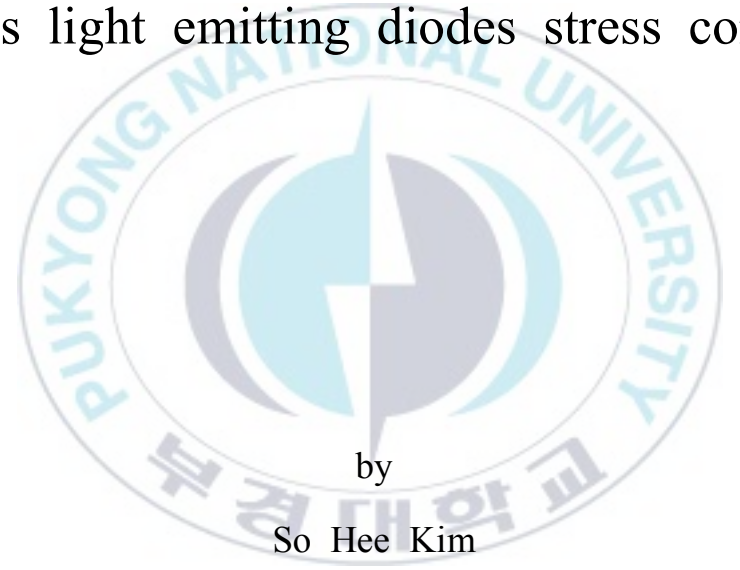
Thesis for the Degree of Master of Engineering

Enhancement of unsaturated fatty acid productivity from three microalgae under various light emitting diodes stress conditions



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February 2020

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(LED를 이용한 다양한 스트레스 조건에서 미세조류의 불포화지방산 생산성 향상)

Advisor: Prof. Sung-Koo Kim

by

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A dissertation

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해양 미세조류의 불포화지방산 생산성 향상

김소희

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요약

3세대 바이오매스인 미세조류는 빠른 성장, 높은 바이오매스 생산량, 높은 지질함량 및 경작지에 대한 제한이 거의 없다는 장점이 있다. 또한, 미세조류에서 생산되는 불포화지방산은 건강보조제로써 중요한 기능을 한다. 본 연구는 LED를 이용한 미세조류의 바이오매스 생산량을 증가시키고, 다양한 스트레스 조건에서 지질 및 불포화지방산의 함량을 증가시키는데 목적을 두었다. 1장에서는, *Pavlova lutheri, Chlorella vulgris* 및 *Porphyridium cruentum*를 F/2배지를 이용하여 질산염의 농도에 따른 바이오매스, 지질생산에 대해 최적화를 하였다. *P. lutheri*는 질산염 160 mg/L에서 0.93 g/L의 최적 성장을 보였고, *C. vulgaris*는 240 mg/L 에서 1.13 g/L의 최적 성장을 나타내었다. *P. cruentum*은 160 mg/L에서 1.25 g/L의 최적 성장을 보였다. 질산염의 농도를 최적화한 후, LED 파장에 따른 성장 및 지질 축적 함량을 알아보았다. *P. lutheri*는 청색파장 (465 nm)에서 1.09 g/L, *C. vulgaris*는 적색파장 (625 nm)에서 1.23 g/L, *P. cruentum*은 녹색파장 (520 nm)에서 1.28 g/L의 가장 높은 바이오매스 함량을 나타내었다. *P. lutheri*의 지질함량은 황색파장 (590 nm)에서 49.1%, *C. vulgaris*는 녹색파장 (520 nm)에서 46.2%, *P. cruentum*은 적색파장 (625 nm)에서 37.8%로 가장 높은 지질함량을 나타내었다. 또한, 지방산 조성 분석을 통해 *P. lutheri, C. vulgais* 및 *P. cruentum*은 각각 최대 eicosapentaenoic aicd (EPA) 10.35%, 10.14%, 그리고 14.61% (w/w), docosahexaenoic

acid (DHA) 6.09%, 8.95%, 그리고 11.29% (w/w)를 함유하고 있음을 나타내었다.

2장은 *Porphyridium cruentum*을 이용하여 14L 광배양생물기에서 대량 배양을 하였다. 바이오매스 생산량 증가를 위하여 1단계로서 녹색파장 (520 nm)을 사용하였고, 2단계로는 지질함량 증가를 위하여 적색파장 (625 nm)을 사용하는 이단 배양을 하였다. 실험 조건으로는 바이오매스 생산량과 지질함량을 증가시키기 위해서 폭기량, 광도와 광주기를 최적화하는 실험을 진행하였다. 또한, 지질과 불포화지방산의 함량을 증가시키기 위해 스트레스 조건인 pH를 조절하여 실험을 진행하였다. *P. cruentum*은 300 μmol/m^2/s 광도, 24:0 h 명/암주기에서 0.91 g/L의 최대 바이오매스 생산량을 나타내었으며, 400 μmol/m^2/s 광도, 18:6 h 명/암주기, pH 6에서 51.8%의 최대 지질함량을 나타내었다. 또한 pH 6에서 *P. cruentum*의 eicosapentaenoic acid (EPA) 함량이 30.6%, docosahexaenoic acid (DHA) 함량이 14.5%까지 증가하였다. pH 6에서 전체 불포화지방산의 함량은 35.1%에서 56.2%로 21.1% 증가하였다.

Chapter I. General introduction

1.1. Introduction

Unsaturated fatty acids are the essential components of higher eukaryotic cells (Ward and Singh, 2005). The unsaturated fatty acid omega-3 (ω-3) consists of α-linolenic acid ($C_{18}H_{30}O_2$, ALA), eicosapentaenoic acid ($C_{20}H_{30}O_2$, EPA), and docosahexaenoic acid ($C_{22}H_{32}O_2$, DHA). ALA can lower blood cholesterol levels and thus reduce the risk of cardiovascular diseases and protect the function of the arteries (Brenna et al., 2009). EPA promotes blood cholesterol and triglyceride levels to decrease and suppresses blood clot formation while also stimulating brain function. It is effective in the prevention of diseases, such as arteriosclerosis and lung diseases (Simopoulos, 1991). DHA is one of the major components of the membranes of all cell types and is primarily found in the brain and retina. Additionally, it is one of the important components of the brain (Swanson et al., 2012). Currently, the major source of EPA and DHA is marine fish, such as mullet, salmon, and mackerel (Adarme-Vega et al., 2012). However, overfishing has exponentially increased since the 1980s worldwide. Therefore, the use of fish as the unsaturated fatty acid resource is no longer a sustainable option (Ryckebosch et al., 2014). Consequently, microalgae have increasingly attracted attention due to their EPA and DHA contents.

Microalgae are unicellular photosynthetic organisms that grow by using sun light and carbon dioxide and produce lipids that can be applied as biofuels, food, feed, and high value bioactive agents based on nutrient sources such as nitrogen, phosphorus, and gallium (Wu et al., 2017; Hoekman., 2012;

Shimizu., 1996). Microalgae grow rapidly fix carbon dioxide 10-50 times more efficiently than land plants (Tredici., 2010; Li et al., 2008; Lam et al., 2012). Among different microalgal species, *Pavlova lutheri*, *Chlorella vulgaris,* and *Porphyridium cruentum* were used in this study. *P. lutheri* and *C. vulgaris* have a high lipid content of 30%-40% per dry cell weight and are suitable for commercial use with lipids (Ryckebosch et al., 2014; Liang et al., 2009). In addition, *P. lutheri* and *P. cruentum* produce high content of unsaturated fatty acid and contain omega-3 fatty acids such as EPA and DHA (Ryckebosch et al., 2014).

Microalgal growth and lipid production are affected by various physical and chemical stresses as well as environmental conditions. Temperature (Renaud et al., 2002), nutrients (Yang et al., 2011), light intensity (Cheiirsilp et al., 2012), salt concentration (Takagi et al., 2006), L/D photoperiod cycle (Wahidin et al., 2013), and LED wavelengths (Ra et al., 2016) affect the biomass and lipid production of microalgae. When *C. vulgaris* was cultured under LED light emitted at appropriate wavelengths, the biomass yields increased when light was emitted at red (660 nm) wavelength (Yan et al., 2013).

Optimum light wavelength using light emitting diodes (LEDs) in the narrow spectrum band is essential for microalgal culture (Teo et al., 2014). The advantage of LEDs is low energy consumption and low heat generation with sufficient light emission to facilitate maximum growth of heat sensitive microalgae. LEDs have a longer life than fluorescent lamps and has high conversion efficiency (Chen et al., 2011). The lifetime of LEDs is 500% and 941% longer than that of a fluorescent light. LEDs can emit uniform light to

the bioreactor owing to the dispersion of lights, and microalgae can be cultured by adjusting light intensity (Teo et al., 2014).

Most microalga strains have been isolated and cultured under controlled conditions for efficient cultivation. Photobioreactors (PBRs) can adjust the culture conditions for microalga strains and control the metabolism of microalgae (Sierra et al., 2008). Bioreactors with light-emitting diodes (LEDs) have been used for the efficient cultivation of microalgae. However, it is not clear whether the LED chip can supply enough light energy that can penetrate the 20-mm thick bioreactor wall and enter into the chamber (Taisir et al., 2016; Sirisuk et al., 2018b). To solve this issue, an internal light illumination source was installed in this study. Optimization of the LED light intensity and photoperiod improve lipid accumulation as well as the growth of microalgae (Jung et al., 2019).

In chapter 2 study, cell growth and lipid production were improved through two-phase culture system using wavelength stress. Two-phase culture system produced the highest biomass in the first phase culture and provide light that can not be absorbed by wavelength stress when reaching the stationary phase, leading to high lipid production and efficient results (Ra et al., 2016). In the following two-phase culture system, microalgae grew to blue (465 nm), red (625 nm), and green (520 nm) LED wavelengths to produce maximum biomass. In the second phase, yellow (590 nm), green (520 nm), and red (625 nm) LED wavelengths stresses were applied to increased lipid production. This study aimed to improve cell biomass production in the first phase and lipid production in the second phase by applying a two-phase culture system, and ultimately increase the content of

unsaturated fatty acid. The first trial of culture was performed to determine optimum nitrate concentrations for the three microalgae, namely as *P. lutheri*, *C. vulgaris* and *P. cruentum*. After optimizing the nitrate concentration, the first phase of the second experiment was carried out, by employing the wavelengths of the LED light determined during the first trial, in order to increase cell biomass. This experiment facilitates an increase in lipid production during the second phase of culture when the microalgae could not absorb LED light stress. Therefore, when microalgae are cultured in a two-phase culture system, lipid production can be increased in second phase culture compared with the lipid production in the first phase culture (Figure 1).

In this study, a two-phase culture system involving 14-L PBRs was used for the cultivation of *P. cruentum*. The first phase comprised the green (520 nm) LED to increase the biomass production, and then the LED wavelength was switched to red (625 nm) during the second phase to stimulate lipid accumulation. The optimization of the aeration rate, light intensity, and photoperiod increased the biomass production, while the optimization of the light intensity, photoperiod, and pH increased the lipid accumulation. Finally, the effect of pH on the accumulation of ω-3 fatty acids was evaluated.

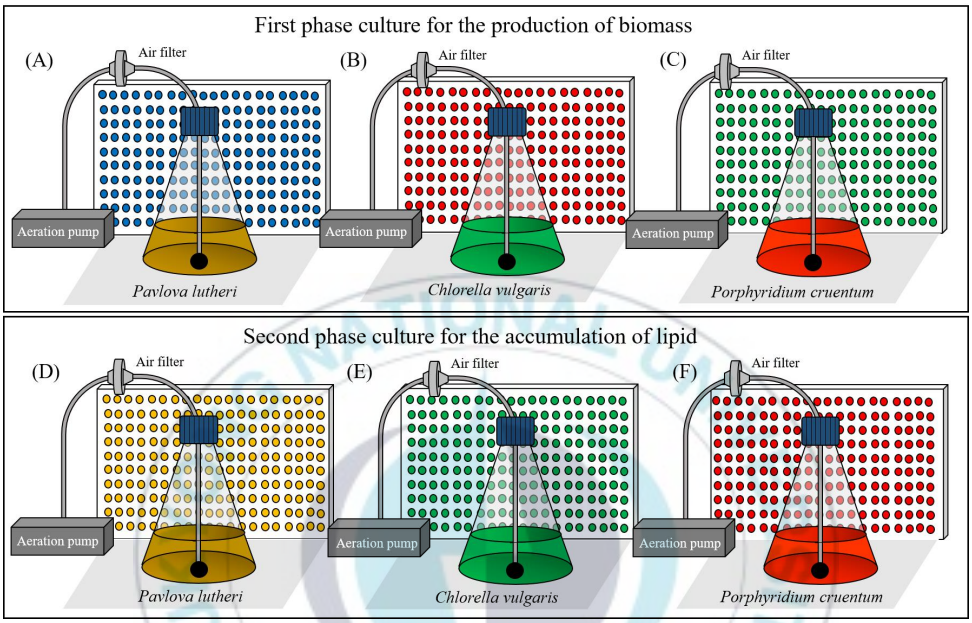

Figure 1. Graphical views of two-phase culture system setting. LED panels with dimensions of $28.5 \times 38.6 \times 4.4$ cm^3 (Luxpia Co. Ltd., Suwon, Korea) were arranged in strips in this experiment. Each LED strip comprised 10 diodes spaced vertically and 20 diodes spaced horizontally at 1-cm intervals. The LED light panel is composed of red, yellow, green, blue, and purple. (A)-(C) first phase cultures and (D)-(F) second phase culture system. (A) and (D), (B) and (E), and (C) and (F) are *Pavlova lutheri*, *Chlorella vulgaris*, and *Porphyridium cruentum*, respectively.

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Chapter II. Lipid and unsaturated fatty acid productions from three microalgae using nitrate and light emitting diodes with complementary LED wavelength in a two-phase culture system

2.1. Materials & methods

2.1.1. Microalgae and culture conditions

Pavlova lutheri, Chlorella vulgaris, and *Porphyridium cruentum* were obtained from the Korea Institute of Ocean Science & Technology (Southern Sea Research Institute of Korean Institute of Ocean Science and Technology (KIOST), Geoge-si, Korea) and cultured under LED light emitted at various wavelengths, as shown in Figure 2. The algae were pre-cultured for 12 days in sterilized seawater supplemented with a modified F/2 medium containing 75 mg $NaNO_3$, 5 mg $NaH_2PO_4 \cdot H_2O$, 4.36 mg Na_2EDTA, 3.15 mg $FeCl_3 \cdot 6H_2O$, 0.02 mg $MnCl_2 \cdot 4H_2O$, 0.02 mg $ZnSO_4 \cdot 7H_2O$, 0.01 mg $CoCl_2 \cdot 6H_2O$, 0.01 mg $CuSO_4 \cdot 5H_2O$, 0.006 mg $Na_2MoO_4 \cdot 2H_2O$, 30 mg Na_2SiO_3, 0.2 mg thiamine-HCl, 0.01 mg vitamin B_{12}, and 0.1 mg biotin per liter (Guillard and Ryther., 1962). The initial cell density was 1×10^5cells/mL. The three abovementioned strains were cultured at a temperature of 20℃ and, light intensity of 100 μmol/m^2/s under a photoperiod cycle of 12:12h L/D (Ra et al., 2016). Sodium nitrate was used as the nitrate (Rananan et al., 2013) and concentration were set at 80 mg/L, 160 mg/L, 240 mg/L, and 320 mg/L.

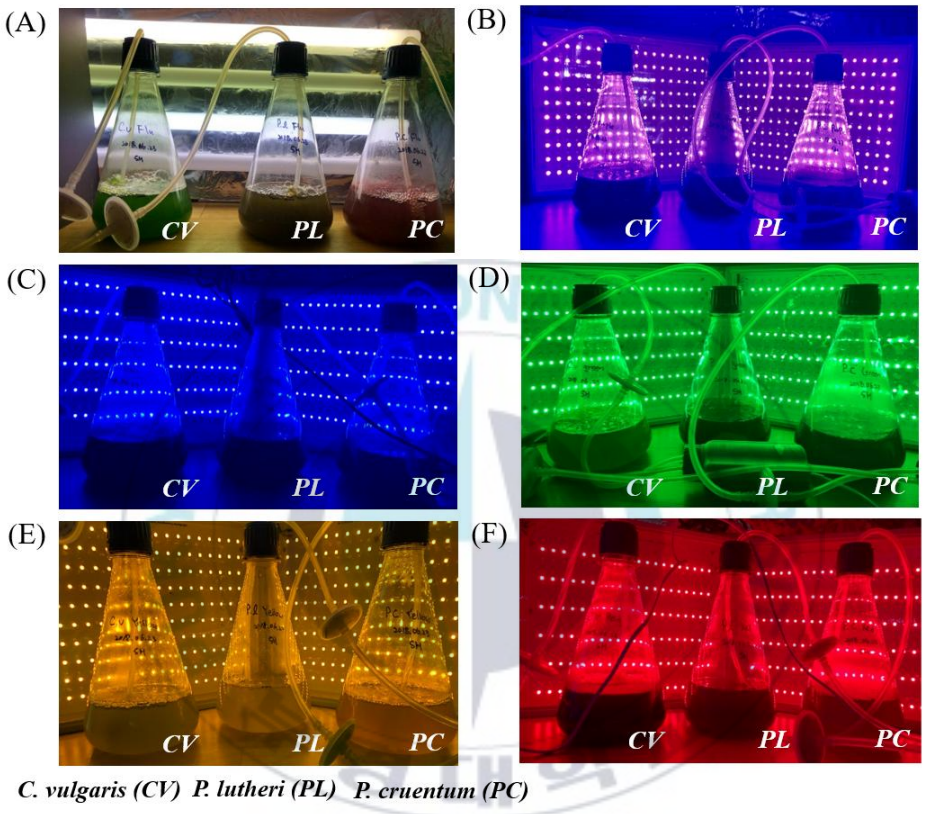

Figure 1. Photographs of microalgae cultured under different wavelengths; *C. vulgaris* (CV), *P. lutheri* (PL), and *P. cruentum* (PC), respectively. Cell culture was carried out under (A) fluorescent light as a control, (B) purple (400 nm), (C) blue (465 nm), (D) green (520 nm), (E) yellow (590 nm), and (F) red (625 nm) LED wavelengths.

2.1.2. LED wavelengths for microalgal culture

LED panels with dimensions of 28.5 x 38.6 x 4.4 cm^3 (Luxpia Co. Ltd., Suwon, Korea) were arranged in strips in this experiment. Each LED strip comprised 20 diodes spaced vertically and horizontally at 1-cm intervals. LED wavelengths used for the growth of microalgae were purple (400 nm), blue (465 nm), green (520 nm), yellow (590 nm), and red (625 nm). The light intensity was measured using a light sensor (TES-1339; UINS Ins., Busan, Korea) at the centerline of the flask filled with culture medium. The control culture was maintained for 17 days under fluorescent light, not natural solar light. Natural solar light is more economical than fluorescent light. However, since the light intensity and the photoperiod are not controllable, the reproducibility of the experiment is degraded and can not be performed in comparison to other conditions such as LED wavelength (Borowitzka., 1999).

2.1.3. Measurement of microalgal biomass growth

Dry cell weight was determined using an ultraviolet–visible spectrophotometer (Ultrospec 6300 Pro; Biochrom Ltd., Cambridge, UK) at an optical density of 680 nm (OD_{680}) and 540 nm (OD_{540}) (Collos et al., 1999; Maksimova et al., 2000).

The correlation between the optical densities (680 nm and 540 nm) of the three microalgae and their dry cell weights was determined by Eq. (1–3);

Dry cell weight of *P. lutheri* (g dcw/L) = $0.35(OD_{680})$ (R^2=0.99) Eq.(1)

Dry cell weight of *C. vulgaris* (g dcw/L) = $0.41(OD_{680})$ (R^2=0.98) Eq.(2)

Dry cell weight of *P. cruentum* (g dcw/L) = $0.77(OD_{540})$ (R^2=0.99) Eq.(3)

2.1.4. Total lipid measurement

Cell harvesting was carried out by centrifuging (Supra R22; Hanil Scientific Inc., Gimpo, Korea) at 9,946 × g for 10 min and washed twice using distilled water. The cell biomass was dried using a freeze dryer (SFDSM-24L; SamWon Industry, Seoul, Korea). Subsequently, 5 mL of distilled water was added to 10 mg of the dried cell biomass, and cells were sonicated for 10 min using a sonicator (100 W, 20 kHz, 550 Sonic Dismembrator; Fisher Scientific Inc., Pittsburgh, PA, USA). The total lipid content was determined using methanol and chloroform following a modified solvent-based method (Blight and Dyer., 1959), as shown in Eq. (4);

$$Lipid content\,(\%\ of\ dcw) = \frac{(W_2 - W_1)\times 100}{DCW} \qquad Eq.\ (4)$$

where lipid content is the cellular lipid content of the microalgae (% of DCW). W_1 (g) is the weight of an empty 20-mL glass tube and W_2 (g) is the weight of a 20-mL glass tube containing the extracted lipid. DCW (g) is the dried microalgal cell biomass.

2.1.5. Fatty acid methyl ester measurement

The direct transesterification method (Dhup and Dhawan., 2014) was used to convert extracted lipids to FAMEs. FAMEs were then analyzed using gas chromatography (GC, YL 6100; Young Lin Inc., Anyang, Korea) by employing a flame ionization detector (FID) and a silica capillary column (30 m × 0.32 mm × 0.5 μm; HP-INNOWAX; Agilent Technologies, Santa Clara, CA, USA). The column temperature adjustments were as follows: 140℃ for

5 min followed by a temperature increase to 240℃ at 5℃/min, which was subsequently maintained for 10 min. The injector and FID temperatures were set at 250℃. FAMEs were identified by comparing their retention times against those of authentic standards.

2.1.6. Statistical analyses

Each experiment was conducted in triplicate. The statistical significance of cell biomass and lipid content was evaluated by one-way analysis of variance (ANOVA) and Duncan's multiple range test ($P < 0.05$) using SPSS software (ver. 23.0; SPSS Inc., Chicago, IL, USA).

2.2. Results and Discussion

2.2.1. Effect of nitrate concentration on cell growth

P. lutheri, C. vulgaris, and *P. cruentum* were cultured in 2-L flasks with a 1-L working volume at 20℃ and at an aeration rate of 2.5 L/min. Nitrate concentration of microalgae was controlled to determine optimal biomass production. Nitrate concentrations of 80 mg/L, 160 mg/L, 240 mg/L, and 320 mg/L were prepared for cultures with a fluorescent light intensity of 100 μ mol/m^2/s. Figure 2(A), (B), and (C) demonstrates that *P. lutheri, C. vulgaris* and *P. cruentum* cultured at nitrate concentration of 160 mg/L, 240 mg/L, and 160 mg/L showed maximum biomass production levels of 0.93 g dcw/L, 1.13 g dcw/L, and 1.25 g dcw/L, respectively, over 15 days. Increase in nitrate concentration resulted in higher cell biomass production. For the cultures of *Phaeodactylum tricornutum, Dunaliella tertiolecta,* and *Isochrysis galbana*, nitrate concentration contributing to generation of maximum biomass were different (Jung et al., 2018). Here, *P. lutheri* and *P. cruentum* showed maximum biomass production at the same nitrate concentration of 160 mg/L, whereas the nitrate concentration required for facilitating. Maximum biomass production by *C. vulgaris* was different, i.e., 240 mg/L. A further increase in nitrate concentration to 160 mg/L for *P. lutheri* and *P. cruentum* led to a reduction in biomass production levels; *C. vulgaris* also showed the same trend for nitrate concentration exceeding 240 mg/L, as shown in Figure 2 (A), (B), and (C). This indicates that high concentrations of nitrate have an inhibitory effect on algal growth. Microalgae increase the activity of nitrate reductase at high concentrations of nitrate, leading to an enhanced production of nitrite and ammonia; thus, the accumulated nitrite and ammonia may act

as inhibitory compounds in biomass production (Jeanfils et al., 1993; Ra et al., 2016).

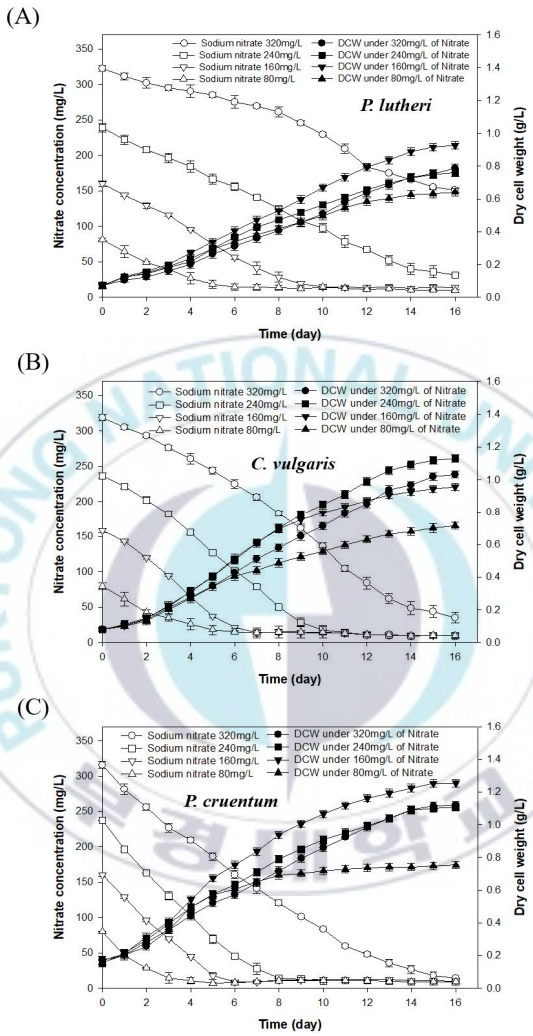

Figure 2. Biomass production by various initial nitrate concentrations and fluorescent light intensity of 100 μmol/m^2/s. (A) *P. lutheri*, (B) *C. vulgaris,* and (C) *P. cruentum*.

2.2.2. Effects of LED wavelengths on cell growth and lipid accumulation in the first phase of culture

Figure 3 show the complementary LED wavelengths. The complementary wavelength was based on complementary color. A complementary color is a pair of colors that make up the color contrast. It is a complementary color to each other in the color wheel. Using complementary colors, the difference between the two colors is called complementary color contract (Bruno et al., 2005).

An important factor in determining the optimal photosynthetic activity of microalgae is the wavelength. Cell growth and lipid production in microalgae are affected by LED wavelengths. The LED lights were emitted at the following wavelengths: purple (400 nm), blue (465 nm), green (520 nm), yellow (590 nm), and red (625 nm). Fluorescent light was used for the control culture.

Figure 4 shows dry cell weight achieved using the purple (400 nm) to red (625 nm) wavelengths at optimal nitrate concentrations of 160 mg/L for *P. lutheri* and *P. cruentum*, and 240 mg/L nitrate for *C. vulgaris*. Figure 4(A) show that among these wavelengths, blue LED wavelength facilitated *P. lutheri* to produce the highest biomass of 1.09 g dcw/L on day 14, followed by purple LED (0.99 g dcw/L), red LED (0.98 g dcw/L), fluorescent light (0.89 g dcw/L), green LED (0.86 g dcw/L), and yellow LED (0.62 g dcw/L). These results indicated that the use of blue LED wavelength as a light source could enhance the biomass production by *P. lutheri*. *Phaeodactylum tricornutum*, a brown-colored microalgae similar to *P. lutheri*, showed the highest biomass production under blue LED (Costa et al., 2013). This is

because *P. tricornutum* possesses chlorophyll a, chlorophyll c1+c2 and fucoxanthin as primary pigments and some carotenoids that absorb blue light (Kosakowska et al., 2004). Thus, for *P. lutheri,* blue LED wavelength was chosen as the wavelength for biomass production in the first phase of culture.

Figure 4(B) shows that *C. vulgaris* produced maximum biomass at 1.23 g dcw/L on day 14 under red LED wavelength, followed by blue LED (1.15 g dcw/L), fluorescent light (1.10 g dcw/L), purple LED (1.06 g dcw/L), yellow LED (1.04 g dcw/L) and green LED (0.88g dcw/L). Therefore, red LED wavelength was used to increase the biomass production of *C. vulgaris*. This result is consistent with that reported (Yan et al., 2013). When *C. vulgaris* was cultured at various wavelengths of red, white, yellow, purple, blue and green LEDs, *C. vulgaris* generated the highest biomass under red LED. The reason for this is that *C. vulgaris*, green microalgae, possesses chlorophyll a, chlorophyll b and the accessory pigment carotenoid for photosynthesis and that all chlorophylls have a maximum absorption band at red (600-700 nm) and blue (400-500 nm) wavelengths (Kubin et al., 1983). Thus, red LED wavelength was a suitable wavelength for biomass production of *C. vulgaris* in the first phase of culture.

The main carotenoids of microalgae with various LED wavelength produced different amounts of biomass. The supply of undesired wavelengths to carotenoids caused in photo-oxidation, reduction of photosynthesis, and decrease of cell division leading to a reduction of biomass production (Severes et al., 2017). However, proper light with desired wavelengths to main carotenoids of microalgae increases the activity of cell and biomass production (Duarte et al., 2019).

Figure 4(C) shows that *P. cruentum* yielded the highest biomass at 1.28 g dcw/L under green LED wavelength, following by purple LED (1.23 g dcw/L), blue LED (1.23 g dcw/L), fluorescent light (1.22 g dcw/L), yellow LED (1.20 g dcw/L) and red LED (1.17 g dcw/L). Similar biomass production levels were also achieved by *Porphyridium purpureum* at the abovementioned wavelengths (Coward et al., 2016). *P. purpureum* is a red microalga belonging to the same genus as *P. cruentum*. *P. purpureum* showed the highest biomass production under green LED among red, green, blue wavelengths as well as on being exposed to a combination of red, green, and blue. According to these results, *P. cruentum* was cultured for increasing biomass production at green LED wavelengths. Phycobiliprotein has been reported to be a major harvest pigment of red microalgae. Phycobiliprotein is mainly composed of phycoerythrin and small amounts of phycocyanin and allophycocyanin (Guiheneuf and Stengel., 2015). Phycoerythrin absorbs light efficiently at green wavelength with a range of absorption bands of 450-600 nm (Coward et al., 2016). Thus, green LED wavelength was selected as a suitable wavelength for biomass production of *P. cruentum* in the first phase of culture.

According to the results of Figure 4, the brown microalgae *P. lutheri*, the green microalgae *C. vulgaris*, and the red microalgae *P. cruentum* generate the highest biomass yields in complementary LED wavelength to the microalgae color blue (465 nm), red (625 nm) and green (520 nm) LEDs, respectively. This indicates that the microalgae were able to increase absorption of light at complementary LED wavelengths.

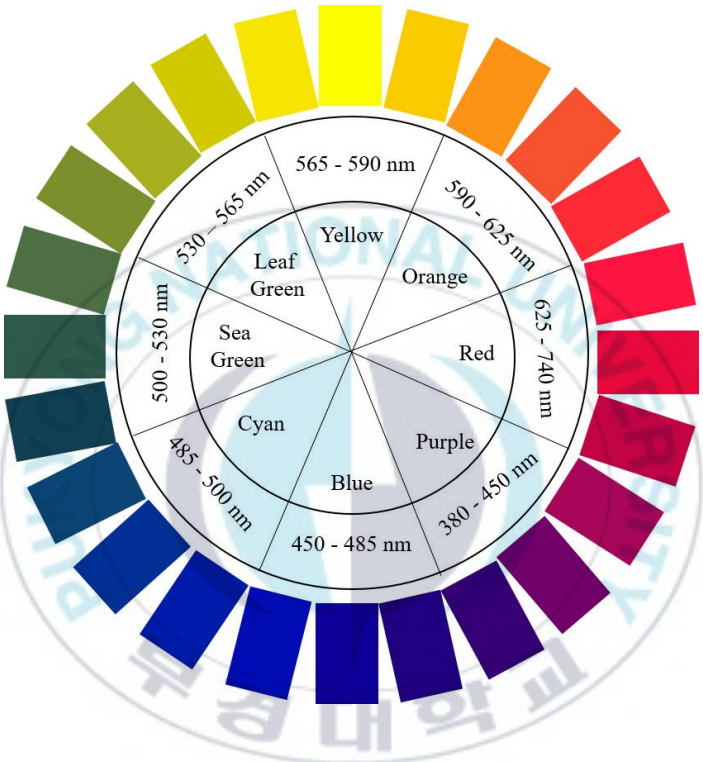

Figure 3. Complementary wavelength of LED lights. When complementary lights with opposite light colors are overlapped, the light color becomes white (Bruno et al., 2005).

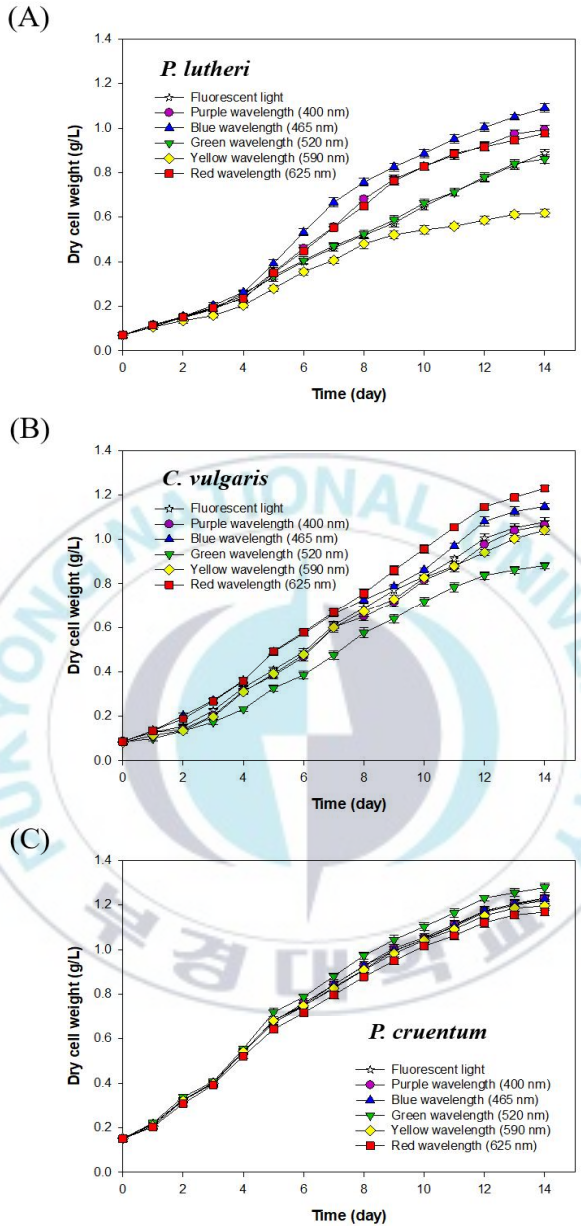

Figure 4. Biomass productions under different LED wavelengths during the first phase of culture (A*) P. lutheri*, (B) *C. vulgaris* and (C) *P. cruentum.*

Figure 5 shows the lipid content on day 14 of the first phase of culture. *P. lutheri* showed the highest lipid content at 52.0% (w/w) under yellow wavelength as same wavelength of the cell color. *C. vulgaris* showed the highest lipid content at 50.5% (w/w) under green LED wavelength. *P. cruentum* showed the highest lipid content at 36.7% (w/w) under red LED wavelength. The highest lipid production can be obtained at the wavelength that generates the lowest biomass. Lipid production is occurred by protein and carotenoid biodegradation. As a results, photosynthesis does not occur and the biomass production is low (Chen et al., 2017). Microalgae accumulates lipids under stress condition because they reflect light without absorbing it (Jung et al., 2019). Lipid accumulation is induced by energy imbalance of microalgae and the exposure to stress factor. In addition, cells produced lipid from self-defense mechanisms by photo-oxidation of light (Shin et al., 2018). The lipid production was carried out by the enzymatic synthesis of ribulose bisphosphate carboxylase/oxygenase (RuBPCase) and carbonic anhydrase (Roscher and Zetsche., 1986). Thus, yellow, green, and red LEDs were selected as optimal wavelengths for lipid production in the second phase of culture of *P. lutheri, C. vulgaris* and *P. cruentum*, respectively.

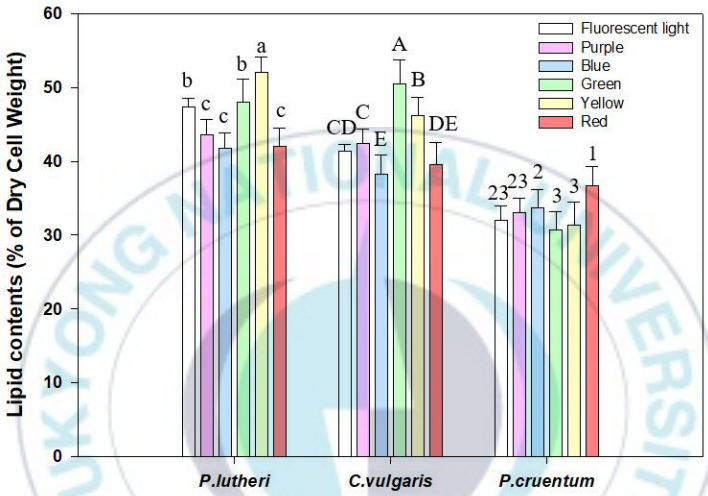

Figure 5. Lipid production at different LED wavelengths during the end of the first phase of culture to enable wavelength selection during the second phase of culture. Different letters and numbers indicate significant differences ($P < 0.05$, Duncan's test).

2.2.3. Biomass and lipid production by two-phase culture

The two-phase cultures of microalgae were carried out under optimal nitrate concentrations and LED wavelengths as shown in Figure 6.

Figure 6(A) shows the two-phase culture for biomass and lipid production by *P. lutheri*. *P. lutheri* used blue (465 nm) LED wavelength in the first phase of culture for biomass production and yellow (590 nm) LED wavelength in the second phase of culture for lipid production. The second phase of culture was started on day 14. Red (625 nm) LED wavelength was used for the first phase of culture of *C. vulgaris* for biomass production and green (520 nm) LED wavelength was used for the second phase of culture for lipid production. Green (520 nm) LED wavelength was used for biomass production by *P. cruentum* in the first phase of culture, while red (625 nm) LED wavelength was used in the second phase for lipid production.

LED light stress in the second phase of culture was exerted for 3 days to determine the optimum culture time to obtain maximum lipid content. The lipid content of *P. lutheri* cultured in two-phase culture was shown in Figure 6(B). On day 2 of the second phase of culture, *P. lutheri* generated the highest lipid content at 49.1% under yellow LED, while *C. vulgaris* and *P. cruentum* generated the highest lipid content at 46.2% and 37.8%under green and red LED wavelengths, respectively. Under stress-induced conditions, lipid content was highest on day 2 and slightly decreased on day 3. Day 3 is associated with excessive and prolonged stresses that decrease lipid production. Stress is required to generate high lipid content. However, the duration of light exposure increases, the lipid production decreases due to oxidative stress (Lucas et al., 2013). Similar results were reported regarding

the effect of green wavelength stress on lipid synthesis in *Nannochloropsis oculata, Nannochloropsis salina*, and *Nannochloropsis oceanica* (Ra et al., 2016). The results revealed that lipid production could be improved in the second phase of culture when LED light under stress-induced condition was employed in the two-phase culture. According to these results, the abovementioned condition is critical to increase lipid production in the two-phase culture.

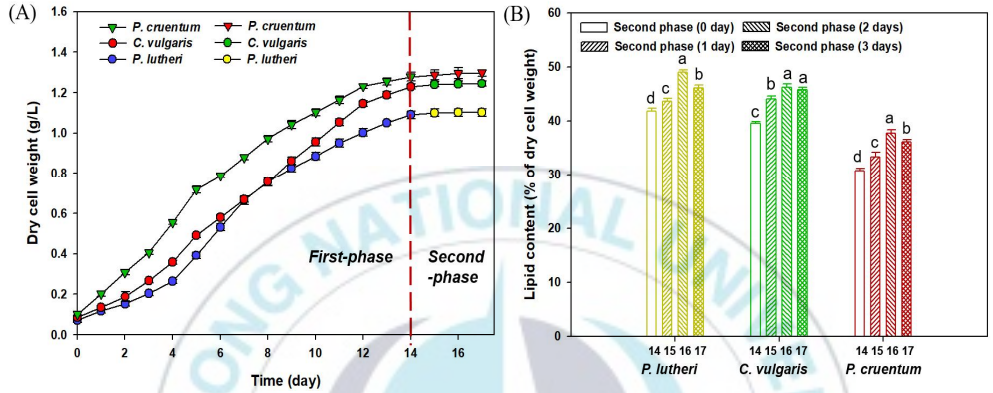

Figure 6. Microalgal culture under blue, red, and green LED wavelengths for biomass production and under yellow, green, and red LED wavelengths for lipid production by *P. lutheri*, *C. vulgaris*, and *P. cruentum*, respectively. (A) Two-phase cultures of *P. lutheri, C. vulgaris*, and *P. cruentum* involving biomass production in the first phase and lipid production in the second phase and (B) lipid production by the three microalgae under LED wavelength-induced stress at the stationary phase of the second phase of culture. The vertical line in (A) indicates the start of the second phase of the cultures. Different letters indicate significant differences in lipid content ($P < 0.05$, Duncan's test).

2.2.4. Effect of LED wavelength on fatty acid composition in lipids produced from 3 microalgae

Table 1. presents fatty acid composition of *P. lutheri, C. vulgaris*, and *P. cruentum* cultures. Table 1(A) shows the fatty acid composition at the end of the first phase of culture, and Table 1(B) shows the fatty acid composition on day 2 of the second phase of culture. As shown in Table 1(A), all three microalgae contain the highest stearic (C18:0) acid content, ranging from 34.25% (w/w) to 43.77% (w/w) for *P. lutheri*; 47.26% (w/w) to 63.47% (w/w), *C. vulgaris*; and 45.64% (w/w) to 56.76% (w/w), *P. cruentum*. The most common components of biodiesels are palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2) methyl esters (Ramos et al., 2009). Stearic acid (C18:0) could be used as a biodiesel because of its high cetane number (Knothe et al., 2003). As shown in Table 1(B), the content of unsaturated fatty acids in the three microalgae after the second phase of culture was higher than that in the first phase of culture. In the second phase of culture, the highest unsaturated fatty acid content of *P. lutheri, C. vulgaris*, and *P. cruentum* was determined to be 55.64% (w/w), 45.91% (w/w), and 43.38% (w/w) on being exposed to yellow, green, and red LED wavelengths, respectively. This suggests that unsaturated fatty acid content was increased under stress-induced by wavelength radiation during the second phase of culture.

Table 1. Composition of fatty acid methyl esters (FAMEs) as fatty acids in *P. lutheri, C. vulgaris*, and *P. cruentum* cultured under purple, blue, green, yellow, and red LED wavelengths and under fluorescent light.

(A) First phase in which the three microalgae were cultured under purple (400 nm), blue (465 nm), green (520 nm), yellow (590 nm), and red (625 nm) LED wavelengths as well as under fluorescent light for 14 days.

(B) Second phase in which the three microalgae were cultured under purple (400 nm), blue (465 nm), green (520 nm), yellow (590 nm), and red (625 nm) LED wavelengths as well as under fluorescent light on day 2 of the second phase. The LED wavelengths used during the first phase of culture of *P. lutheri*, *C. vulgaris*, and *P. cruentum* were blue, red, and green, respectively.

(A)

Free fatty acid (% of total fatty acid)	*Pavlova lutheri*						*Chlorella vulgaris*						*Porphyridium cruentum*					
	Purple	Blue	Green	Yellow	Red	Flu	Purple	Blue	Green	Yellow	Red	Flu	Purple	Blue	Green	Yellow	Red	Flu
Myristic acid (C14:0)	6.19	**6.93**	3.70	3.57	6.82	4.32	0.14	0.16	0.06	0.15	**0.12**	0.22	0.10	0.25	**0.43**	0.21	0.43	0.30
Palmitic acid (C16:0)	16.86	**16.89**	16.61	9.89	17.45	15.14	6.80	6.11	9.88	9.78	**6.71**	7.08	11.28	14.64	**15.26**	12.32	14.32	19.24
Palmitoleic acid (C16:1)	2.94	**3.63**	3.12	2.31	5.33	3.42	0.68	0.36	0.48	1.60	**0.32**	0.66	0.87	3.01	**2.05**	2.02	0.54	0.38
Stearic acid (C18:0)	41.88	**43.77**	42.24	34.25	35.41	41.40	60.07	58.56	47.26	60.30	**63.47**	57.07	56.76	53.90	**56.38**	54.89	45.64	56.20
Oleic acid (C18:1)	13.14	**11.47**	13.83	21.68	18.56	12.36	13.36	20.60	10.62	11.80	**14.08**	14.44	12.81	8.54	**8.15**	8.02	9.16	6.66
Linoleic acid (C18:2)	4.00	**3.76**	2.60	4.57	3.36	3.02	2.16	2.10	3.98	2.09	**0.92**	1.83	2.56	2.39	**2.73**	3.25	3.27	2.53
Linolenic acid (C18:3)	3.20	**2.18**	4.65	5.00	2.13	6.07	8.92	5.51	1.66	4.86	**8.78**	10.68	1.67	1.80	**1.97**	2.85	1.27	2.05
Arachidic acid (C20:0)	0.33	**0.28**	0.34	0.48	0.29	0.79	0.11	0.09	2.32	0.35	**0.09**	0.11	0.18	0.17	**0.09**	0.12	0.16	0.22
Eicosapentaenoic acid (C20:5)	5.09	**3.48**	4.71	9.84	5.11	6.22	4.10	3.26	11.45	3.71	**1.99**	4.06	4.62	5.62	**4.26**	7.33	12.61	4.89
Behenic acid (C22:0)	1.12	**0.85**	1.90	2.01	1.23	2.76	0.65	0.80	3.90	0.89	**0.66**	0.41	0.35	1.64	**2.03**	4.62	2.31	1.15
Docosahexaenoic acid (C22:6)	5.24	**6.75**	6.29	6.40	4.31	4.51	3.00	2.45	8.40	4.46	**2.86**	3.44	8.79	8.03	**6.64**	4.36	10.29	6.38
Unsaturated fatty acid	33.62	**31.28**	35.21	49.81	38.79	35.60	32.22	34.27	36.58	28.53	**28.96**	35.11	31.33	29.39	**25.82**	27.85	37.13	22.90
Saturated fatty acid	66.38	**68.72**	64.79	50.19	61.21	64.40	67.78	65.73	63.42	71.47	**71.04**	64.89	68.67	70.61	**74.18**	72.15	62.87	77.10

(B)

Free fatty acid (% of total fatty acid)	*Pavlova lutheri*						*Chlorella vulgaris*						*Porphyridium cruentum*					
	Purple	Blue	Green	Yellow	Red	Flu	Purple	Blue	Green	Yellow	Red	Flu	Purple	Blue	Green	Yellow	Red	Flu
Myristic acid (C14:0)	4.16	5.33	3.19	**2.57**	4.45	3.26	0.11	0.12	**0.06**	0.12	0.11	0.12	0.11	0.12	0.13	0.12	**0.14**	0.13
Palmitic acid (C16:0)	12.26	13.68	11.44	**8.54**	14.09	12.15	5.02	5.08	**5.36**	6.81	6.30	6.81	10.46	12.14	13.16	12.32	**11.31**	17.24
Palmitoleic acid (C16:1)	4.85	3.36	4.10	**5.13**	4.46	4.39	0.68	0.34	**0.77**	0.97	0.76	0.01	2.87	3.01	2.05	2.02	**2.21**	2.84
Stearic acid (C18:0)	34.28	37.09	36.05	**32.32**	33.01	36.23	58.44	56.63	**48.26**	57.06	58.01	55.69	54.01	53.44	54.12	54.27	**44.13**	53.79
Oleic acid (C18:1)	18.70	17.15	18.90	**20.97**	19.19	17.45	14.61	19.60	**16.20**	14.64	15.62	15.39	13.10	10.14	9.53	11.08	**10.09**	7.66
Linoleic acid (C18:2)	6.36	5.64	6.40	**6.81**	5.54	5.98	3.59	3.18	**4.10**	3.20	2.24	3.37	2.13	2.14	2.54	2.41	**2.92**	2.17
Linolenic acid (C18:3)	5.41	4.18	5.73	**6.29**	5.98	6.22	7.20	4.06	**5.75**	4.85	6.07	8.87	2.66	2.20	2.47	2.52	**2.27**	2.21
Arachidic acid (C20:0)	0.88	0.85	0.61	**0.82**	0.95	0.59	0.11	0.11	**0.32**	0.25	0.19	0.11	0.12	0.12	0.08	0.11	**0.12**	0.12
Eicosapentaenoic acid (C20:5)	6.43	5.83	6.20	**10.35**	6.05	7.04	6.10	5.26	**10.14**	5.71	5.90	5.06	5.62	7.22	7.26	7.63	**14.61**	5.89
Behenic acid (C22:0)	0.24	0.33	0.49	**0.11**	0.27	0.57	0.13	0.18	**0.10**	0.10	0.17	0.12	0.13	0.43	0.03	0.16	**0.92**	0.15
Docosahexaenoic acid (C22:6)	6.43	6.57	6.88	**6.09**	6.02	6.11	4.00	5.45	**8.95**	6.29	4.64	4.44	8.79	9.03	8.64	7.36	**11.29**	7.80
Unsaturated fatty acid	48.18	42.72	48.22	**55.64**	47.24	47.19	36.17	37.88	**45.91**	35.66	35.23	37.14	35.17	33.74	32.49	33.03	**43.38**	28.57
Saturated fatty acid	51.82	57.28	51.79	**44.36**	52.76	52.81	63.83	62.12	**54.09**	64.34	64.77	62.86	64.83	66.26	67.52	66.97	**56.62**	71.43

2.3. Conclusion

Microalgal culture using two-phase culture system increases biomass and lipid production. In the first phase, the growth of *P. lutheri, C. vulgaris,* and *P. cruentum* was increased by selecting the complementary LED wavelength of microalgae colors, blue (465 nm), red (625 nm) and green (520 nm), respectively. The lipid content was increased in the second phase of culture. *P. lutheri, C. vulgaris*, and *P. cruentum* produced 31.3% (w/w), 29.0% (w/w), and 25.8% (w/w) of unsaturated fatty acids in the first phase, which increased to 42.7% (w/w), 35.2% (w/w), and 32.5% (w/w) during the second phase by exposing microalgae to the same color LED wavelength as a stress condition, respectively.

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Chapter III. Improvement of unsaturated fatty acid, and biomass productivity from *Porphyridium cruentum* using a pH-controlled two phase system in a bioreactor with light emitting diodes

3.1. Materials & methods

3.1.1. Microalga strain and culture conditions

P. cruentum was obtained from the Korea Institute of Ocean Science & Technology (KIOST, Geoge-si, Gyeongsangnam-do, Korea) and pre-cultured for 10 days in sterilized seawater with modified F/2 medium containing 75 mg $NaNO_3$, 5 mg $NaH_2PO_4 \cdot H_2O$, 4.36 mg Na_2EDTA, 3.15 mg $FeCl_3 \cdot 6H_2O$, 0.02 mg $MnCl_2 \cdot 4H_2O$, 0.02 mg $ZnSO_4 \cdot 7H_2O$, 0.01 mg $CoCl_2 \cdot 6H_2O$, 0.01 mg $CuSO_4 \cdot 5H_2O$, 0.006 mg $Na_2MoO_4 \cdot 2H_2O$, 30 mg Na_2SiO_3, 0.2 mg thiamine-HCl, 0.01 mg vitamin B_{12}, and 0.1 mg biotin per liter (Guillard and Ryther, 1962). The experiment was carried out with a working volume of 10 L in 14-L PBRs at 20 °C and 150 rpm. The initial cell density was 1×10^5 cells/ml. The cultivation process was aimed at maximum biomass production in the first phase of the culture, and maximum lipid production and polyunsaturated fatty acid (PUFA) synthesis in the second phase.

3.1.2. PBR and illumination system

Each PBR was made of Pyrex glass with an internal diameter and height of 200 mm and 450 mm, respectively (FMT ST series, Fermentec Co. Ltd.,

Chungbuk, South Korea) as shown in Figure.1 (A). Filtered air was supplied through a ring sparger at the bottom of the tank, and the aeration rate was controlled by a rotameter. The agitation system consisted of 2-disk turbine impellers and a foam breaker. To enhance mixing, three baffles were set at the bottom of the PBR. The external LED panels used in this experiment had a size of 28.5 × 38.6 × 4.4 cm^3 (Luxpia Co. Ltd., Suwon, Korea) and arranged in strips as shown in Figure. 1 (B) and (C). Each LED strip comprised 20 diodes spaced at 1-cm intervals vertically and horizontally. Additionally, three LED sticks in a glass tube were installed inside the PBR for internal illumination as shown in Figure. 1 (D). The green LED wavelength (520 nm) was used during the first phase for biomass production [Figure. 1 (E)], and the red LED wavelength (625 nm) during the second phase for lipid production [Figure. 1(F)]. Light intensity was measured at the center of the PBR by a light sensor (HD2102.2; Delta Ohm S.R.L., Padova, Italy).

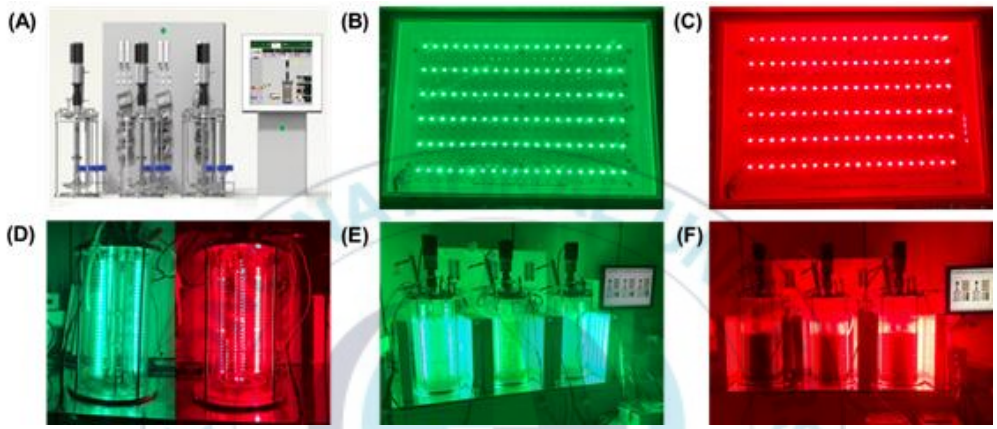

Figure 1. Application of three 14-L LED bioreactors. (A) Graphical photographs of the 14-L LED bioreactors. Photographs of the microalgae grown with external (B) green (520 nm) and (C) red LED (625 nm) panels and (D) internal LED light during the (E) first phase under the green LED wavelength for biomass culture and the (F) second phase under the red LED wavelength for lipid accumulation

3.1.3. Experimental design

A two-phase culture system was used to enhance the biomass, lipid, and PUFA yields from *P. cruentum*. Toward this end, culture conditions were separately optimized for the two phases. In the first phase, various growth conditions, including the aeration rate (0.25, 0.50, and 0.75 vvm), light intensity (100, 200, 300, 400, 500, and 600 μmol/m^2/s), and photoperiod cycle (12:12, 18:6, and 24:0 h) based on the green LED wavelength (520 nm) were evaluated for the maximum cell growth. After determining the optimal aeration rate, light intensity, and photoperiod cycle for the cell growth in the first phase of the culture, the conditions for the lipid production were optimized in the second phase by controlling the light intensity (100, 200, 300, 400, 500, and 600 μmol/m^2/s), pH (5.0, 6.0, 7.0, 8.0, 9.0), and photoperiod cycle (12:12, 18:6, and 24:0 h) using the red LED wavelength (625 nm). The pH was adjusted to 5.0 using the citrate buffer, to 6.0, 7.0, or 8.0 using the potassium phosphate buffer, and to 9.0 using the tris hydroxymethyl aminomethane buffer as previously described (Sakarika and Kornaros, 2016). Untreated in culture is defined as the control.

3.1.4. Assessment of microalgal growth

Dry cell weight was determined by an ultraviolet–visible spectrophotometer (Ultrospec 6300 Pro; Biochrom Ltd., Cambridge, UK) at an optical density of 540 nm (OD_{540}) (Maksimova et al., 2000).

The correlation equation (R^2 = 0.99) for *P. cruentum* dcw at OD_{540} was as follows as shown in Eq. (1).

Dry cell weight of *P. cruentum* (g dcw/L)

$$= 0.77(OD_{540}) \quad (R^2=0.99) \qquad \text{Eq.(1)}$$

3.1.5. Assessment of total lipid content

Cell harvesting was carried out using a centrifuge (Supra R22; Hanil Scientific Inc., Gimpo, Korea) at 8000rpm for 10 min. The precipitated biomass was washed twice using distilled water. Then, the biomass was dried using a freeze dryer (SFDSM-24L; SamWon Industry, Seoul, Korea). Then 5 mL of distilled water was added to 10 mg of dried cell biomass, and the cell were sonicated for 10 min using a sonicator (100 W, 20 kHz, 550 Sonic Dismembrator; Fisher Scientific Inc., Pittsburgh, PA, USA). Total lipid content was determined using methanol and chloroform following a modified solvent-based method (Bligh and Dyer, 1959) as shown in Eq. 2;

$$Lipid content\,(\%\ of\ dcw) = \frac{(W_2 - W_1)\times 100}{DCW} \qquad \text{Eq.(2)}$$

where lipid content is the cellular lipid content of the microalgae (% of DCW). W_1 (g) is the weight of the empty glass tube of 20-mL, and W_2 (g) is the glass tube weight of the 20-mL with extracted lipid. DCW (g) is the dried microalgae cell biomass.

3.1.6. Fatty acid methyl ester (FAME) measurement

The direct transesterification method of Dhup and Dhawan (2014) was used to convert extracted lipids to FAMEs. FAMEs were then analyzed using gas

chromatography (YL 6100, Young Lin Inc., Anyang, Korea) by employing a flame ionization detector (FID) and a silica capillary column (30 m × 0.32 mm × 0.5 μm, HP-INNOWAX; Agilent Technologies, Santa Clara, CA, USA). The column temperature adjustments were as follows; 140℃ for 5 min, followed by a temperature increase to 240℃ at 5℃/min with subsequently maintained for 10 min. The injector and FID temperatures were set at 250℃. FAMEs were identified by comparing their retention times against those of authentic standards.

3.1.7. Statistical analyses

Each experiment was conducted in triplicate. The statistical significance of cell biomass and lipid content was evaluated by one-way analysis of variance (ANOVA) and Duncan's multiple range test ($P < 0.05$) using SPSS software (ver. 23.0; SPSS Inc., Chicago, IL, USA).

3.2. Results and discussion

3.2.1. Optimization of the first phase culture conditions to improve biomass generation from *P. cruentum*

3.2.1.1. Effect of aeration rate

The photoperiod was set at a 12:12 h L/D cycle. The aeration rates of 0.25, 0.50, and 0.75 vvm were tested with a green LED wavelength (520 nm) and light intensity of 100 μmol/m^2/s. Figure. 2 (A) shows that 0.75 vvm facilitated *P. cruentum* to produce the highest biomass of 0.40 g dcw/L by day 14 among the aeration rates tested, followed by 0.50 vvm (0.38 g dcw/L) and then 0.25 vvm (0.20 g dcw/L). The results indicated that CO_2 supply and culture should be well mixed. Microalgal biomass production is enhanced by sufficient CO_2 supply and aeration (Sánchez Mirón et al., 2000). Sirisuk et al. (2018 b) have shown that increasing the aeration rate from 0.25 vvm to 0.50 vvm increases the amount of cellular biomass from 0.33 g dcw/L to 0.38 g dcw/L in *Nannochloropsis oceanica* cultures. To obtain large microalgal biomasses, high levels of CO_2 are required, necessitating high aeration rates (Ugwu et al., 2008). The maximum biomass in this study was achieved with a 0.75 vvm aeration rate, and thus the CO_2 aeration rate was set to this optimal value to obtain the largest microalgal biomass from our culture.

3.2.1.2 Effect of green LED light intensity

After determining the optimal aeration rate, the effect of light intensity on biomass production was evaluated. *P. cruentum* was cultured at the optimum aeration rate of 0.75 vvm with the green LED wavelength. The light intensities of 100, 200, 300, 400, 500, and 600 μmol/m^2/s were tested in this study. Figure 2(B) showed that P. cruentum produced the maximum biomass of 0.75 g dcw/L by day 12 with 300 μmol/m^2/s of light intensity, followed by 0.74, 0.73, 0.72, 0.50, and 0.40 g dcw/L of biomass with 400, 500, 600, 200, and 100 μmol/m^2/s of light intensities, respectively. Wahidin et al. (2013) have reported a correlation between light intensity and microalgal biomass. *Nannochloropsis* sp. produces the largest biomass with 100 μ mol/m^2/s of light intensity than with 50 or 200 μmol/m^2/s. Thus, microalgae produce large biomass at high light intensities; however, when the intensity increases above the saturated levels, the light inhibits cell growth, thus decreasing biomass production. In the current study, the highest biomass was achieved with 300 μmol/m^2/s light intensity. This light intensity was determined optimal because the growth rate decreased at the higher as well as lower intensities.

3.2.1.3 Effect of photoperiod under the green LED wavelength

After determining the optimal aeration rate of 0.75 vvm and light intensity of 300 μmol/m^2/s, the photoperiod was optimized. Photoperiods of 12:12, 18:6, and 24:0 h L/D cycle were used under the green LED wavelength. Figure 2(C) shows that *P. cruentum* yielded the largest biomass of 0.91 g dcw/L by day 8 with the photoperiod of 24:0 h L/D cycle, followed by 18:6

h (0.79 g dcw/L) and then 12:12 h (0.63 g dcw/L). The biomass productivity of *P. cruentum* was 0.0625 g/L/day at day 12 of cultivation when light intensity was optimized. However, by optimizing the light intensity and photoperiod, it reached 0.113 g/L/day on the day 8 of culture to have high biomass productivity. In addition, the incubation time with the 24:0 h L/D cycle was shorter than with the 12:12 h L/D cycle. The stationary phase was reached by days 12, 9, and 8 with the 12:12 h, 18:6 h, and 24:0 h L/D cycles, respectively. The growth rate changed according to photoperiod. Sirisuk et al. (2018 a) have reported that *Phaedactylum tricornutum* and *Nannochloropsis salina* grow faster with a 24:0 h L/D cycle than with a 12:12 h L/D cycle. However, *Isochrysis galbana* proliferate the most with an 18:6 h L/D cycle versus 12:12, and 24:0 h L/D cycles. Although excess light has an inhibitory effect on cell growth, *P. cruentum* was not negatively affected by the 24:0 h L/D cycle in this study. Therefore, the 24:0 h L/D cycle was selected to be optimal to produce the highest biomass yield.

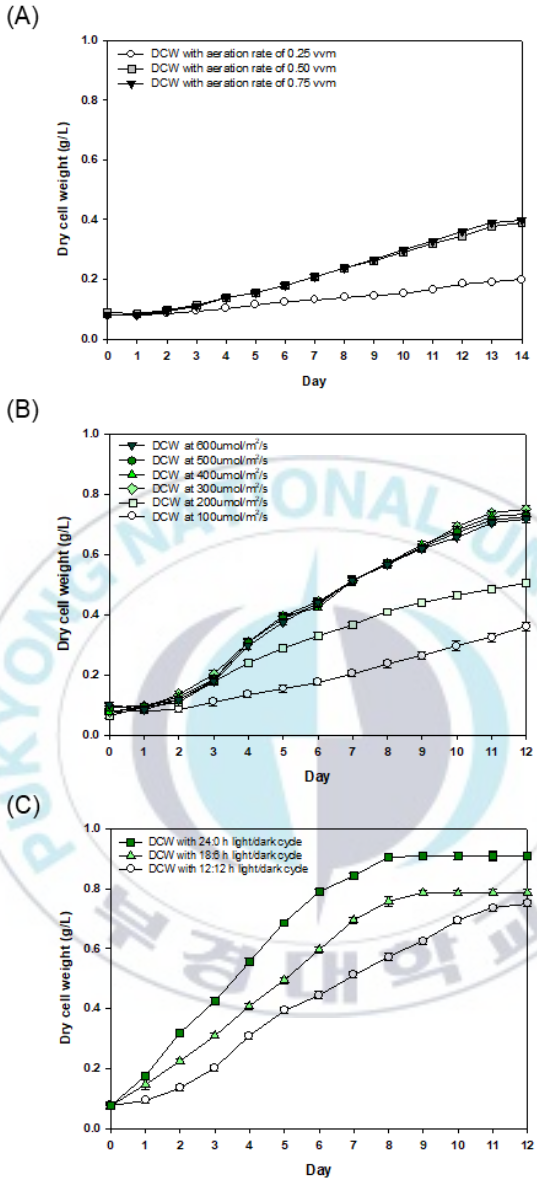

Figure 2. Optimization of maximum biomass production of *P. cruentum* cultured in 14-L LED bioreactors under the green LED wavelength with various (A) aeration rates, (B) light intensities alongside 0.75 vvm aeration rate, and (C) photoperiods alongside 0.75 vvm aeration rate and 300 μmol/m^2/s light intensity

3.2.2. Optimization of the second-phase culture conditions for lipid accumulation in *P. cruentum*

3.2.2.1. Effect of red LED light intensity in the second phase of culture

The optimal conditions for the biomass production in the first phase of the culture were set as 0.75 vvm aeration rate, 300 μmol/m^2/s light intensity, and a photoperiod of 24:0 h L/D cycle under the green LED wavelength. Lipid synthesis of *P. cruentum* was induced with the red LED wavelength in the second phase. Light intensities of 100, 200, 300, 400, 500, and 600 μ mol/m^2/s were used under red LED wavelength. The light intensity in the second phase of the culture was evaluated for 3 days to determine the optimal culture time for the maximum lipid production. Figure 3(A) shows that the lipid content increased under various light intensities. *P cruentum* showed the highest lipid production of 43.3% (w/w) with 400 μmol/m^2/s of light intensity. Maximum total lipid productions of 38.0, 39.6, 41.3, and 43.3% (w/w) were obtained at the light intensities of 100, 200, 300, and 400 μmol/m^2/s, respectively. However, light intensities above 500 μmol/m^2/s suppressed lipid production. The increase in lipid production upon adjustment of the light intensity is mediated by fatty acid biosynthesis enzymes, such as acetyl CoA carboxylase and ATP/citrate lyase, which are highly active at high light-energy levels. However, the lipid biosynthesis pathway is inhibited when the light energy exceeds the saturation intensity, thus decreasing lipid synthesis (Mondal et al., 2017). In addition, Dunstan (1973) has reported a direct correlation between light intensity and lipid accumulation in microalgae

and indicated that the relationship between the light intensity and photosynthesis depends on the chlorophyll type and content of microalgae.

3.2.2.2. Effect of photoperiod under the red LED wavelength in second phase of the culture

After determining the optimal light intensity of 400 μmol/m^2/s in the second phase of the culture, the photoperiod was optimized for the highest lipid production. Photoperiods of 12:12, 18:6, and 24:0 h L/D cycles were used under the red LED wavelength. The microalgae were cultured up to 3 days during the second phase to determine the optimal culture time for the maximum lipid production. The lipid production of *P. cruentum* using the two-phase culture is shown in Figure 3 (B). By day 2 of the second phase of the culture, *P. cruentum* generated the highest lipid production of 49.1% (w/w) and lipid productivity of 0.0447 g/L/day with the 18:6 h L/D cycle photoperiod. This result showed a similar trend with that of Wahidin et al. (2013). *Nannochloropsis* sp. has the highest lipid content of 31.3% with an 18:6 h L/D cycle among 12:12, 18:6, and 24:0 h L/D cycles. Photoperiods have been reported to induce significant changes in the total chemical compositions, pigment contents, and photosynthetic activities of microalgae (Richardson et al., 1983). A light source is an essential requirement for the production of triacylglycerides. The required light intensity and photoperiod vary from species to species (Harwood, 1998).

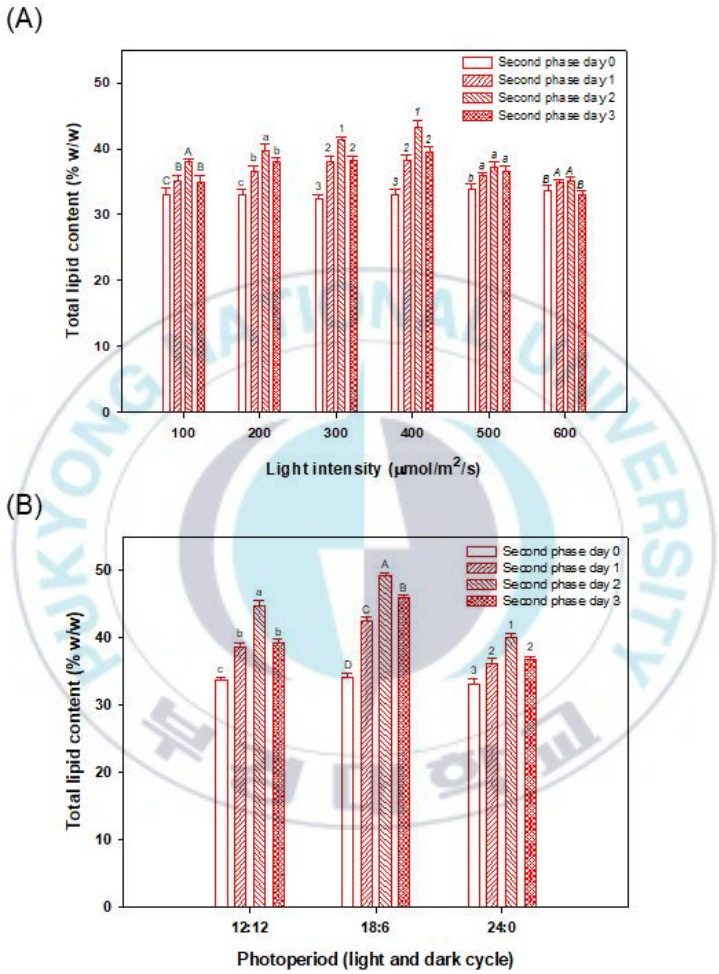

Figure 3. Total lipid content during the second phase of *P. cruentum* culture under various (A) light intensities and (B) photoperiods of the red LED illumination. Different letters and numbers indicate the significant differences ($P < .05$, Duncan's test)

3.2.2.3. Effect of pH on the lipid content of *P. cruentum*

The two-phase culture system was performed following the optimization of aeration rate, light intensity, and photoperiod. *P. cruentum* was cultured under green LED wavelength at 0.75 vvm aeration rate, 300 μmol/m^2/s light intensity, and a photoperiod of 24:0 h L/D cycle for 8 days in the first phase of the culture. Subsequently, the LED wavelength was switched from green to red light at the intensity of 400 μmol/m^2/s, and the cells were cultured with a photoperiod of 18:6 h L/D cycle for 2 days in the second phase of the culture to obtain the highest total lipid production as shown in Figure 4 (A). Control culture conditions were not treated for pH changes and pH ranged from 8.3 to 8.5. Various pH values (5.0, 6.0, 7.0, 8.0, and 9.0) were tested for the maximum lipid production alongside the control for 3 days in the second phase. Figure 4 (B) shows that the highest lipid production of 51.8% (w/w) was obtained by day 2 of the second phase of the *P. cruentum* culture at pH 6.0. *P. cruentum* lipid content increased from 33.5% to 51.8%, also lipid productivity increased from 0.0381 g/L/day on day 0 to 0.0471 g/L/day on day 2 of second phase culture at pH 6.0 by optimizing the light intensity, photoperiod, and pH.

The pH of the culture affects the lipid synthesis of microalgae, which increases lipid synthesis under pH stress (Mandotra et al., 2016). This outcome is most likely because the activity of ACCase, one of the key enzymes in lipid biosynthesis, is known to be pH-dependent (Thampy and Wakil, 1985) and inhibited at pH 5.0. In line with this data, adjusting the pH from the control's pH of 8.5 to pH 6.0 increased the lipid accumulation in this study.

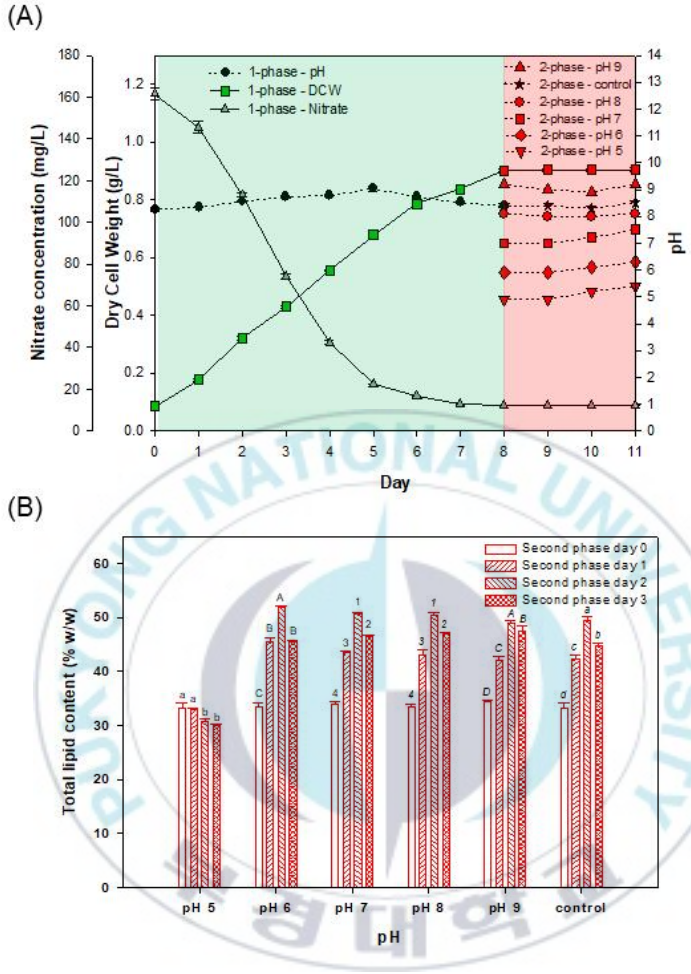

Figure 4. (A) Time course profile of *P. cruentum* biomass production (line) versus pH (dotted line) in the second phase. The green and red shades represent the first and second phases of the culture, respectively, and the culture pH was set to 5.0, 6.0, 7.0, 8.0, or 9.0. (B) The total lipid content during the second phase with various pH values. Different letters and numbers indicate the significant differences ($P < .05$, Duncan's test)

3.2.3. Effect of pH change on the fatty acid composition of *P. cruentum*

The effect of the pH during the second-phase culture on the fatty acid composition of *P. cruentum* biomass was studied. The fatty acid compositions of *P. cruentum* cultured at different pHs (5.0, 6.0, 7.0, 8.0, and 9.0) alongside the control for up to 3 days are shown in Table 1. Relative to the control, there was no significant change for 3 days at pH 5.0 in the fatty acid composition [Table 1 (A) and (B)]. However, the unsaturated fatty acid levels at pHs 6.0 and 7.0 were higher than that of the control, and the saturated fatty acid levels, such as that of stearic acid (C18:0), were lower [Table 1 (C) and (D)]. The unsaturated fatty acid level reached approximately 50% (w/w). At pH 6.0, the total unsaturated and saturated fatty acid levels were estimated to be 56.2% (w/w) and 43.85% (w/w), respectively [Table 1 (C)]. Additionally, the EPA level was found to be 30.63% (w/w). At pH 7.0, the saturated fatty acid level decreased to 51.64% (w/w) as shown in Table 1 (D), while the unsaturated fatty acid level increased to 48.36% (w/w). In addition, compared to the day 0 of the second phase culture, the EPA level increased from 8.45 % to 23.58% (w/w) as shown in Table 1 (D). When the culture pH was increased to 8.0, the total saturated fatty acid level decreased from 66.78% to 53.46% (w/w), and the unsaturated fatty acid level increased from 33.22% to 46.24% (w/w) as shown in Table 1 (E). Similar results have been reported for *Isochrysis galbana* at pHs 6.0, 7.0, and 8.0 (Molina Grima et al., 1992). *Isochrysis galbana* produces high levels of polyunsaturated fatty acids, such as EPA, and DHA at pH 6.0 than at pH 7.0 or 8.0. Changes in pH affect the properties of the cell surface, such as adhesion onto the

substrata and biomass aggregation (Jiang and Chen, 2000). As the pH decreases, the carboxylate ions receive protons and can be converted to neutral carboxyl groups, whereby the negative charge of the cells is neutralized and the cell dispersion stability is destroyed, causing the aggregation and sedimentation of the cells (Liu et al., 2013). Such effects alter permeation of ions, acids, and bases into the cell, and affect the biochemical metabolism and conformation of macromolecules, as well as K_m values of enzymes (Jiang and Chen, 2000). Microalgal cell membranes are damaged at rapid pH changes. To prevent this damage, microalgae further accumulates unsaturated fatty acids to increase cell membrane fluidity. Under these cell-membrane–damaging stresses, the three desaturase genes D5-desaturase, plastid acyl-ACP D9 desaturase, and microsomal D12-desaturase are upregulated to increase the unsaturated fatty acid composition (Shekh et al., 2016). Therefore, unsaturated fatty acid composition changes according to the pH of the environment.

Table 1. Fatty acid methyl ester (FAMEs) contents of *P. cruentum* during the second phase of the culture at (A) control pH, (B) pH 5, (C) pH 6, (D) pH 7, (E) pH 8 and (F) pH 9

Free fatty acid (% of total fatty acid)	(A) Control				(B) pH 5				(C) pH 6			
	Day 8	Day 9	Day 10	Day 11	Day 8	Day 9	Day 10	Day 11	Day 8	Day 9	Day 10	Day 11
Myristic acid (C14:0)	0.59	0.24	0.27	0.10	0.82	0.68	0.92	1.05	1.08	1.84	0.11	0.11
Palmitic acid (C16:0)	6.57	6.70	7.00	4.56	5.75	5.75	6.36	7.29	6.53	2.97	2.45	2.88
Palmitoleic acid (C16:1)	0.58	0.37	0.31	1.15	0.95	0.60	0.06	0.07	0.90	2.29	1.36	1.33
Stearic acid (C18:0)	56.84	47.82	43.91	48.48	55.77	55.32	58.60	59.61	53.24	46.81	36.24	38.96
Oleic acid (C18:1)	11.83	12.46	12.96	5.23	12.30	11.45	10.11	11.64	12.20	4.29	2.91	6.07
Linoleic acid (C18:2)	0.71	3.08	3.40	6.76	0.73	0.69	0.35	0.07	0.78	4.29	1.60	5.73
Linolenic acid (C18:3)	0.86	0.07	0.08	0.46	0.89	0.80	1.02	1.17	0.77	0.32	0.14	0.53
Arachicidic acid (C20:0)	2.36	2.77	2.89	5.06	2.46	2.72	2.93	3.42	2.35	4.85	5.16	5.87
Eicosapentaenoic acid (C20:5)	**8.28**	**11.48**	**12.19**	**10.56**	**9.08**	**10.40**	**9.35**	**9.11**	**9.99**	**17.51**	**30.63**	**21.12**
Behenic acid (C22:0)	2.37	3.32	3.50	6.14	2.20	1.83	1.72	1.09	3.21	2.18	4.87	7.11
Docosahexaenoic acid (C22:6)	**9.01**	**11.70**	**13.48**	**11.50**	**9.06**	**9.78**	**8.57**	**5.47**	**8.94**	**12.65**	**14.54**	**10.28**
Saturated fatty acid	67.23	58.15	54.76	59.73	65.42	64.38	68.63	70.22	64.53	54.12	43.80	49.59
Unsaturated fatty acid	32.77	41.85	45.24	40.27	34.58	35.62	31.37	29.78	35.17	45.88	56.20	50.41

Free fatty acid (% of total fatty acid)	(D) pH 7				(E) pH 8				(F) pH 9			
	Day 8	Day 9	Day 10	Day 11	Day 8	Day 9	Day 10	Day 11	Day 8	Day 9	Day 10	Day 11
Myristic acid (C14:0)	0.61	2.03	0.19	0.83	0.63	0.15	0.17	0.09	0.51	0.30	0.18	0.13
Palmitic acid (C16:0)	6.66	3.28	2.84	4.66	6.97	5.92	6.78	4.58	6.61	6.98	7.75	4.45
Palmitoleic acid (C16:1)	0.65	2.77	1.24	1.36	0.62	0.43	0.07	1.28	0.57	0.49	0.07	1.30
Stearic acid (C18:0)	55.30	50.21	43.46	41.07	56.04	52.60	43.20	47.24	58.98	46.42	42.90	47.98
Oleic acid (C18:1)	12.44	4.80	2.70	3.74	11.96	10.39	12.44	4.98	11.39	12.84	12.24	4.92
Linoleic acid (C18:2)	0.64	3.85	2.78	2.78	0.72	2.43	2.90	7.13	0.67	2.84	3.07	6.91
Linolenic acid (C18:3)	0.92	0.29	0.19	0.46	0.88	0.02	0.13	0.43	0.84	0.12	0.07	0.44
Arachicidic acid (C20:0)	2.71	4.59	5.87	5.39	2.32	2.31	3.29	5.03	2.24	2.72	2.79	4.98
Eicosapentaenoic acid (C20:5)	**8.45**	**14.57**	**23.58**	**23.54**	**8.34**	**12.18**	**3.03**	**10.97**	**7.13**	**11.90**	**12.54**	**10.24**
Behenic acid (C22:0)	2.43	2.12	4.96	3.96	2.26	2.25	3.49	5.93	2.26	3.36	3.54	6.50
Docosahexaenoic acid (C22:6)	**9.20**	**11.50**	**12.20**	**12.42**	**9.27**	**11.31**	**14.51**	**12.34**	**8.80**	**12.02**	**14.85**	**12.15**
Saturated fatty acid	65.92	57.92	51.64	50.97	66.78	60.95	53.76	58.27	69.20	57.19	54.44	59.50
Unsaturated fatty acid	34.08	42.08	48.36	49.03	33.22	39.05	46.24	41.73	30.80	42.81	45.56	40.50

According to these results, the optimal culture conditions for the maximum biomass yield during the first phase was determined to be 0.75 vvm aeration rate, 300 μmol/m^2/s light intensity, and 24:0 h photoperiod under green (520 nm) LED light, achieving maximum biomass of 0.91 g/L. The second phase culture aimed to increase the lipid content, and the optimal conditions were obtained with the red LED wavelength (625 nm), 400 μmol/m^2/s light intensity, 18:6 h photoperiod, and pH 6.0. The maximum lipid content reached 51.8% (w/w). In addition, the EPA and DHA contents were increased to 30.6% (w/w) and 14.5% (w/w) at pH 6.0, and the unsaturated fatty acid content reached 56.2% (w/w).

3.3. Conclusion

Optimal aeration rate, light intensity, and photoperiod of green and red wavelengths under a two-phase culture system were found to be the most important determinants for improved cell growth and lipid accumulation in *P. cruentum*. Low pH conditions exhibited a positive effect on the EPA synthesis in *P. cruentum*. With the optimized conditions, the two-phase culture system provides up to 0.91 g dcw/L *P. cruentum* biomass and 51.8% (w/w) lipids with the EPA and DHA contents reaching to 30.6% and 14.6%, respectively.

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