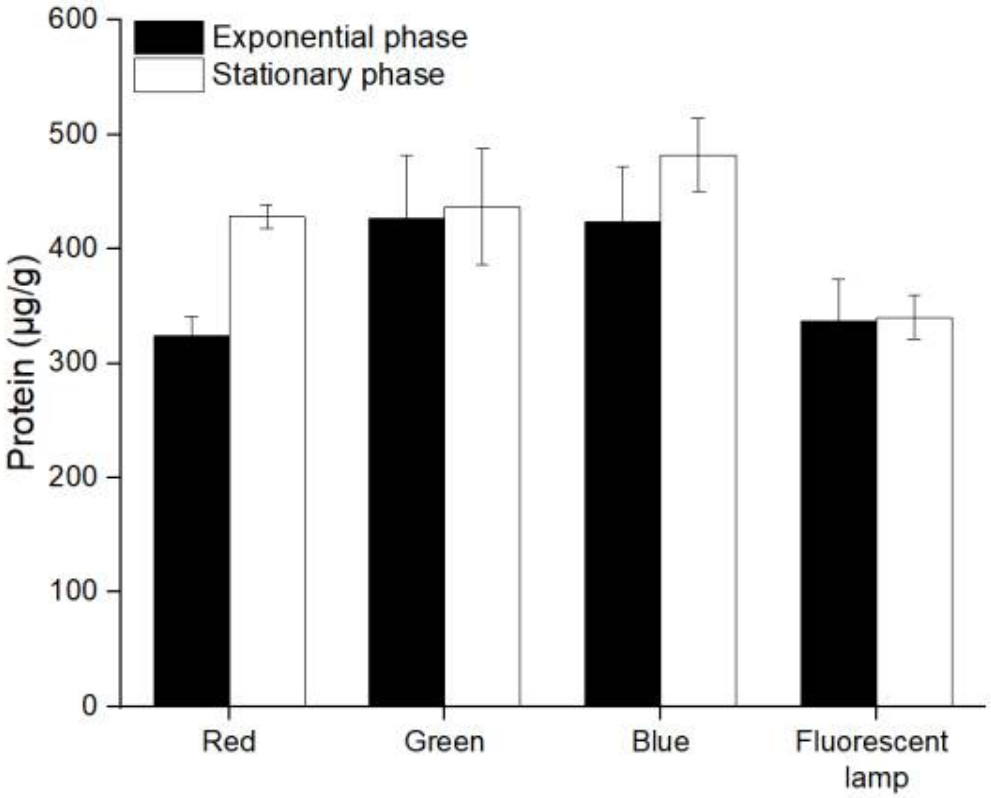

Figure 15. Protein changes in *Chlorella vulgaris* (transformed PKVL7422) cultured under red LED, green LED, blue LED and fluorescent lamp.

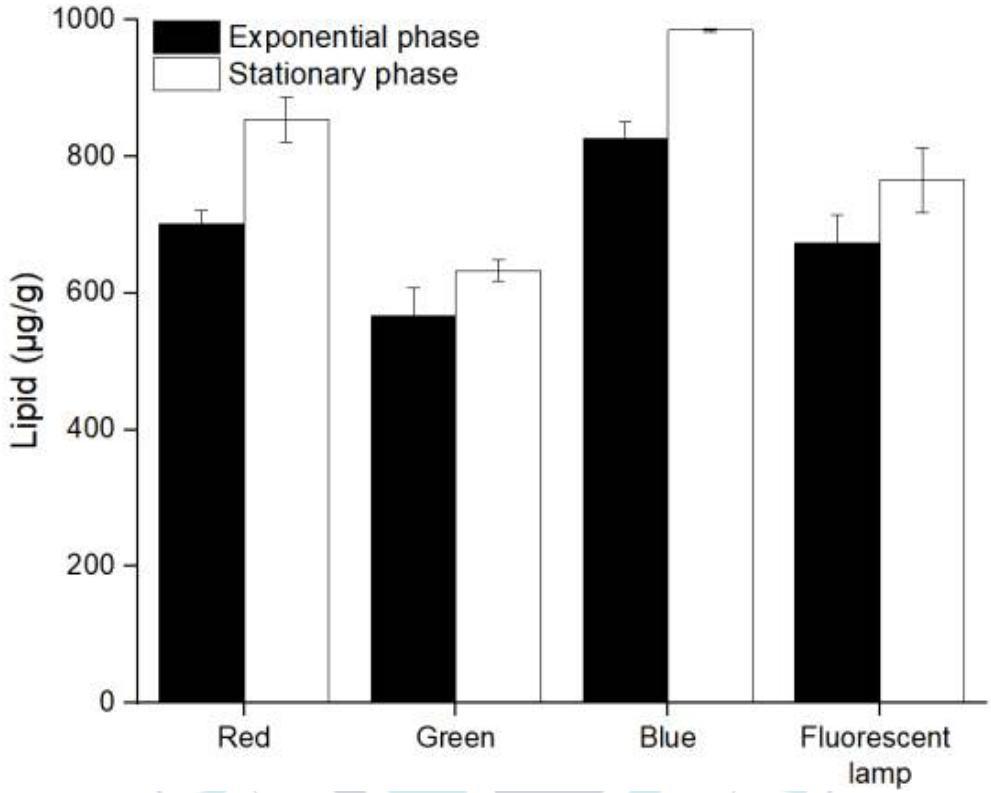

Figure 16. Lipid changes in *Chlorella vulgaris* (transformed PKVL7422) cultured under red LED, green LED, blue LED and fluorescent lamp.

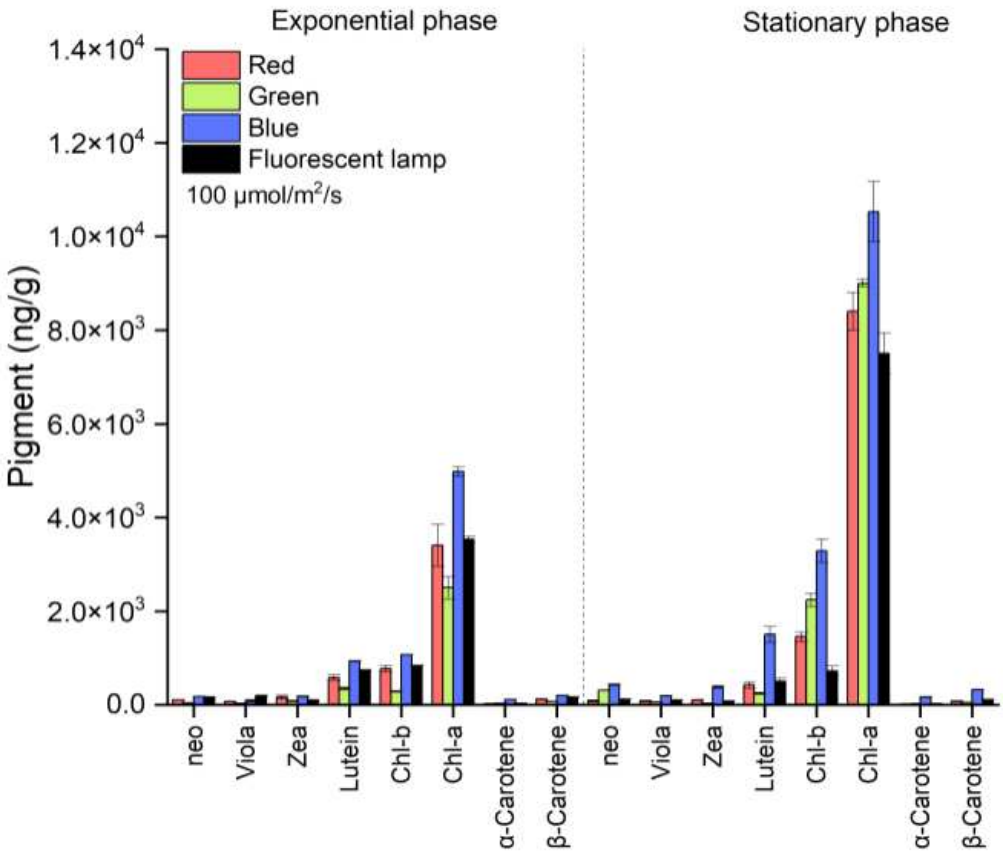

Figure 17. Pigment contents of *Chlorella vulgaris* (transformed PKVL7422) under red LED, green LED, blue LED and fluorescent lamp at 100 μ mol/m^2/s.

3.2.2 Two-phased Light Culture

In the two-phased culture, the carbohydrate content showed no statistically significant difference between the first and second phases ($P>0.05$), averaging about 133 μg/g (Fig. 13). Protein content varied throughout the phases, appearing at 334 μg/g on the 11th day (first phase), increasing to 361 μg/g on the 15th day (second phase), 455 μg/g on the 16th day (second phase), and peaking at 512 μg/g on the 17th day (second phase), before decreasing to 467 μg/g on the 18th day (second phase). The protein content on the 17th day was about 1.45 times higher than that under the red wavelength (355 μg/g) at a stationary phase light intensity of 100 μmol/m^2/s, similar to the blue wavelength (509 μg/g). Lipid content also varied, with 457 μg/g on the 11th day (first phase), 595 μg/g on the 15th day (second phase), 660 μg/g on the 16th day (second phase), peaking at 715 μg/g on the 17th day (second phase), and then dropping to 680 μg/g on the 18th day (second phase). The lipid content on the 17th day was about 1.2 times higher than that under the red wavelength (577 μg/g) at a stationary phase light intensity of 100 μmol/m^2/s, comparable to the blue wavelength. In this two-phased culture, the main pigments chl-*a* and chl-*b* increased by 1.18 times each after switching to the blue wavelength but showed a lower content compared to the red and blue single wavelengths (Fig. 19). However, compared to the conventional mass culture light source, the fluorescent lamp, the content of the auxiliary pigment violazanthin was 4.41 times higher.

Ra et al. (2016) reported that the microalgae *N. salina*, *N. Oceanica*, and *N. oculata* exhibited effective biomass enhancement with blue wavelength

injection until the late exponential growth phase, and in two-phase cultivation with green wavelength injection from the exponential growth phase, the cellular lipid content was found to be highest. Similarly, Sirisuk et al. (2018) reported that *N. salina*, *P. tricornutum*, and *I. galbana* showed the highest lipid content in two-phased cultivation when red wavelength was injected until the late exponential growth phase, followed by green wavelength injection from the exponential growth phase. This observation is in line with the fact that different light wavelenghths have been reported to alter lipid metabolism in microalgae, leading to changes in lipid profiles (Harwood, 1998).

Furthermore, stress induces various metabolic responses in microalgae, such as the breakdown of cellular compounds like proteins, chlorophyll, and DNA, as well as the accumulation of energy-rich compounds like lipids and carbohydrates. Therefore, in two-phase cultivation, it is inferred that more valuable substances accumulate compared to single-wavelength conditions, as stress triggers various metabolic reactions in microalgae (Jung et al., 2019).

Furthermore, numerous studies have reported that pigments, similar to proteins and lipids, are more effectively accumulated through two-phased cultivation. Sui et al. (2019) observed the accumulation of beta-carotene using a two-phased cultivation method involving increased light intensity and nutrient deficiency. Similarly, García-López et al. (2020) found an increase in phycocyanin production in *S. maxima* when exposed to sunlight until the late exponential growth phase, followed by blue light injection during the stationary phase. However, Pagels et al. (2020) found that under two-phased cultivation using white and red wavelengths, the production of auxiliary pigments, such as

carotenoids, increased in *Cyanobium* sp., while the primary pigment showed minimal changes. This observation aligns with our study's results.

Two-phased cultivation proved to be more effective in accumulating proteins, lipids, and pigments compared to traditional fluorescent lights. However, compared to single-wavelength blue light, two-phased cultivation was not as effective in accumulating valuable substances, excluding the auxiliary pigment violazanthin. Therefore, it is considered that injecting a single wavelength is more economically beneficial and contributes to increased productivity in establishing a phototrophic cultivation system in the future. Nevertheless, considering the specificity depending on the microalgae species or strains, it is deemed crucial to investigate the physiological status regarding light for various strains in future studies.

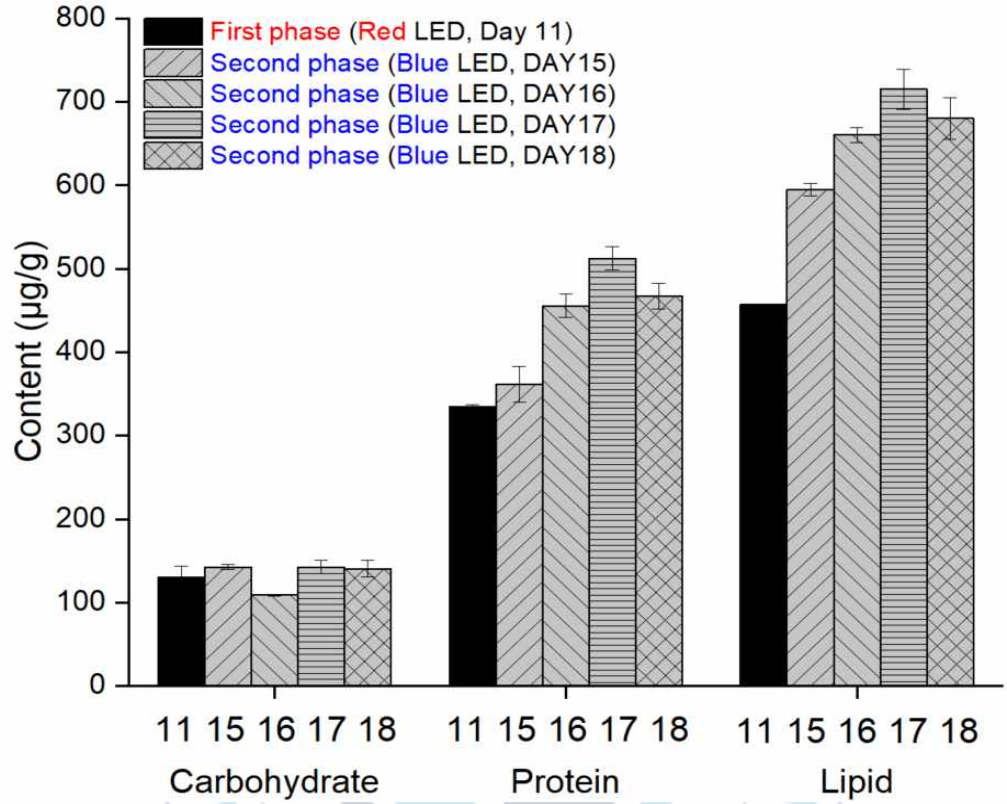

Figure 18. Biochemical composition changes in *Chlorella vulgaris* (PKVL7422) cultured at two-phased light culture.

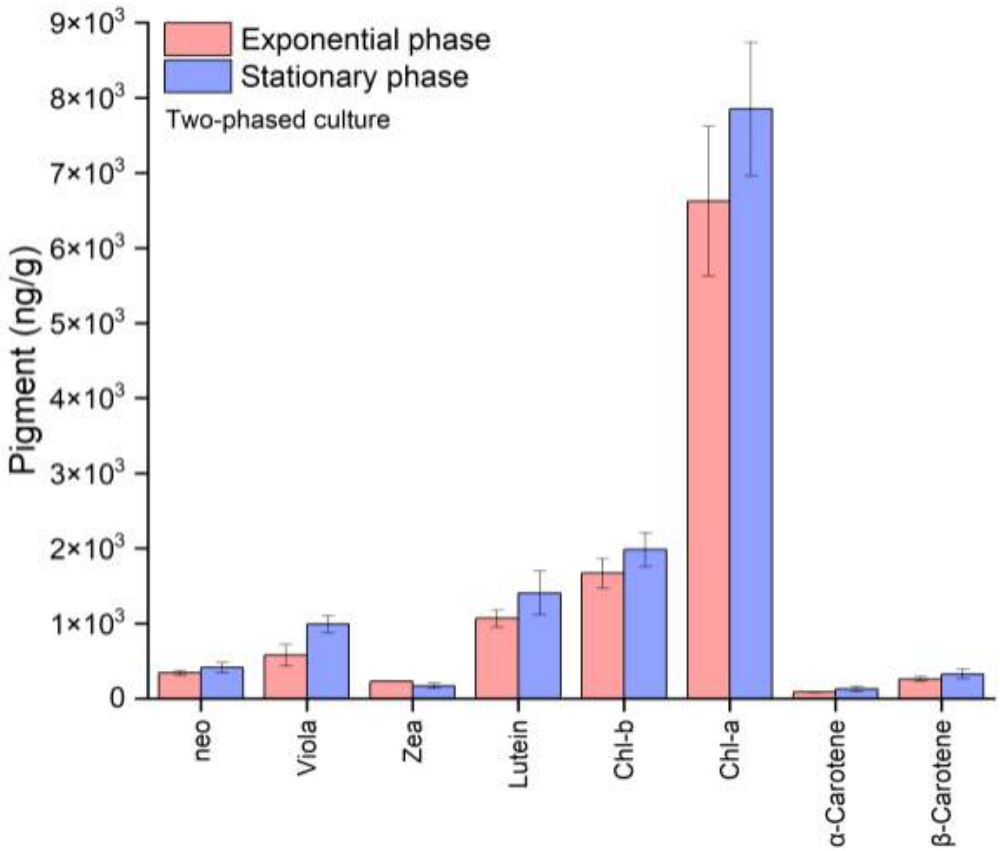

Figure 19. Pigment contents of *Chlorella vulgaris* (PKVL7422) cultured at two-phased light culture.

3.2.3 LED Photoperiod

The carbohydrate content was highest at 146 μg/g in the red wavelength of the exponential phase, 12L:12D, and there was not much difference between 16L:08D and 24L:00D (Fig. 14). Except for the carbohydrate content of the red wavelength, 12L:12D of the exponential phase, there was no statistically significant difference in carbohydrate content in all light-dark cycles ($P>0.05$). The protein content of the red wavelength of the exponential phase was highest at 16L:08D, followed by 24L:00D and 12L:12D (Fig. 14). The lipid content was highest at 24L:00D followed by 16L:08D and 12L:12D (Fig. 14), but there was no statistically significant difference in protein and lipid content of the red wavelength of the exponential phase ($P>0.05$). In the stationary phase of the red wavelength, the carbohydrate content was highest at 12L:12D followed by 16L:08D and 24L:00D. Proteins were also highest at 12L:12D followed by 16L:08D and 24L:00D. Lipids were also highest at 12L:12D followed by 16L:08D and 24L:00D. In exponential phase of the blue wavelength, the carbohydrate content was highest at 12L:12D followed by 16L:08D and 24L:00D (Fig. 15). Proteins were highest at 16L:08D followed by 12L:12D and 24L:00D (Fig. 15). Lipids were highest at 12L:12D (496 μg/g), followed by 24L:00D and 16L:08D (Fig. 15). In the stationary phase of the blue wavelength, the carbohydrate, protein, and lipid contents showed similar trends in all light-dark cycles. The carbohydrate content was highest at 12L:12D followed by 16L:08D and 24L:00D (Fig. 15). Proteins were also highest at 12L:12D followed by 16L:08D and 24L:00D. Lipids were also highest at 12L:12D followed by 16L:08D and 24L:00D (Fig. 15). As a result

of the light-dark cycle control experiment, the main pigments chl-*a* and chl-*b* and auxiliary pigments generally showed a high content when the light-dark cycle was appropriately controlled to 12L:12D (Fig. 21; Fig. 23).

Che and Kim (2023) reported that when the light-dark cycle for the cultivation of *Chlamydomonas hedleyi* was set at 12L:12D, the biomass was lower compared to 24L:00D, and adjusting the light-dark cycle was deemed ineffective for lipid accumulation, similar to the findings of this study. These results suggest that sustaining a long light period could act as prolonged stress on microalgae (Sirisuk et al., 2018; Che et al., 2019), leading to a reduction in photosynthesis and energy efficiency, ultimately hindering the production of valuable substances. However, Wahidin et al. (2013) also noted that the lipid content of *Nannoclopsis* sp. was highest with a longer light period of 18L:06D, and He et al. (2015) reported that the lipid content of *Ankistrodesmus fusiformis* was highest when the light-dark cycle was set at 24L:00D.

To evaluate ideal light regimes, it is necessary to consider biomass and lipid productivity of microalgae, as well as the energy demand for power, as Maroneze et al. (2016) pointed out. Since this study focused only on the total lipid content and did not find significant differences in valuable substance content according to the light cycle, future evaluations of phototrophic cultivation systems should consider both the energy demand for power and lipid productivity of microalgae according to the light cycle.

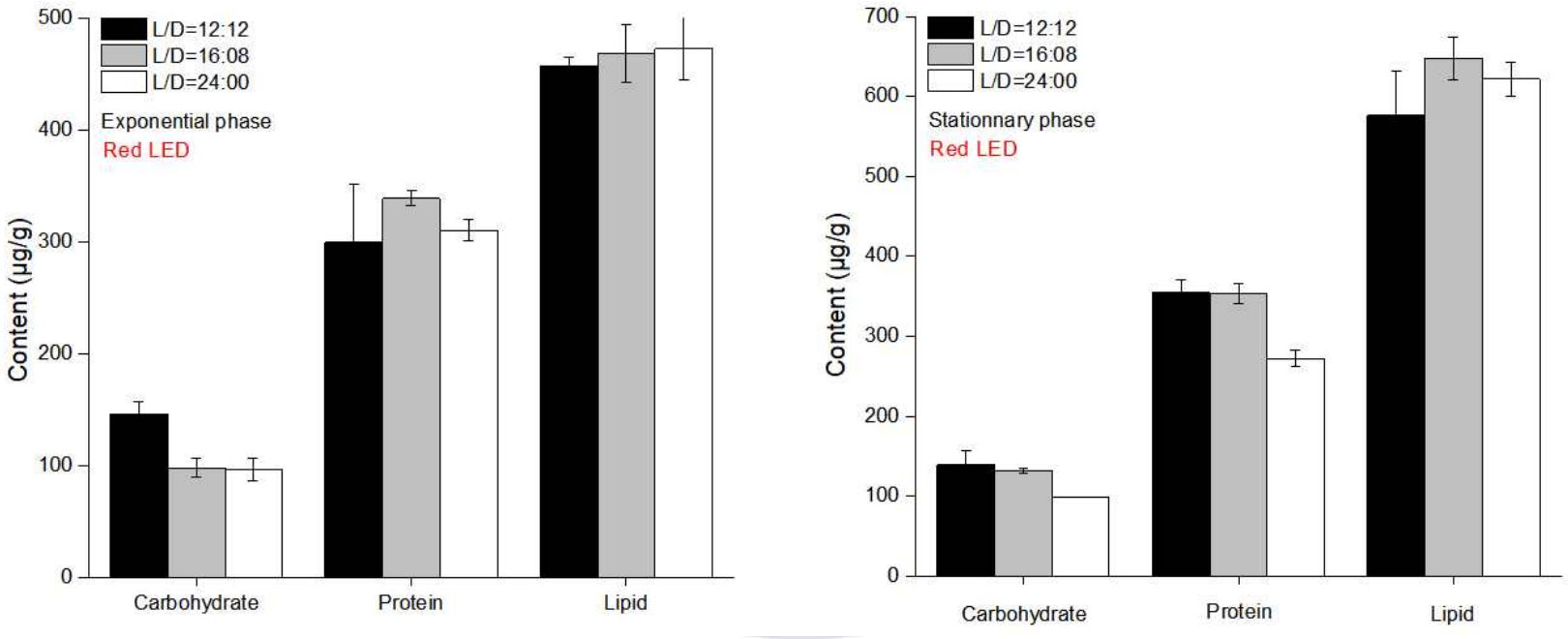

Figure 20. Biochemical composition changes in *Chlorella vulgaris* (PKVL7422) were observed under red LED and photoperiods of 12:12, 16:08, 24:00 h L:D.

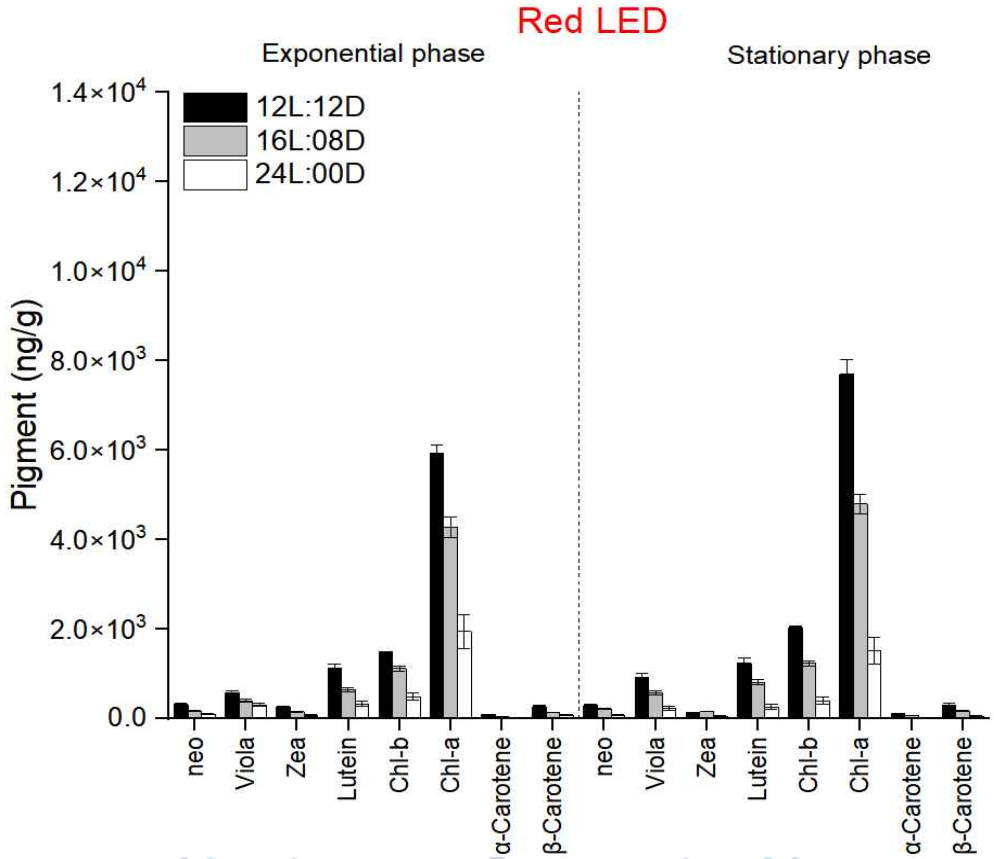

Figure 21. Pigment contents of *Chlorella vulgaris* (PKVL7422)were observed under red LED and photoperiods of 12:12, 16:08, 24:00 h L:D.

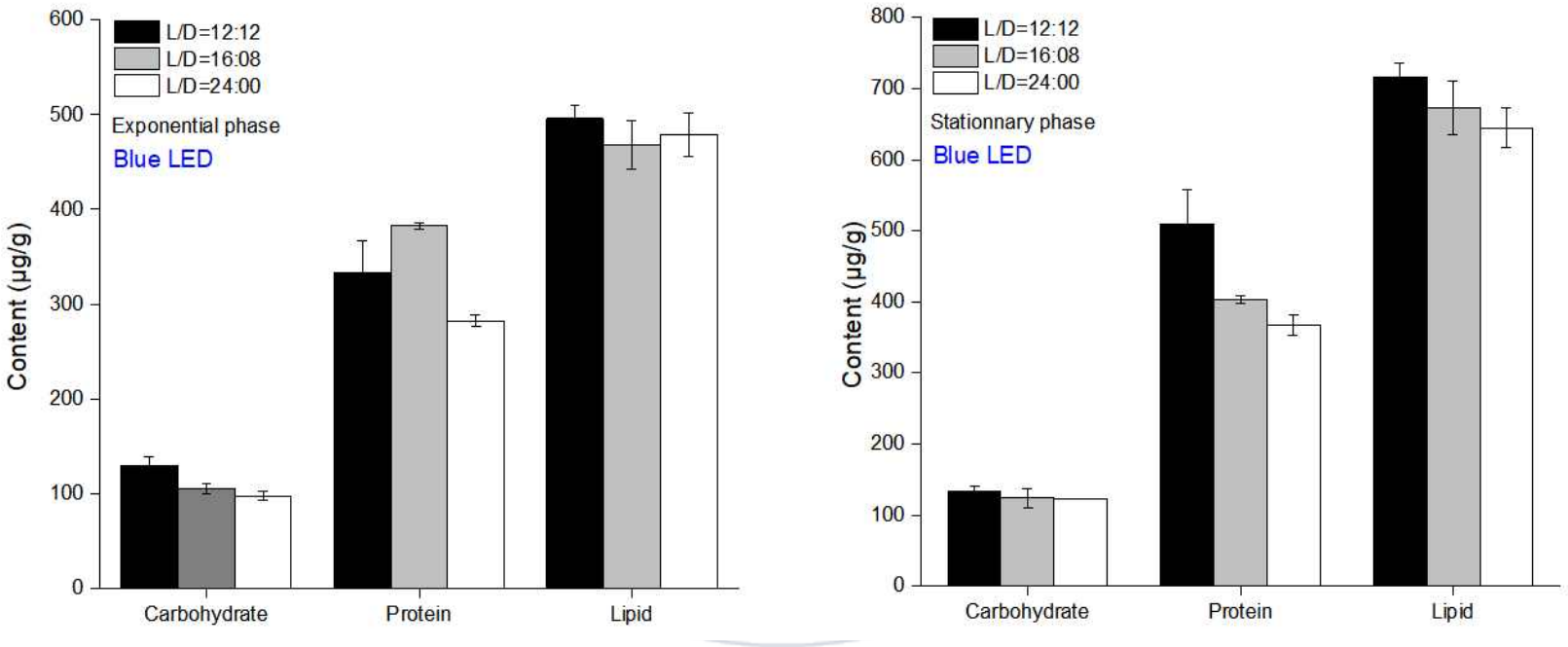

Figure 22. Biochemical composition changes in *Chlorella vulgaris* (PKVL7422) were observed under blue LED and photoperiods of 12:12, 16:08, 24:00 h L:D.

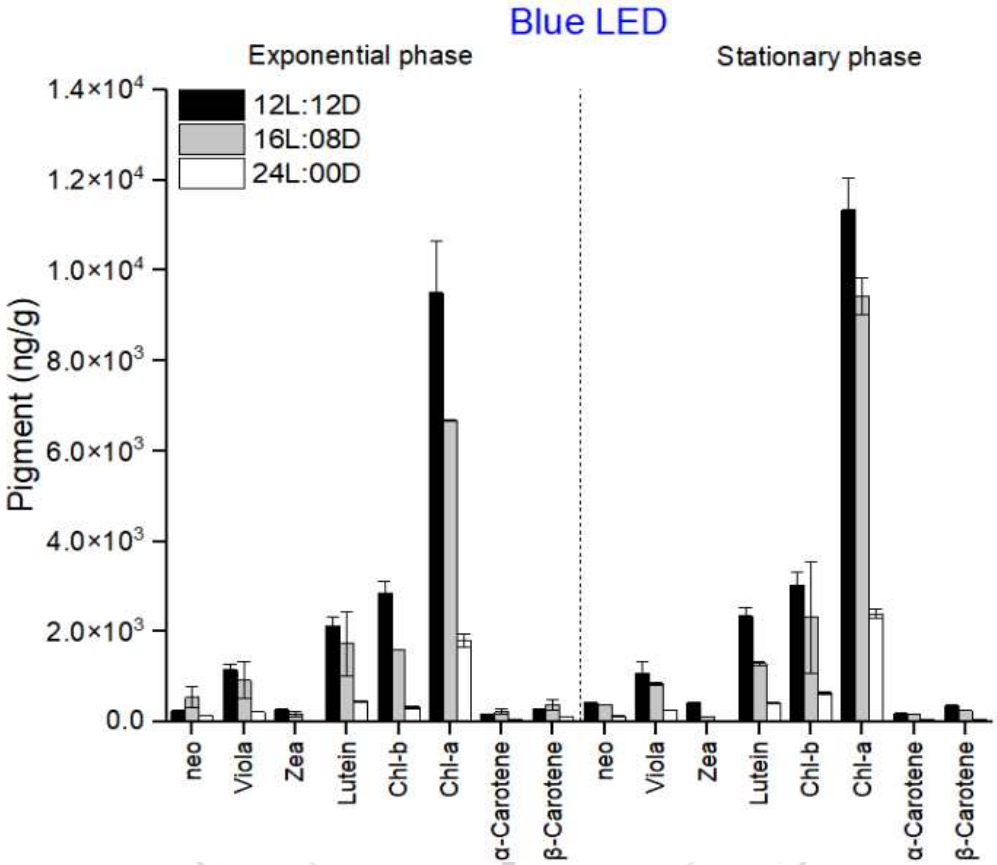

Figure 23. Pigment contents of *Chlorella vulgaris* (PKVL7422) were observed under blue LED and photoperiods of 12:12, 16:08, 24:00 h L:D.

3.2.4 Mixed Wavelengths

In the wild type, there was no statistically significant difference in carbohydrate content across all wavelengths (P>0.05) (Fig. 16). The average carbohydrate content was approximately 104 μg/g, which was higher in the exponential phase. This content was about 1.40 times lower than under the red wavelength (146 μg/g) and 1.25 times lower than under the blue wavelength (130 μg/g) at a light intensity of 100 μmol/m^2/s during the exponential phase. In the exponential phase, the carbohydrate content was highest in the 3:7 ratio (123 μg/g), followed by the 5:5 ratio (111 μg/g), and lowest in the 7:3 ratio (94.5 μg/g). In the stationary phase, the 3:7 (106 μg/g) and 5:5 (105 μg/g) ratios showed similar contents, with the 7:3 ratio (86 μg/g) being the lowest.

The protein content in the wild type was higher under the mixed wavelength compared to the single blue wavelength, especially at a red to blue ratio of 7:3 (743 μg/g) (Fig. 17). Comparing the red wavelength at the stationary phase (355 μg/g) and the mixed wavelength 3:7, which showed a high growth rate, the protein content was about 2.1 times higher, and compared to the blue wavelength at the stationary phase (509 μg/g), it was about 1.5 times higher. The lipid content in the wild type was higher in the stationary phase than in the exponential phase. In the exponential phase, the order was 3:7 (500 μg/g), 7:3 (360 μg/g), and 5:5 (332 μg/g), while in the stationary phase, it was 7:3 (691 μg/g), 5:5 (579 μg/g), and 3:7 (577 μg/g) (Fig. 18). When the wild type was exposed to mixed wavelengths, carbohydrates and lipids showed higher content than under a single wavelength, but protein content was twice as high under a 7:3 wavelength

with a higher blue wavelength ratio.

For the transformed strain exposed to mixed wavelengths, the carbohydrate content was higher in the exponential phase, with an average content of 96 μg/g (Fig. 19), about 1.08 times lower than the wild type (104 μg/g). This was approximately 1.51 times lower than under the red wavelength (146 μg/g) and 1.25 times lower than under the blue wavelength (129 μg/g) at a light intensity of 100 μmol/m^2/s during the exponential phase. The average protein content of the transformed strain was higher in the stationary phase, with an average of 711 μg/g, about 1.13 times higher than the wild type (632 μg/g) (Fig. 20). The lipid content of the transformed strain was also higher in the stationary phase, with an average of 1084 μg/g, about 2.14 times higher than the wild type (507 μg/g). Comparing the red wavelength at the stationary phase (355 μg/g) and the mixed wavelength 3:7, the lipid content was about 4.37 times higher, and compared to the blue wavelength at the stationary phase (509.49 μg/g), it was about 3.04 times higher.

Under mixed wavelengths, both strains exhibited high pigment concentrations, particularly at a blue-dominant ratio of 7:3. In the wild type, chl-a and chl-b showed 1.24 and 1.14 times higher levels, respectively, compared to the blue single wavelength, and 1.65 and 1.60 times higher, respectively, compared to fluorescent lamps. Notably, in the 7:3 ratio during the stationary phase for the trait-transformed strain, the chl-a content was the highest at 18,762 ng/g, a 1.78-fold increase compared to the blue single wavelength. The chl-*b* content was 5,924 ng/g, a 1.60-fold increase. Compared to the blue single wavelength, the concentrations of accessory pigments,

namely neoxanthin, zeaxanthin, lutein, α-carotene, and β-carotene, were 1.50, 1.52, 1.77, 1.49, and 1.88 times higher, respectively.

Li et al. (2020) reported that in *Haematococcus pluvialis*, the content of chl-*a*, chl-*b*, and carotenoids was highest under a 2:1 red to blue wavelength ratio. Mohebi et al. (2023) found that in *Dunaliella salina*, a mixture of red and blue wavelengths (6:4) resulted in higher amounts of lipids, chlorophyll, carotenoids, and β-carotene compared to white single-wavelength light. This is believed to be due to the need for at least 5-10% of photons from blue wavelengths, in addition to red wavelengths, to activate metabolic functions other than photosynthesis during microalgae cultivation (Anderson and South, 2004). The positive effect of blue light on chlorophyll biosynthesis, particularly chl-b, which codes for protochlorophyllide reductase subunit B, may contribute to this phenomenon (Kim et al., 2014).

Moreover, the higher pigment content observed in the mixture with a higher proportion of blue wavelengths is thought to be a result of the stress-inducing role of blue light. This stress could lead to the vigorous formation of carotenoids, such as β-carotene, to protect the cells (Mohebi et al., 2023). Kim et al. (2014) reported that blue light increases cell size, while red light enhances cell division. Therefore, when a mixed wavelength is applied, it is inferred that the simultaneous enhancement of cell division by red light and an increase in cell size by blue light lead to substantial accumulation of valuable substances within the cells, resulting in higher protein and lipid content than with single-wavelength conditions. Hence, it is deemed necessary for future research to measure cell size, assess the expression of the

minD gene that inhibits cell division, and measure reactive oxygen species (ROS) levels according to the mixture ratio.

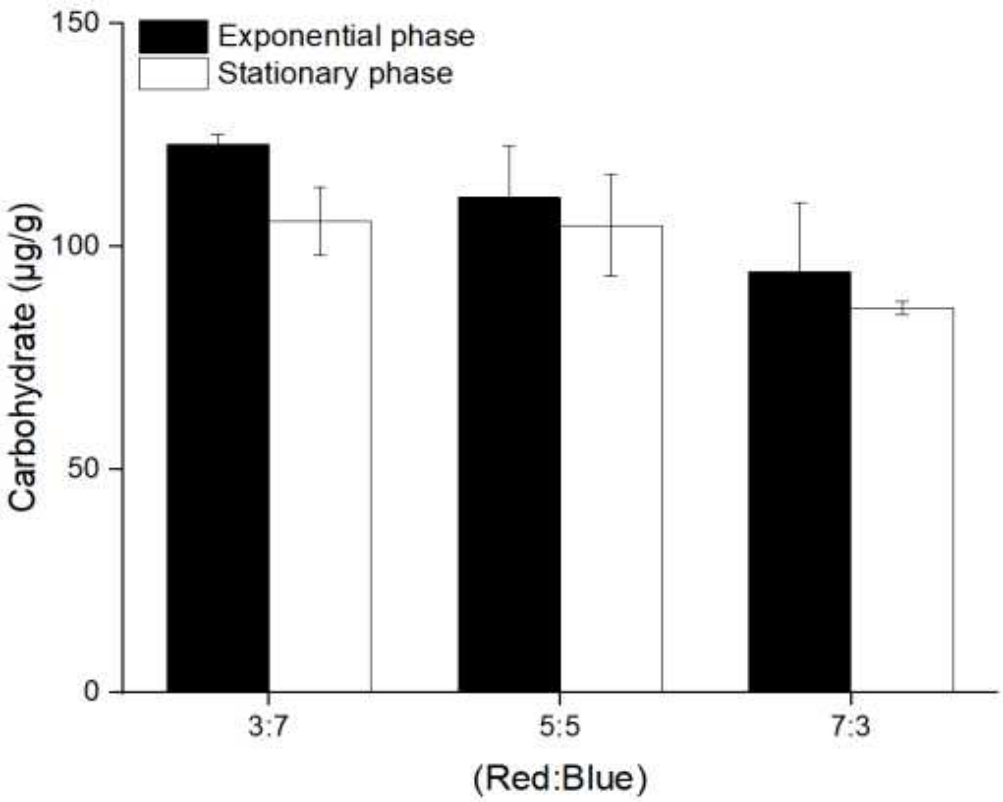

Figure 24. Cabohydrate contents of *Chlorella vulgaris* (PKVL7422) cultured at different mixed wavelength ratios.

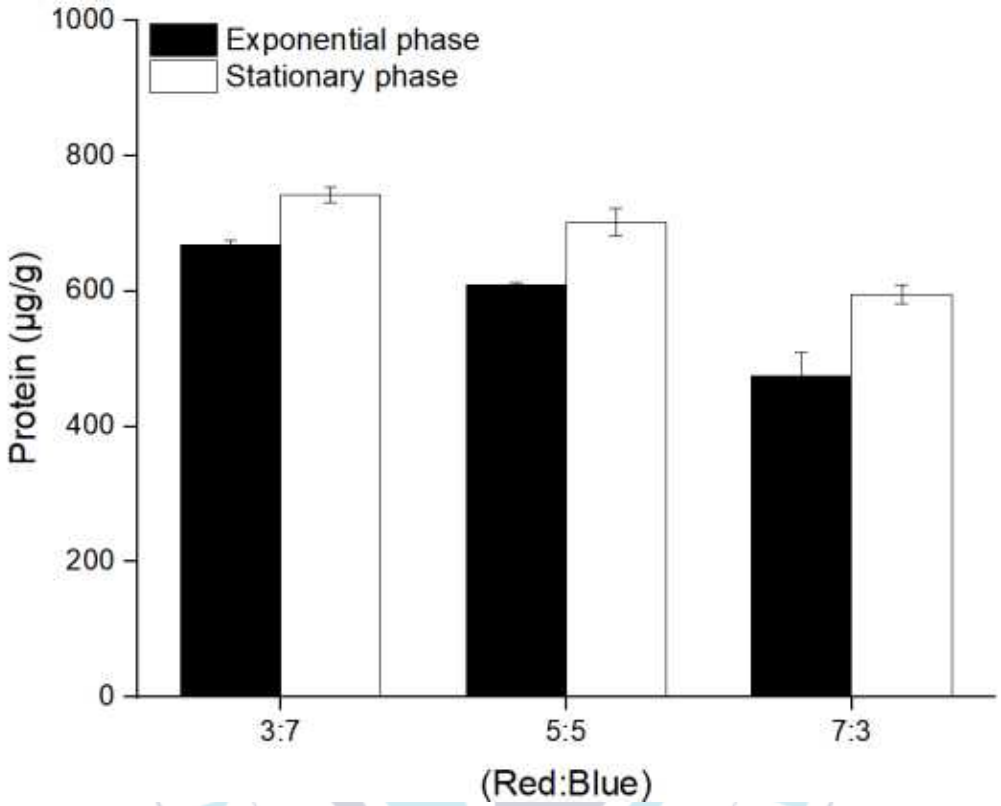

Figure 25. Protein contents of *Chlorella vulgaris* (PKVL7422) cultured at different mixed wavelength ratios.

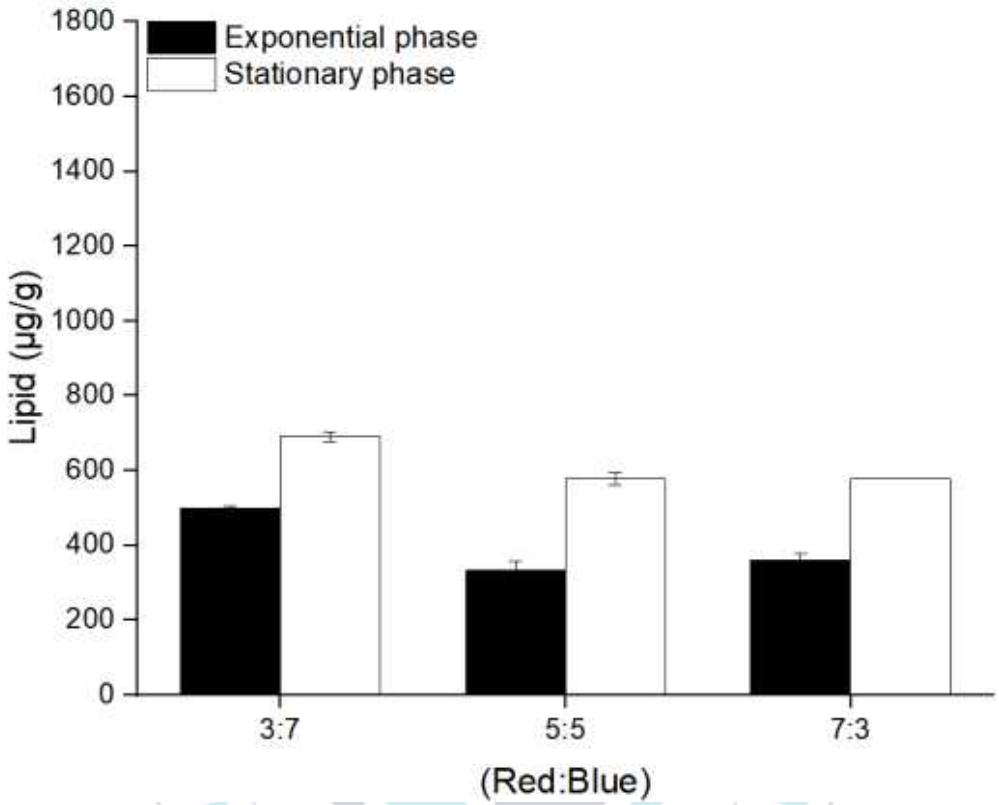

Figure 26. Lipid contents of *Chlorella vulgaris* (PKVL7422) cultured at different mixed wavelength ratios.

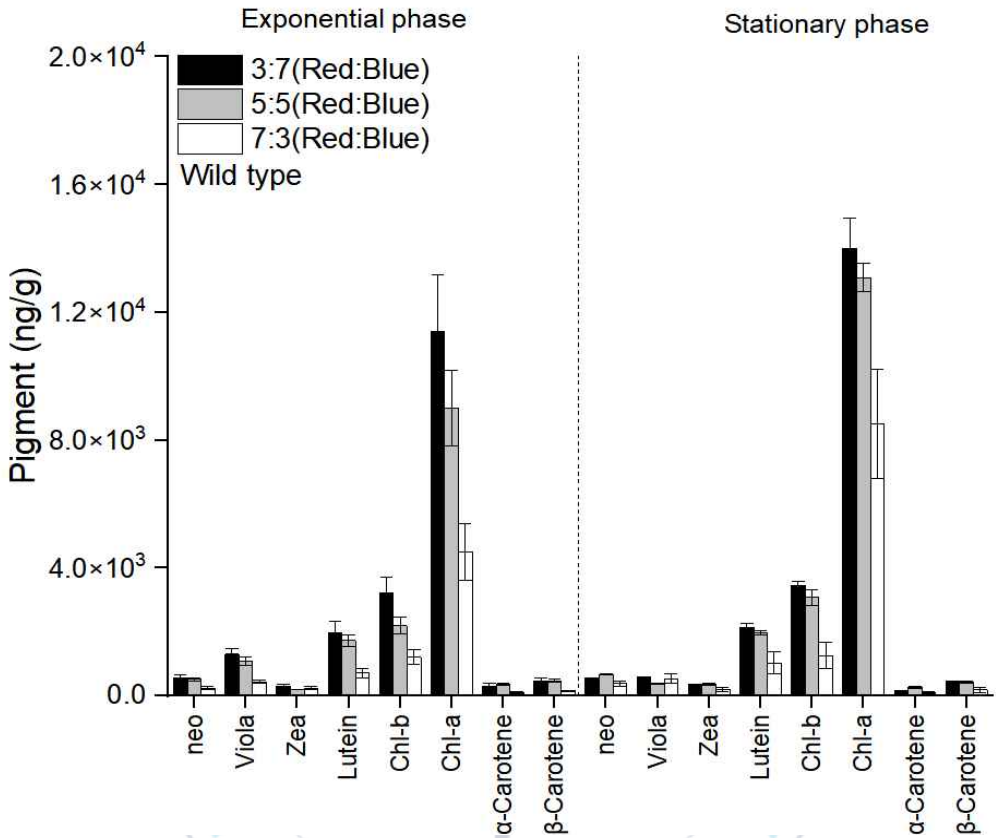

Figure 27. Pigment contents of *Chlorella vulgaris* (PKVL7422) cultured at different mixed wavelength ratios.

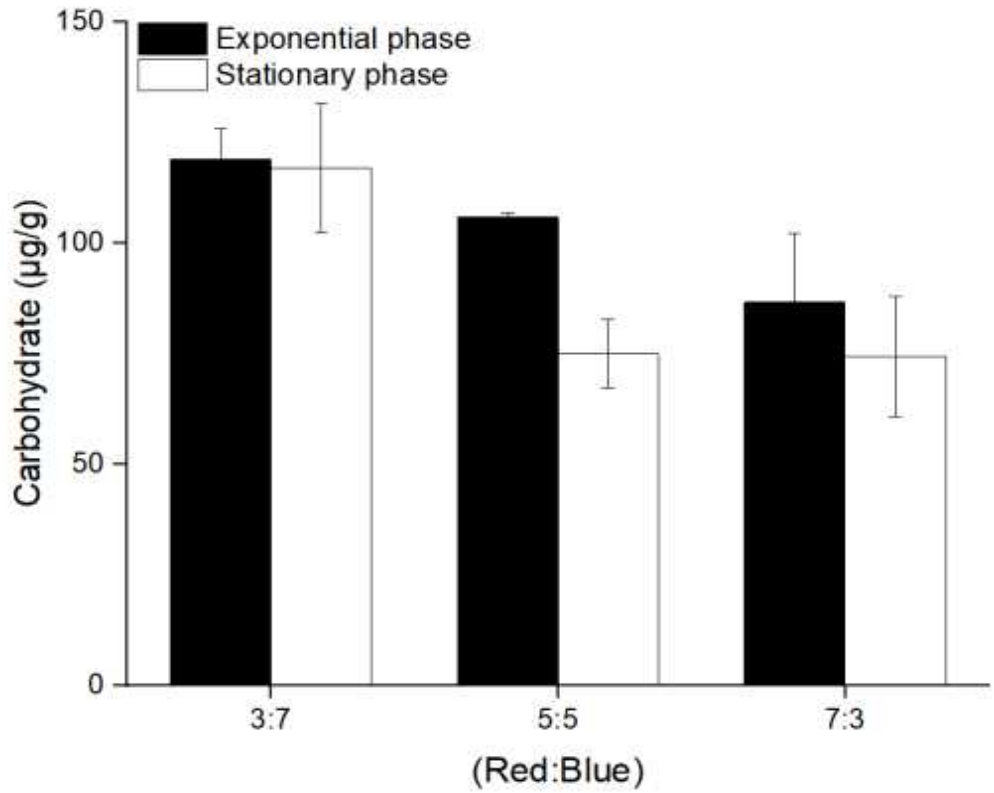

Figure 28. Carbohydrate contents of *Chlorella vulgaris* (transformed PKVL7422) cultured at different mixed wavelength ratios.

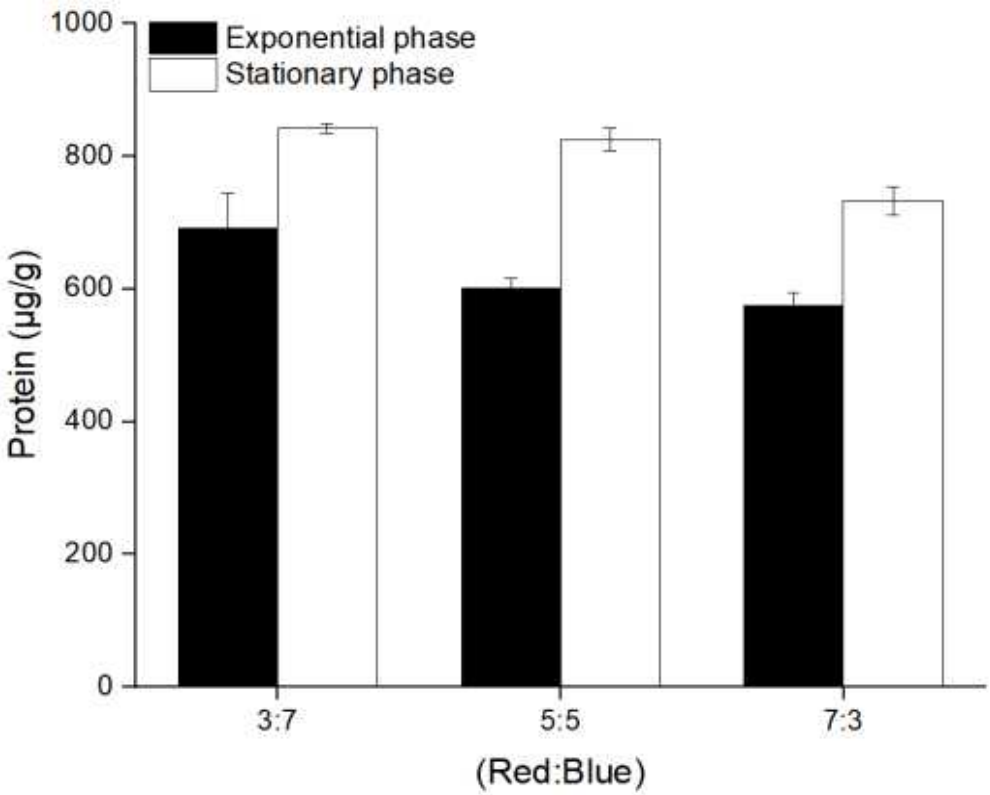

Figure 29. Protein contents of *Chlorella vulgaris* (transformed PKVL7422) cultured at different mixed wavelength ratios.

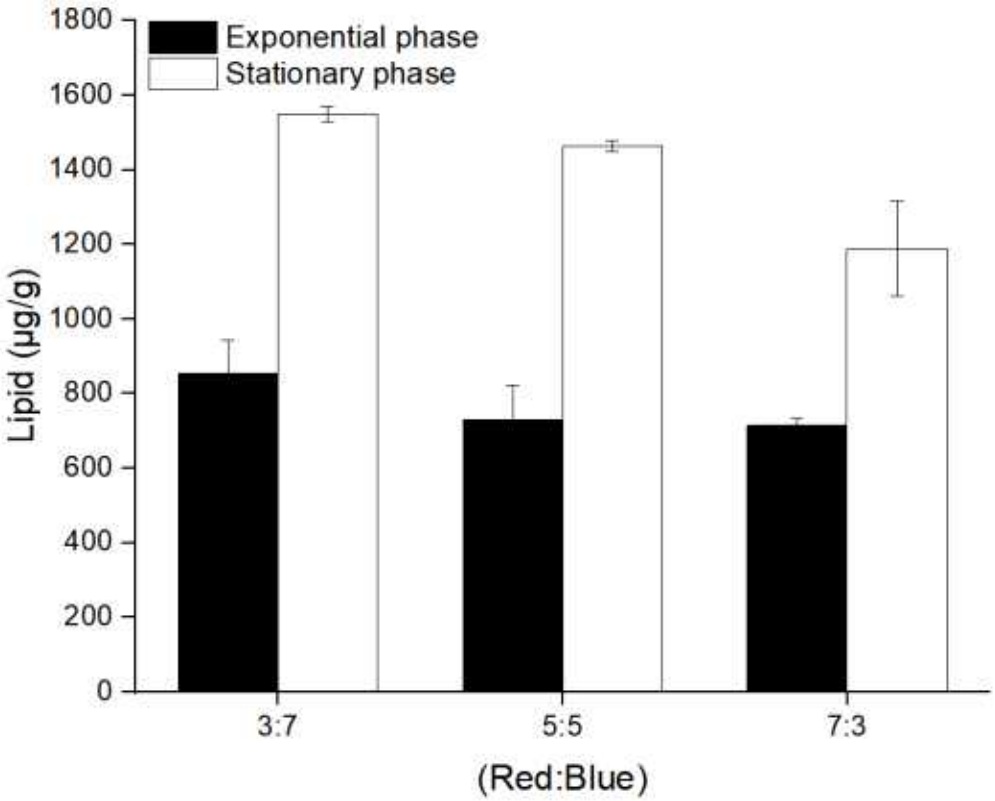

Figure 30. Lipid contents of *Chlorella vulgaris* (transformed PKVL7422) cultured at different mixed wavelength ratios.

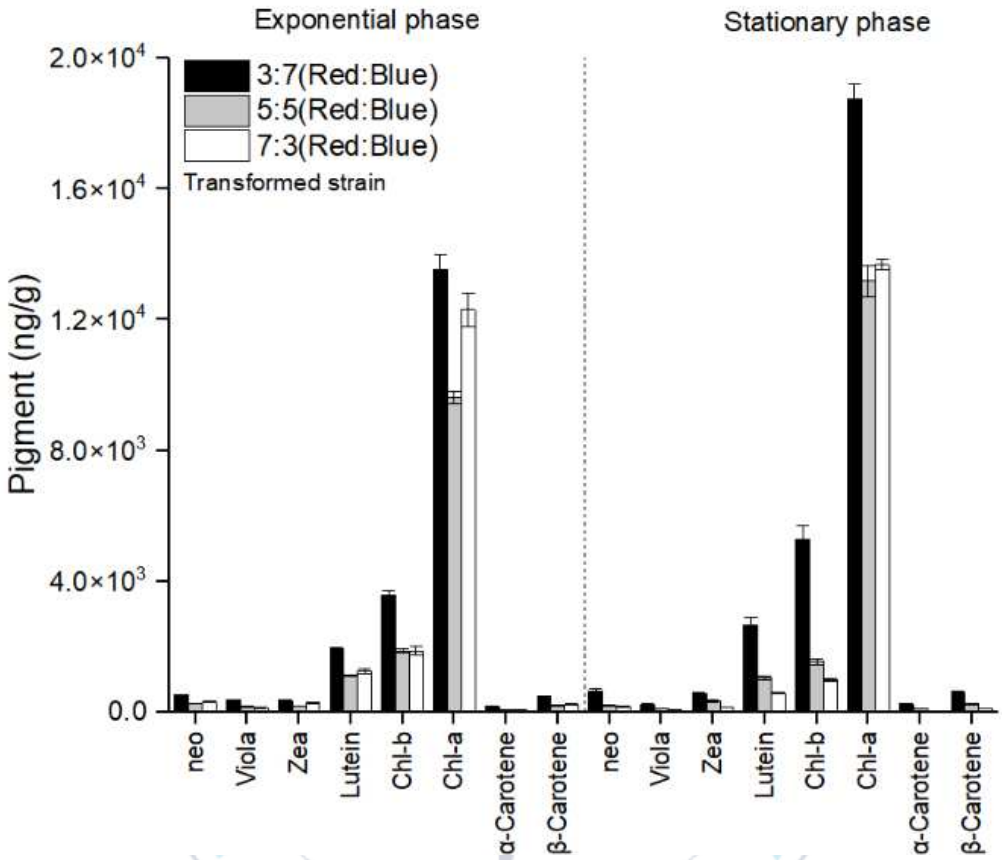

Figure 31. Pigment contents of *Chlorella vulgaris* (transformed PKVL7422) cultured at different mixed wavelength ratios.

Ⅳ. Conclusion

The experimental species in this study, *C. vulgaris*, accumulates valuable substances such as carbohydrates, proteins, lipids, and pigments within its cells and is rich in various nutrients including minerals, vitamins, and amino acids. It is utilized in diverse fields such as future food resources, medicines, and cosmetics. Additionally, the growth-promoting factor (*Chlorella* Growth Factor; CGF) contained in *C. vulgaris* has been reported to have medical benefits, such as improving and preventing strokes, anti-cancer effects, promoting growth in plants and animals, and preventing adult diseases. Consequently, research on its efficacy, as well as the separation and application of its physiologically active components, is actively underway.

In this study, we first investigated the effects of LED light intensity and wavelength on the growth of *C. vulgaris*, a promising BT (Biotechnology) material, and identified the optimal wavelength for its growth. Furthermore, we measured the content of carbohydrates, proteins, and lipids, which vary according to LED light intensity and wavelength, to identify the wavelength effective for promoting the synthesis of these useful substances and discussed our findings.

Firstly, we identified the growth specificity of *C. vulgaris* in response to changes in LED light intensity and wavelength. The results indicated a high growth rate under red and blue wavelengths among the LED wavelengths. This can be attributed to the auxiliary pigment chl-*b*, which absorbs light in the

blue (450 nm) and red (662 nm) wavelengths and transfers the light energy to chl-*a*, thereby promoting a high growth rate under these wavelengths. Additionally, the K_s was approximately three times lower under the red wavelength compared to the blue wavelength. This suggests that the red wavelength is an economical light source that can enhance the cell density of *C. vulgaris*. Therefore, it seems advantageous to use the red wavelength initially to induce rapid growth, followed by the blue wavelength, which is beneficial for promoting the synthesis of useful biochemical substances such as proteins and lipids. Based on these findings, three experiments were conducted using these two wavelengths.

A two-phase culture was conducted, initially irradiating *C. vulgaris* with red wavelength light, optimal for growth in the early stages of culture, followed by blue wavelength light to induce stress and promote the synthesis of biochemical substances. The experiment revealed that while this method effectively accumulated useful substances compared to the red wavelength, it was not as effective in accumulating proteins and lipids as the blue wavelength. Regarding pigments, there was a significant increase, approximately fourfold, in specific auxiliary pigments like violazanthin compared to fluorescent lamps. However, this method was deemed less efficient compared to other wavelengths for pigment production.

The second experiment, involving light-dark cycle control, demonstrated that the content of carbohydrates, proteins, lipids, and pigments was generally higher when the light-dark cycle was controlled to 12 hours of light (L) and 12 hours of dark (D). This suggests that an appropriate light-dark cycle creates

optimal conditions for photosynthesis, generating more energy and aiding in the accumulation of useful substances. Thus, light-dark cycle control alone was not found to be significantly effective in enhancing the synthesis of these substances.

In the mixed wavelength experiment, the production of proteins and lipids was 2-4 times higher than under the blue wavelength, which is known to be effective in promoting the synthesis of useful substances. For pigments, the primary pigments increased by 1.1-1.8 times, and auxiliary pigments by 1.5-1.9 times compared to the blue wavelength. It was observed that both strains showed a high accumulation of pigments at a 7:3 ratio of mixed wavelengths, where the blue wavelength was dominant.

Thus, it is postulated that employing a mixed wavelength of red and blue lights, particularly one with a higher ratio of blue wavelengths, could significantly enhance the economic efficiency and productivity of future light culture systems. This specific blend, favoring blue wavelengths, is anticipated to be more effective than a simple mixed wavelength approach. However, continuous database construction is essential for not only *Chlorella vulgaris* but also for determining the optimal culture light intensity and wavelength to promote the synthesis of useful substances in each microalgae species. Further research is necessary for practical application in industrial settings beyond laboratory environments.

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발광다이오드 광량 및 파장에 따른 *Chlorella vulgaris*의 생장 및 생화학적 조성 변화 연구

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요약

녹조류 *Chlorella vulgaris*는 세포 내 탄수화물, 단백질, 지질 및 색소와 같은 유용물질을 축적하고, 미네랄, 비타민, 무기질 및 아미노산 등 각종 영양소가 풍부하여, 미래 식량자원의 유력한 후보로 평가되고 있다. 또한 *C. vulgaris* 내에 함유되어 있는 성장 촉진 인자(Chlorella Growth Factor; CGF)는 뇌졸중 개선 및 예방, 항암효과, 성인병 예방 등에 의학적인 효과가 있는 것으로 보고된 바 있어, 그 효능에 대한 연구와 생리활성 성분들의 분리와 응용이 활발하게 진행중이다. 따라서 본 연구는 효과적인 BT 재료인 *C. vulgaris*를 이해하기 위해서, 먼저 생장에 미치는 LED 광량과 파장의 영향을 조사하여, *C. vulgaris*의 생장에 대한 최적의 파장을 파악하였다. 또한 LED 광량 및 파장에 따라 달라지는 탄수화물, 단백질과 지질 함량을 측정하여 유용물질 증진에 효과적인 파장을 파악하고 토의하였다.

먼저 LED 광량과 파장 변화에 따른 *C. vulgaris*의 생장특이성을 파악하였다. LED 파장 중 적색파장과 청색파장에서 높은 생장속도를 보였으며, 적색파장에서 청색파장보다 약 3배 낮은 광친화성지수를 나타냈다. 이는 적색 파장이 *C. vulgaris*의 세포 밀도를 촉진시킬 수 있는 경제적인 광원임을 의미한다. 또한 청색 파장은 단백질 및 지질과 같은 유용한 생

화학적 물질의 촉진에 유용한 파장영역임을 알 수 있었다. 따라서 세포 밀도를 증진시킬 수 있는 경제적인 광원인 적색 파장과 유용물질 증진에 효과적인 청색파장을 이용하여 세 가지 실험을 진행하였다.

첫 번째로 배양 초기에 생장을 위한 최적 환경조건인 적색파장을 주사하고, 이후 생장에 스트레스를 주는 환경조건으로 생화학적 물질을 유도할 수 있는 청색파장을 주사하는 다단 배양 실험을 진행하였다. 실험 결과, 다단 배양 실험은 기존의 대량배양 광원인 형광등과 비교하여, 단백질과 지질은 1.2, 1.4배 높은 함량을 나타냈으며, 색소의 경우 특정 보조색소인 violazanthin의 함량이 4.4배 높게 나타났다. 실험 결과, 형광램프와 비교하여서 단백질, 지질 및 색소와 같은 유용물질을 효과적으로 축적하였으나, 청색 단일파장과 비교하여서 유용물질 증진에는 비효율을 가질 수 있다고 생각된다. 두 번째로 명암주기 조절 실험 결과, 탄수화물, 단백질, 지질 및 색소의 함량은 명암주기를 12L:12D로 조절하였을 때 유용물질의 함량이 높게 나타났다. 이러한 결과는 긴 명기를 지속하는 것이 미세조류에게 장기간 스트레스로 작용하여, 광합성과 에너지 효율을 떨어트려 오히려 유용물질 생성에 방해가 된 것으로 판단된다. 따라서 명암주기에 따른 유용물질의 극대화는 효과적이지 않다고 판단된다. 마지막으로 청색 파장과 적색 파장을 일정비로 혼합한 혼합파장 실험 결과, 유용물질 촉진에 효과적인 청색 파장보다 2~4배 많은 단백질 및 지질을 생산하였으며, 색소의 경우 주색소는 1.2~1.7배, 보조색소는 2배 높은 함량을 나타냈다. 특히 청색 파장의 비가 높은 3:7(적색:청색) 파장비에서 유용물질 촉진이 가장 효과적으로 나타났다.

따라서 본 연구 결과 유용물질 축적에 있어 혼합파장, 그 중에서도 청색 파장의 비가 높은 혼합파장을 이용한다면, 추후 광배양시스템의 경제적인 효과 및 생산성 증대에 기여할 수 있을 것으로 판단된다. 하지만 미세조류의 배양 산업을 효과적으로 성장시키기 위해서, *Chlorella vulgaris* 한 종만이 아닌 배양하는 미세조류 별 최적 배양 조건 및 유용물질을 증진시킬 수 있는 파장에 대한 지속적인 연구가 필요할 것으로 판단된다.

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그리고 바쁘신 와중에도 아낌없는 조언과 논문 지도 및 고견을 해주신 김태진 교수님과 최태진 교수님께도 감사을 마음을 전합니다. 더하여, 책에서는 배울 수 없었던 가르침과 깨달음을 주셨던 여러 교수님께도 지면으로 감사의 인사를 드립니다.

우리 CEES 실원인 페이진언니, 승진이오빠, 민규에게도 감사의 인사를 전합니다. 막내부터, 처음해본 방장자리까지 부족한 점도 미흡한 점도 많았지만 늘 응원하고 지지해줘서 정말 감사했습니다. 항상 든든한 지지목이 되어준 혜빈, 현주, 영은, 소희 그리고 늘 옆에서 응원해준 선배이자, 남자친구인 준용이에게도 감사의 마음을 전하고 싶습니다.

마지막으로 부족한 딸을 항상 믿어주시고, 응원해주신 저희 부모님께 감사의 말을 전합니다. 어떠한 감사의 말씀을 전해도 부족하지만, 항상 제 편이 있다는 생각에 힘들다가도 다시금 힘을 낼 수 있었습니다. 이 마음 잊지 않고, 더욱 자랑스러운 딸이 되도록 하겠습니다. 진심으로 사랑하고 감사드립니다.

이렇게 많은 분들이 도와주셨기에 지금의 제가 있을 수 있었습니다. 제게 주셨던 도움을 밑바탕으로 앞으로도 최선을 다하겠습니다.