Thesis for the Degree of Master of Engineering

Enhanced biomass and lipid productions from various marine microalgae using light-emitting diodes (LEDs) and two-phase culture system

by

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Department of Biotechnology

The Graduate School

Pukyoung National University

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(LED광원과 배양시스템을 이용한 해양미세조류 바이오매스와 지질생산 향상에 대한 연구)

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LED광원과 배양시스템을 이용한 해양미세조류 바이오매스와 지질생산 향상에 대한 연구

정 장 현

부 경 대 학 교 대 학 원 생물공학과

요 약

3세대 바이오매스인 미세조류는 빠른 성장, 높은 바이오매스 생산량, 높은 지질 함량, 경작지에 대한이점을 가지고 있다. 이 연구는 LED광원과 배양시스템을 이용하여 여러가지 미세조류의 바이오매스, 지질, 지방산 생산을 향상하였다. 1장은 *Phaeodatylum tricornutum*, *Dunaliella teritolect,* and *Isocrhysis galbana* 를 F/2배지를 이용하여 청색 LED파장 (465 nm)와 형광등을 12:12 LD cycle로 20℃에서 100 μmol/m^2/s의 광량을 사용하며, 2.5L/m의 공기를 공급하여 배양하였다. 또한 질소원 (8,12,16,20,24mg/L)을 각각 2L 배양하여 최적 질소원 농도를 확립하였다. 최적 질소원 농도에 따른 20 mg/L의 질소원 배지에서 *P. tricornutum*는 1.35g/L의 성장을 보였으며, *D. teritolect*는 12 mg/L에서 0.63 g/L의 성장하였고, *I. galbana*는 16 mg/L에서 1.47 g/L의 성장을 보였다. 지질의 향상을 위하여 배지 내 질소원을 제거하는 방법과 0.5 M의 NaCl을 첨가하는 방법, LED 녹색광원 (520 nm)으로 광원을 교체하는 방법을 이용하여 3일간 실험하였다. 이러한 2단계 배양 방법에서 미세조류는 높은 바이오매스 생산량, 높은 지질축적 과 높은 불포화지방산의 분포를 나타내었다. 가장 높은 효과를 보인 배양 방법은 LED 녹색광원를 이용한 것이며, 2일차에 각각의 미세조류에서 기존 1단계 배양 보다 51, 64, 70%의 지질 향상을 보였으며, 불포화 지방산 분포 또한 다른 배양 방법보다 높은 것을 확인 할 수 있었다. 2장은 *Phaeodactylum tricornutum*, *Isochrysis galbana*, *Nannochloropsis salina*, *N. oceanica* 그리고 *Picochlorum* atomus를 이용하여 적색과 청색을 혼합한 LED광원을 이용하여 배양을 하여 바이오매스와 지질 함량을 증가시키는 연구이다. 또한 적색과 청색을 혼합한 LED광원을 이용하여 배양한 미세조류는 형광등을 이용한

배양에서 보다 높은 색소 (chlorophyll a and b, and carotenoids)함량을 나타내었다. 배양 이후 3일간의 질소원 저해를 받은 미세조류내 최고 지질생산량은 *P. tricornutum*에서 47.3 mg/L/day, *I. galbana*에서 47.8 mg/L/day, *N. salina*에서 45.7 mg/L/day, *N. oceanica*에서 29.3 mg/L/day 그리고 *P. atomus*에서 34.3 mg/L/day을 보였다. 이 연구에서 *P. tricornutum*와 *I. galbana*가 가장 높은 지질생산량을 보였다. 이러한 적색과 청색을 혼합한 LED광원을 이용한 배양 방법은 형광등 보다 바이오매스 생산에서 31.0%, 지질생산에선 41.7%의 소비전력에 따른 생산량이 높은 것을 확인할 수 있었다. 이러한 배양방법으로 배양한 5종의 미세조류에서 최고 지방산 분포는 palmitic acid (C16:0)였으며, 함량은 총 지방산 함량에 33.8%-56.0% (w/w)를 차지하였다. 이러한 2가지 연구를 통하여서 LED광원을 이용한 미세조류의 배양 방법의 이점과 그에 따른 생산량 증가를 확인 한 결과를 얻었다.

Chapter I Enhanced unsaturated fatty acid production from various marine microalgae using light-emitting diodes (LEDs) and two-phase culture system

1.1. Introduction

Microalgae, 3rd generation biomass has advantage of rapid growth rate, high biomass productivity, high lipid contents, no need for cultivation land and high level of CO_2 absorption (Wang et al., 2008). Those microalgae products such as biomass, carotenoids, lipid, fatty acids can use as a feedstock for food or feed supplements, cosmetics and biodiesel production (Spolaore et al., 2006). In this study, enhanced biomass, lipid and unsaturated fatty acid production can be used as the productions of biodiesel and nutrient feeds for the fish finglings. Microalgae lipids can be used in making biodiesel consisting of triglycerides by which three fatty acid molecules are esterified with methanol. In making biodiesel, triglycerides are reacted with methanol in a reaction known as transesterification or alcoholysis. Transestrification produces fatty acid methyl esters. Those are biodiesel, and glycerol (Chisti, 2007). Unsaturated fatty acids have received growing attention due to their significant roles in human health. Currently, the main source of these nutritionally and medically important fatty acids is marine fish, which could not meet increasing global demand. Marine microalgae are an important alternative source to produce those fatty acids. In this

study, microalgae cultured on light-emitting diodes (LEDs) of blue wavelength 465 nm for growth and green wavelength 520 nm for lipid accumulation. Photoautotrophic microalgae have photosynthesis factor such as chlorophyll a, chlorophyll b and carotenoids. Main factor in photosynthesis, chlorophyll a can absorb high level in blue and red wavelength (400~500nm, 600~700 nm). And green wavelength (500~600 nm) can not be absorbed (Richmond, 2003). Therefore 1^{st} phase culture of microalgae production was with on blue wavelength (465 nm) and 2^{nd} phase culture of lipid accumulation was carried out with green wavelength (520 nm). In this study, the wavelength stress to enhance lipid accumulation from microalgae culture system was compared with other stress methods such as nitrate depletion stress and salt stress. Those three stress methods were called two-stage culture and usually used for lipid accumulation on microalgae research.

1.2. Materials and Methods

1.2.1. Microalgae strains and culture conditions

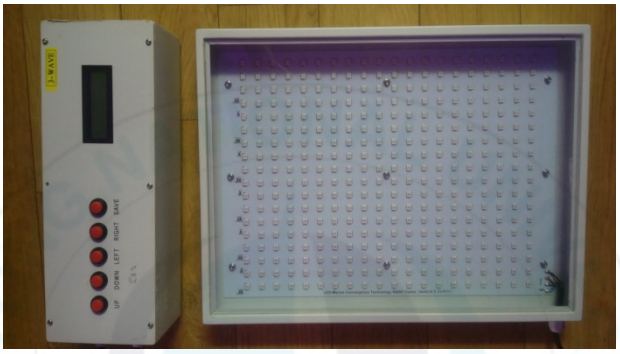
Three microalgae, *Phaeodactylum tricornutum*, *Dunaliella tertiolecta*, *Isochrysis galbana* were obtained from the Korea Marine Microalgae Culture Center (Busan, South Korea). They were cultured in sterilized seawater with modified f/2 medium (Guillard and Ryther, 1962). Seed culture with a working volume of 700 mL in 1-L flask was inoculated to 1.5-L media with the addition of 1×10^5 microalgae cells/mL as an inoculum cell density, cultured in 2-L flasks at 20 ± 1°C under light intensity of 100 μmol/m^2/s, of either LED light with a blue wavelength (465 nm) or fluorescent light. The initial cell density was determined using the standard curve of OD_{680} versus

dry cell weight (DCW) using UV spectrophotometer (Ultrospec 6300 Pro; Biochrom Ltd., Cambridge, UK). Aeration with filtered air was provided through an air stone at a rate of 2.5 L/min. Each of the flasks was then exposed to light with a blue (465 nm) wavelength or fluorescent light under a 12 h:12 h light-dark cycle for 15 days. And optimal nitrate content was chosen using 5 contentration of nitrate (8, 12, 16, 20, 24 mg/L). All experiments were conducted in triplicate.

1.2.2. Light source for microalgae culture

LED square panel lights (28.5 × 38.6 × 4.4 cm^3; LUXPIA Co., Ltd., Suwon, South Korea) were used as the light source for the photoautotrophic growth of microalgae, as shown in Fig. 1. (a) Thirty red (660 nm), green (520 nm), and blue (465 nm) diodes were spaced alternately in six strips at 1-cm intervals in both rows and columns. The high biomass of microalgae can be produced with blue (465 nm) wavelength and high lipid accumulation with green (520 nm) wavelength (Ra et al., 2016). In this study, LED light was blue (465 nm) wavelength on 1^{st} phase for high biomass production and green (520 nm) wavelength on 2^{nd} phase for lipid accumulation as shown in Fig. 1 (b). The LED square panel lights were placed in parallel to illuminate the 2-L flask culture at the distance of 15 cm and separated from other lighting sources. The light intensity was measured with a light sensor (TES-1339; UINS Inc., Busan, South Korea) as the photon flux at the center line of the flask filled with culture medium. A control experiment was conducted under the same intensity of fluorescent light and operating conditions for 15 days.

(a)



(b)

Fig. 1. Image of (a) Controller and LED light (b) Green and blue LED light at algae cultivation

1.2.3. Two-stage and two-phase culture system

After 1st culture for high biomass production in 12 days, 4 samples were cultured 4 different cultivation environments of 1 control and 3stress for lipid accumulation. Sample 1 was same environment of uninterrupted 1st culture using for control. Sample 2 was nitrate depletion stress using centrifugation (994 × *g*, 10 min) and added media of removal nitrate source. Sample 3 was added 0.5 M of NaCl on media for salt stress. Those 2 culture systems were using LED blue (465 nm) wavelength of same light intensity and light-dark cycle on 1st culture. Those cultures methods called two-stage and usually used for lipid accumulation on microalgae research (Griffiths and Harrison, 2009). And last sample was changed LED light wavelength of green (520 nm) wavelength for light stress. This method could be carried out as an easy lipid accumulation method without changing media (Ra et al., 2016).

1.2.4. Lipid extraction and transesterification

After the culture of microalgae, suspended cells were harvested by the centrifugation (994 × *g*, 10 min) and lyophilized by freeze drier (SFDSM-24L; SamWon Industry, Seoul, South Korea). Total lipids were extracted with chloroform/methanol (2:1, v/v) and quantified gravimetrically (Bligh and Dyer, 1959). The lipid content in dry weight was calculated using Eq. (1):

$$\text{Lipid content (\% of DCW)} = \frac{(W_2 - W_1)}{\text{DCW}} \times 100 \qquad \text{Eq. (1)}$$

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

The lipid composition was determined as fatty acid methyl esters (FAMEs) via the direct transesterification method reported by Dhup et al. (2014). FAME profiling was performed by gas chromatography (GC; YL 6100; YoungLin Inc., Anyang, Korea) with a flame ionization detector (FID) and a silica capillary column (HP-INNOWAX; 30 m × 0.32 mm × 0.5 μm; Agilent Technologies, Santa Clara, CA, USA). The column temperature adjustments were carried out as follows: 140°C for 5 min, followed by a temperature increase to 240°C at a rate of 5°C/min; this temperature was maintained for 5 min. Both injector and FID detector temperatures were set at 250°C. The FAMEs were identified by comparing their retention times against those of authentic standards.

1.2.5. Analyses

The numbers of inoculated microalgae cells were determined by counting using a hemocytometer and a microscope (CK40-SLP; Olympus, Tokyo, Japan). The cultures were sampled at 24-h intervals. Based on the standard curve of OD_{680} and DCW, one unit of OD_{680} corresponded to 0.42, 0.34 and 0.42 g DCW/L dry biomass weights of *P. tricornutum*, *D. tertiolecta* and *I. galbana*, respectively. Nitrate concentration was determined according to standard methods using an ultraviolet-visible (UV-Vis) spectrophotometer (Ultrospec™ 6300 Pro; Biochrom Ltd.) (Collos et al., 1999). Chlorophyll a (Chl a), chlorophyll b (Chl b), and total carotenoid contents have been well-established (Lichtenthaler et al., 1987). The determination of their levels in whole pigment extract of green plant tissue using a UV-Vis spectrophotometer was performed using Eqs. (2–4).

Chlorophyll a (Chl a, μg/mL) = Eq. (2)

11.24 × Absorbance (661.6 nm) – 2.04 × Absorbance (644.8 nm)

Chlorophyll b (Chl b, μg/mL) = Eq. (3)

20.13 × Absorbance (644.8 nm) – 4.19 × Absorbance (661.6 nm)

Total carotenoids (TC, μg/mL) = Eq. (4)

[1,000 × Absorbance (470 nm) – 1.90 × Chl a (μg/mL) – 6.314 × Chl b (μg/mL)]/214

1.3. Results and Discussion

1.3.1. Effect of growth rate on LED blue wavelength

Three microalgae, *P. tricornutum, D. tertiolecta* and *I. galbana* were cultured in 2 L-flask with 1.5 L working volume on F/2 media (8 mg/L of nitrate) at 20 ± 1°C, an aertion rate of 2.5 L/min and 12 h:12 h light-dark cycle for 15 days. And those cultures were using different light sources of LED blue (465 nm) wavelength and fluorescent light. Fig. 1 (a), (b) and (c) are cell growth rate and nitrate content on LED blue (465 nm) wavelength and fluorescent light for *P. tricornutum, D. tertiolecta* and *I. galbana*, respectively. As show on, Fig.2 (a) *P. tricornutum* were 0.66 g/L of dry cell weight on fluorescent light and 0.94 g/L on LED blue at 12 days of ended cell growth. Fig.2 (b), *D. tertiolecta* were 0.37, 0.51 g/L of dry cell weight each condition, (c) *I. galbana* were 0.75, 0.75 g/L of dry cell weight. Three microalgae were consumed all nitrate at 6 days and ended growth phase at 12 days. Fig.2 (d) is biomass content on LED blue and fluorescent light at 12 days of ended cell growth phase. *I. galbana* was not different biomass production. It was high biomass productivity on LED blue and fluorescent light. But the others, *P. tricornutum* and *D. tertiolecta* were higher biomass content 36%, 39% on LED blue than fluorescent light. Atta et al. (2013) reported *C. vulgaris* exhibited higher growth rate, dry cell weight and lipid content on LED blue than fluorescent light. Also, Teo

et al. (2014) reported *Tetraselmis* sp. and *Nannochloropsis* sp. showed higher growth rate and lipid content by LED blue than those by fluorescent light. Ra et al. (2016) reported 1st phase culture of two-phase culture for microalgae (*Nannochloropsis* sp.). It was chosen LED blue for high biomass production. So, in this study, 3 microalgae 1st phase cultures were chosen on blue (465 nm) wavelength.

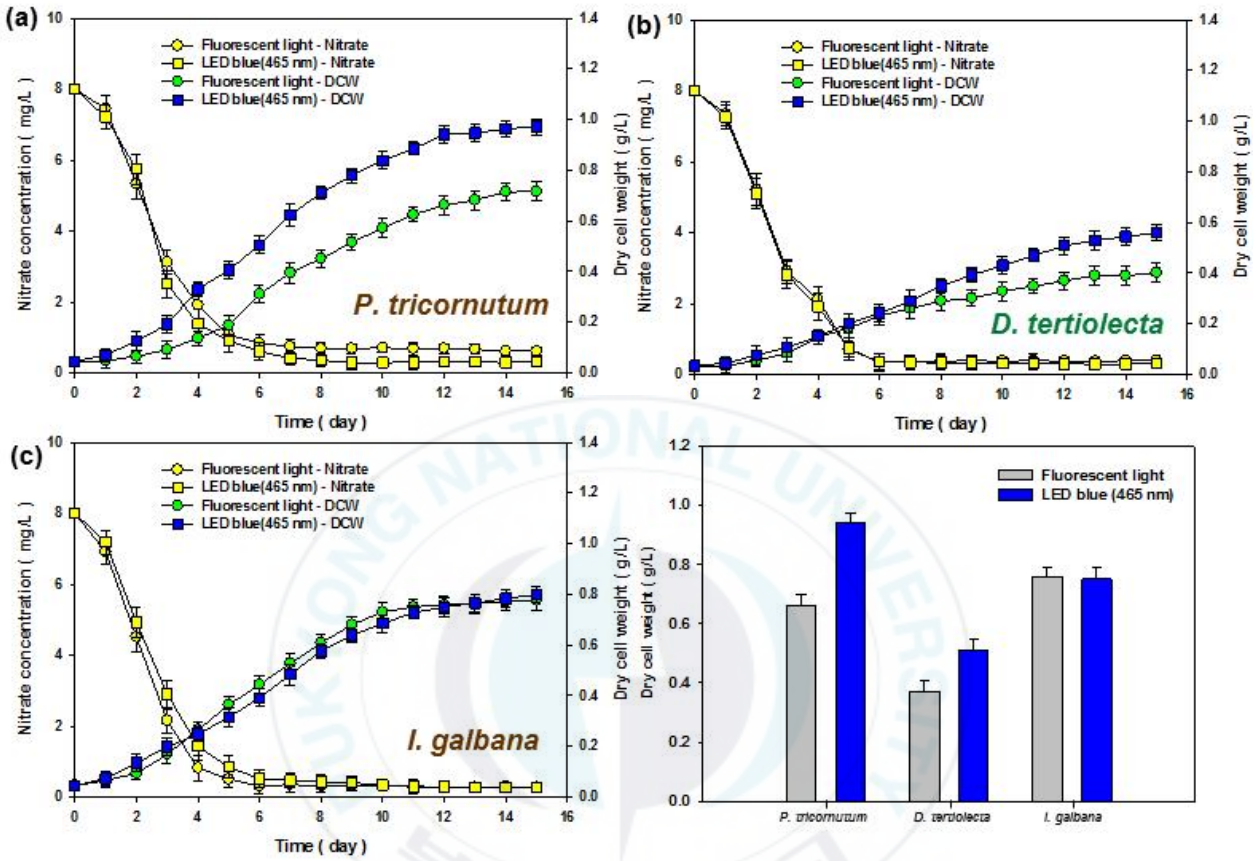


Fig. 2. Cell growth of 3 microalgae on fluorescent light and LED blue (465 nm) wavelength.

(a) *P. tricornutum* (b) *D. tertiolecta* (c) *I. galbana* (d) biomass production on 12days

1.3.2. Optimal nitrate content on LED blue wavelength

Three microalgae were cultured by different nitrate contents on modified F/2 media with 8, 12, 16, 20, 24 mg/L nitrate concentrations. Those microalgae cultured 20 ± 1°C under light intensity of 100 μmol/m^2/s of blue LED. Fig.3 (a), *P. tricornutum* were cultured 5 different nitrate content of 8, 12, 16, 20, 24 mg/L. Also on Fig.3 (a), *P. tricornutum* showed highest cell growth of 1.35 g dcw/L with 20 mg/L nitrate content media. Nitrate was consumed all at 11 days and ended growth at 12 days. *P. tricornutum* cultured on 24 mg/L was could consume all nitrate. Fig 3 (b), *D. tertiolecta* showed highest growth of 0.63 g dcw/L with 12 mg/L nitrate content media. It consumed all nitrate at 8 days and ended growth at 12 days. Also, *D. tertiolecta* cultured with 20 and 24 mg/L nitrate could not consume all. As shown in Fig 3 (c), *I. galbana* showed the highest growth of 1.47 g dcw/L with 16 mg/L nitrate content media. It consumed all nitrate at 9 days and ended growth at 12 days. Also, *I. galbana* cultured on 24 mg/L was could consume all nitrate. White symbols in Fig.3 (a), (b) and (c) were cell growth with fluorescent light at those optimal nitrate content media. The cell growth with fluorescent light as light source showed lower growth rate than these with LED blue. Three microalgae were could consume all nitrate on 24 mg/L however, those culture showed lower cell growth than these of optimal nitrate content of *P. tricornutum* 20 mg/L, *D. tertiolecta* 12 mg/L and *I. galbana* 16 mg/L. More than 8 mg/L of nitrate content (F/2 media contented 8 mg/L) on media could enhance growth rate of microalgae however high nitrate concentrations provide an inhibitory effect on algal growth. Jeanfils et al. (1993) and Li et al. (2008) reported that microalgae increase the activity of nitrate reductase at high concentrations of nitrate, leading to enhanced production of nitrite and ammonia; thus, accumulated nitrite and ammonia may act as inhibitory compounds in biomass production. As

indicated by these results, optimal nitrate content were chosen in 20 mg/L for *P. tricornutum*, 12 mg/L for *D. tertiolecta* and 16 mg/L for *I. galbana*.

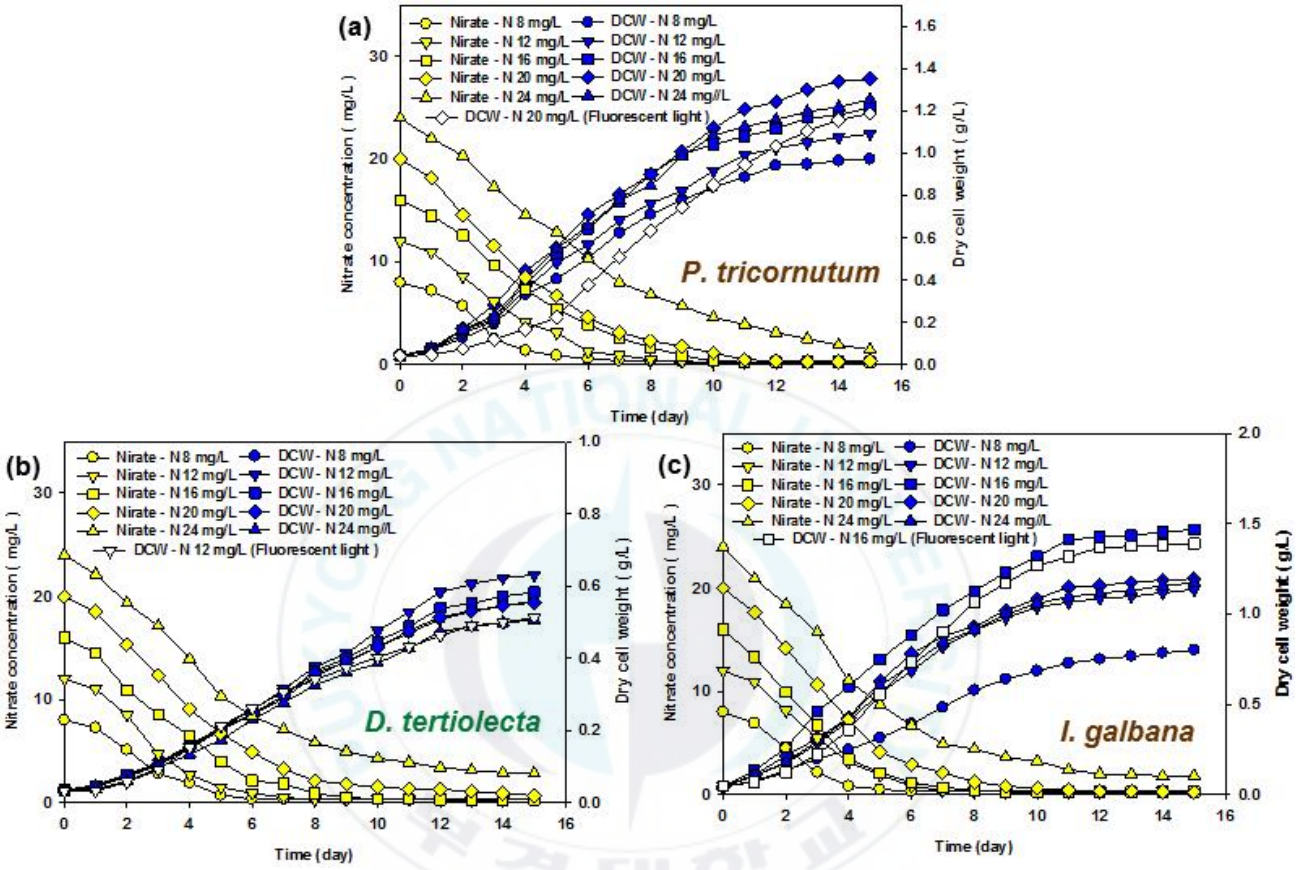


Fig. 3. Cell growth of 3 microalgae on LED blue(465 nm) on various nitrate concentrations.

(a) *P. tricornutum* (b) *D. tertiolecta* (c) *I. galbana*

1.3.3. Microalgae culture using two-stage and two-phase culture systems

The 1st culture was chosen on LED blue (465 nm) wavelength of 100 μmol/m^2/s with modified F/2 media of each optimal nitrate content (*P. tricornutum*; 20 mg/L, *D. tertiolecta*; 12 mg/L and *I. galbana*; 16 mg/L) for high biomass in 12 days. The 2nd cultures of three microalgae were using 4 different culture systems for lipid accumulation in 3 days. Sample 1 was same environment of uninterrupted 1st culture using for control. Sample 2 was nitrate depletion stress using centrifugation and addition of media with out nitrate source. Sample 3 was added 0.5 M of NaCl on media for salt stress. Those 2 culture system were using LED blue (465 nm) wavelength of same light intensity and light-dark cycle on 1st culture. And last sample was LED light wavelength change to green (520 nm) wavelength as light stress. Fig.4 (a) showed 3 microalgae growth using optimal culture system as 1st culture. After 12 days, those microalgae entered stationary phase. Second cultures started after 12 days of culture as 1st culture. Bar graphs in Fig.4 (b), (c) and (d) were lipid content 4 different culture systems in 3 days. White bars were lipid content after 12 days of 1st culture. Those lipid contents were 39.5% in *P. tricornutum*, 27.5% in *D. tertiolecta* and 29.7% in *I. galbana*. As show in Fig. 4 (b), lipid content of *P. tricornutum* showed highest lipid content in 2 days of second phase showing 43.5% in control, 50.1% in nitrate stress, 58.1% in salt stress and 59.6% in wavelength stress. Fig. 4 (c), lipid content of *D. tertiolecta* showed highest lipid content each samples on 2 days after second phase showing 30.9% in control, 39.5% in nitrate stress, 43.4% in salt stress and 45.1% in wavelength stress. Also Fig. 4 (d), lipid content of *I. galbana* showed highest lipid content each samples on 2 days after second phase showing 35.6% in control, 47.5% in nitrate stress, 48.9% in salt stress and 50.5% in wavelength stress. The highest content of lipid was obtained with wavelength stress of LED green (520

nm) wavelength at 14 days of culture as 2 days with two phase culture system. As show on Table 1, four culture conditions of three microalgae showed highest lipid content at 14 days. Wavelength stress of second phase culture systems (LED green 520 nm) showed highest lipid content as 2 days of second phase. Those lipid contents were enhanced 51% in *P. tricornutum*, 64% in *D. tertiolecta* and 70% in *I. galbana* from the control of first phase culture. Su et al. (2011) reported two-stage culture systems. In which, lipid contents of (*Nannochloropsis oculata*) enhanced on two-stage culture with nitrate depletion stress and salt stress; 35% in 0 g/L salt content, 41% in 35 g/L and 43.2% in 70 g/L(10% of lipid content in 1st culture). Line and plot graphs in Fig.4 (b), (c) and (d) were chlorophyll a contents on each culture systems. Those chlorophyll a contents were decreased on all stress condition. One chlorophyll a molecule contains four nitrogen atoms. Therefore, it becomes difficult for the cell organelles to synthesize chlorophyll in nitrate depletion condition (Pisal and Lele, 2005). Also, chlorophyll molecules could be used as a nitrogen source for cell survival and growth (Li et al., 2008). All nitrogen sources on culture condition were consumed at first phase culture. Therefore, chlorophyll a contents were decreased on second phase culture. Also other pigments, chlorophyll b and total carotenoids were decreased on second phase culture as shown Table1.

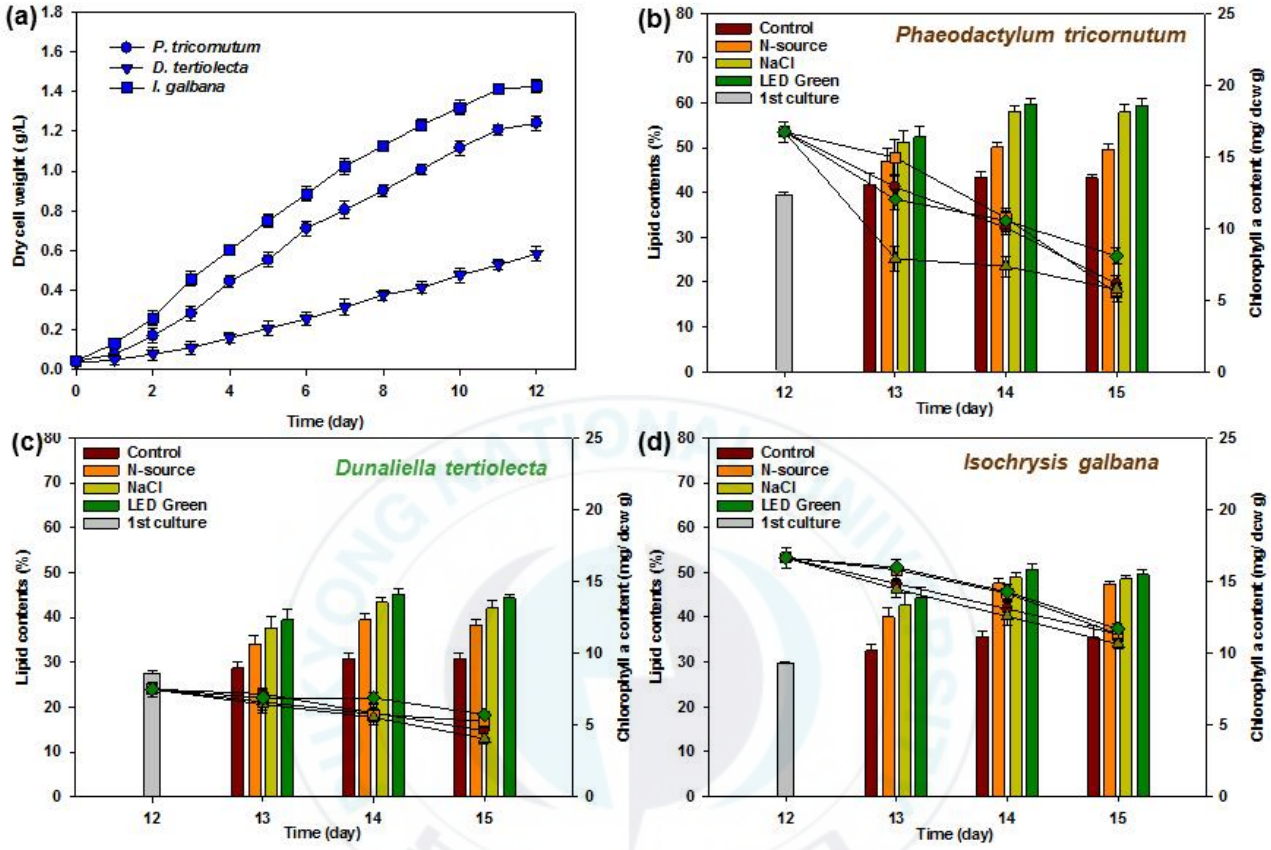


Fig. 4. Cell growth of first phase or stage, lipid and chlorophyll a contents of 3 microalgae on second phase or stage for 3 days (a) Cell growth rate of 1st culture (b) *P. tricornutum* (c) *D.tertiolec* (d) *I. galbana*

Table 1. Lipid and pigments contents of 3 microalgae on second phase or stage culture (12 to 15 days of whole culture). (Chl a - chlorophyll a, Chl b - chlorophyll b, TC - total carotenoids)

	P.tricornutum					*D. tertiolecta*					*I. galbana*				
	Time (day)	Lipid (%)	Chl a (mg/g)	Chl b (mg/g)	TC (mg/g)	Time (day)	Lipid (%)	Chl a (mg/g)	Chl b (mg/g)	TC (mg/g)	Time (day)	Lipid (%)	Chl a (mg/g)	Chl b (mg/g)	TC (mg/g)
Control	12	39.5	16.73	0.41	9.18	12	27.5	7.50	2.93	3.03	12	29.7	16.63	1.57	9.14
	13	41.8	12.88	0.20	7.62	13	28.7	7.18	2.55	3.03	13	32.5	14.84	0.95	8.96
	14	43.5	10.06	0.08	6.40	14	30.9	5.84	2.24	2.35	14	35.6	13.08	1.03	7.34
	15	43.3	6.11	0.02	4.59	15	30.9	4.62	1.37	1.84	15	35.5	11.31	0.93	6.73
Nitrate	12	39.5	16.73	0.41	9.18	12	27.5	7.50	2.93	3.03	12	29.7	16.63	1.57	9.14
	13	47.1	14.93	0.42	7.92	13	34.1	6.61	2.44	2.83	13	40.1	15.80	1.38	8.49
	14	50.1	10.73	0.45	5.63	14	39.5	5.76	2.02	2.34	14	47.5	14.16	0.86	8.22
	15	49.6	5.49	0.06	3.49	15	38.4	5.25	2.03	1.93	15	47.2	11.31	0.47	6.89
NaCl	12	39.5	16.73	0.41	9.18	12	27.5	7.50	2.93	3.03	12	29.7	16.63	1.57	9.14
	13	51.3	7.89	0.42	4.58	13	37.7	6.44	1.98	3.00	13	42.6	14.45	0.79	7.97
	14	58.1	7.35	0.38	4.33	14	43.4	5.53	1.77	2.45	14	48.9	12.56	0.62	7.36
	15	58	5.76	0.19	3.06	15	42.1	4.05	1.22	1.89	15	48.6	10.63	0.51	6.71
Green	12	39.5	16.73	0.41	9.18	12	27.5	7.50	2.93	3.03	12	29.7	16.63	1.57	9.14
	13	52.4	12.04	0.40	7.45	13	39.5	6.90	2.21	2.96	13	44.3	15.96	1.27	8.54
	14	59.6*	10.53	0.44	6.84	14	45.1*	6.89	2.20	2.60	14	50.5*	14.27	1.01	7.99
	15	59.4	8.07	0.26	4.71	15	44.3	5.74	1.80	2.27	15	49.5	11.69	0.97	6.79

1.3.4. Fatty acid composition using two-stage and two-phase culture systems

Table 2 was fatty acid composition of 3 microalgae on 4 different culture systems at highest lipid content (14 days). As show on Table 2, lipids from *P. tricornutum* contained high composition of palmitic acid (C16:0) and oleic acid (C18:1). Lipids from *D. tertiolecta* and *I. galbana* contained high composition of palmitic acid (C16:0) and arachidic acid (C20:0). Those lipids from microalgae contained 23~32% palmitic acid (C16:0). It is precursor of other fatty acid in fatty acid biosynthesis (Ratledge, 2004). Omega-3 fatty acids were obtained from microalgae such as linolenic acid (C18:3), eicosapentaenoic acid (EPA, C20:5) and docosahexaenoic acid (DHA, 22:6). Lipid from three microalgae contained higher composition of saturated fatty acid (SFA) than unsaturated fatty acid (UFA) on control of first phase culture. However, those lipids from microalgae could increase unsaturated fatty acid on stress condition. Lipid from *P. tricornutum* was enhanced 48.87% to 54.54% on wavelength stress of LED green (520 nm), *D. tertiolecta* from 21.13% to 23.23% and *I. galbana* from 12.14% to 13.9%. Those increases were higher on two-phase culture system of wavelength stress than other two-stage culture systems of salt stress and nitrate depletion stress.

Table 2. Fatty acid compositions in 3 microalgae lipid on second phase or stage culture at 14 days.

	P.tricornutum				*D. tertiolecta*				*I. galbana*			
	Control	Nitrate	NaCl	Green	Control	Nitrate	NaCl	Green	Control	Nitrate	NaCl	Green
C14:0	11.76	10.12	8.22	9.14	11.44	8.27	10.06	11.43	22.07	20.78	19.87	20.37
C16:0	26.09	25.31	24.78	22.95	30.68	31.07	27.35	27.82	31.66	28.69	25.87	24.63
C16:1	17.09	13.82	13.89	15.98	5.17	5.41	6.37	5.41	2.43	3.02	2.44	4.48
C18:0	5.98	3.41	4.10	4.51	12.20	9.16	8.58	11.29	10.02	13.27	11.66	13.95
C18:1	28.53	35.82	35.47	36.49	14.75	15.88	16.14	16.50	6.41	6.49	7.52	5.99
C18:2	2.17	1.79	2.02	0.96	0.72	0.86	0.42	0.68	1.28	0.66	0.66	0.78
C18:3	0.81	0.27	0.23	0.59	0.48	0.41	0.64	0.64	0.68	0.57	0.86	1.03
C20:0	6.30	8.81	10.21	8.61	24.54	28.94	30.45	26.23	23.51	24.80	29.15	27.05
C20:5	0.27	0.25	0.53	0.52	-	-	-	-	0.44	0.36	0.56	0.58
C22:0	1.00	0.39	0.53	0.25	-	-	-	-	0.59	0.30	0.10	0.10
C22:6	-	-	-	-	-	-	-	-	0.90	1.06	1.30	1.03
SFA	51.13	48.04	47.85	45.46	78.87	77.44	76.43	76.77	87.86	87.84	86.66	86.10
UFA	48.87	51.96	52.15	54.54*	21.13	22.56	23.57*	23.23	12.14	12.16	13.34	13.90*

1.4. Conclusions

In this study, *P. tricornutum*, *D. tertiolecta* and *I. galbana* were cultured two-phase system. first phase of two-phase culture could produce high biomass of 1.35 g/L in *P. tricornutum*, 0.63 g/L in *D. tertiolecta* and 1.47 g/L in *I. galbana* on LED blue (465 nm) wavelength with each optimal nitrate content (*P. tricornutum* 20 mg/L, *D. tertiolecta* 12 mg/L and *I. galbana* 16 mg/L). And second phase culture of two-phase culture system on LED green (520 nm) could accumulate lipid of 51% in *P. tricornutum*, 64% in *D. tertiolecta* and 70% in *I. galbana*. Those were higher than two-stage systems of well-known lipid accumulation methods. Such as nitrate depletion and salt increase stress. Also, two-phase culture system could enhance unsaturated fatty acid of high value bioactive substance. These conclusions showed that, two-phase culture system is more effective system than existing two-stage culture systems.

1.5. References

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Chapter II Effects of light-emitting diodes (LEDs) with a mixture of wavelengths on the growth and lipid content of five microalgae

2.1. Introduction

Microalgal biomass is used as a feedstock for food or feed supplements, nutraceuticals, and cosmetics, and has been considered as a promising feedstock for biofuel production (Blanken et al., 2013). In addition, microalgal photosynthesis has been recognized as one of the most cost-effective ways to solve global warning (Wang et al., 2008). The five microalgae *Phaeodactylum tricornutum*, *Isochrysis galbana*, *Nannochloropsis salina*, *N. oceanica*, and *Picochlorum atomus* have desirable features to contribute to resolving environmental and economic issues, namely, their carbon fixation, high biomass productivity, and lipid synthesis (Song et al., 2013; Das et al., 2011; Ra et al., 2016).

Photoautotrophic microalgae absorb light energy (photons) and convert it into chemical energy, such as in the form of ATP and $NADPH_2$. These reactions occur in the microalgal photosystems and the absorption of light energy occurs via chlorophyll pigments and carotenoids (Richmond, 2003). Thus, the wavelength and intensity of light play key roles in the process of photosynthesis for photoautotrophic microalgal growth and also affect lipid production by microalgae (Ugwu et al., 2007). The wavelengths absorbed by microalgae differ depending on the species.

Chlorophylls and carotenoids are the two major classes of photosynthetic pigments found in plants and algae. Chlorophyll a (Chl a) is the primary molecule responsible for photosynthesis, while chlorophyll b (Chl b) is an accessory pigment, the level of which increases upon exposure to a broad spectrum of light that transfers the energy to Chl a. Carotenoids function as photosynthetic pigments that can efficiently dispose of excess energy. The absorbance maxima of chlorophylls and carotenoids are in the red and blue regions of the light spectrum (Richmond, 2003).

The use of specific narrow bands of light, such as from a light-emitting diode (LED), is more economical than using ordinary light sources for providing cost-effective low-wattage irradiance. Indeed, the light absorption ability of photosystem II was shown to be improved by red light, while that of photosystem I was improved by blue light (You and Bernett, 2004). Therefore, LED light with red and blue wavelengths should be more suitable for microalgal growth than other wavelengths and selectively promotes microalgal photosynthesis (Kim et al., 2013).

Against this background, the aim of this study was to use LED light with a mixture of red and blue wavelengths to enhance the formation of components such as biomass, lipids, carotenoids, and fatty acids in five marine microalgae: *P. tricornutum, I. galbana, N. salina, N. oceanica*, and *P. atomus*.

2.2. Materials and Methods

2.2.1. Microalgal strains and culture conditions

Five microalgae, *Phaeodactylum tricornutum, Isochrysis galbana, Nannochloropsis salina, N. oceanica*, and *Picochlorum atomus*, were obtained from the Korea Marine Microalgae Culture Center (Busan, Korea). They were cultured in sterilized seawater

with modified f/2 medium (Guillard and Ryther, 1962). After seed culture in a 1 L flask with a working volume of 700 mL, a 1.5 L working volume ,with the addition of 1×10^5 microalgal cells/mL as an inoculum, was cultured in 2 L flasks at 20 ± 1°C under light intensity of 100 μmol/m^2/s, of either LED light with a mixture of red and blue wavelengths or fluorescent light. The initial cell density was determined using the standard curve of OD_{680} versus dry cell weight (DCW) using a UV spectrophotometer (Ultrospec 6300 Pro; Biochrom Ltd., Cambridge, UK). Aeration with filtered air was provided through an air stone at a rate of 2.5 L/min. Each of the flasks was then exposed to light with a mixture of red (660 nm) and blue (465 nm) wavelengths under a 12 h:12 h light-dark cycle for 13 days.

2.2.2. Light source for microalgal culture

LED square panel lights (28.5 × 38.6 × 4.4 cm^3; LUXPIA Co., Ltd., Suwon, Korea) were used as the light source for the photoautotrophic growth of microalgae, as shown in Fig. 1. Thirty red (660 nm), green (520 nm), and blue (465 nm) diodes were each spaced alternately in six strips at 1 cm intervals in both rows and columns. It is well established that the optimal ratio of LED light is 1:1 for red and blue wavelengths (Kim et al., 2013; Yan and Zheng, 2014). In view of this, LED light at 100 μmol/m^2/s with a mixture of red (660 nm) and blue (465 nm) wavelengths at a 1:1 ratio was used in this study. The LED square panel lights were placed in parallel to illuminate the 2 L flask culture at a distance of 15 cm and separated from other lighting sources. The light intensity was measured with a light sensor (TES-1339; UINS Inc., Busan, Korea) as the photon flux at the center line of the flask filled with culture medium. A control experiment was conducted under the same intensity of fluorescent light and operating conditions for 13 days.

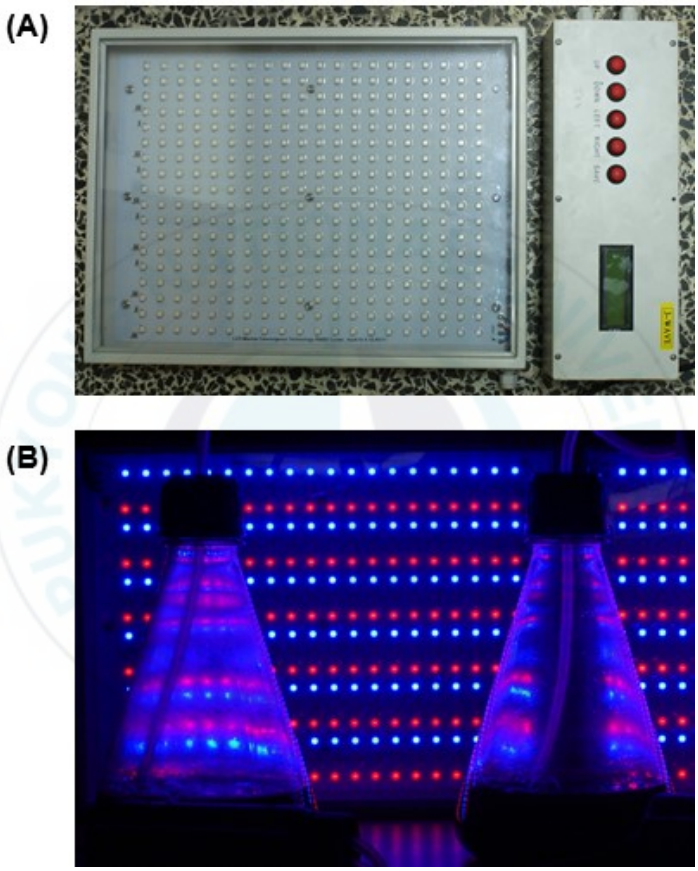

Fig. 1. (A) The setup of light-emitting diode (LED) lights and controller, and (B) algal cultivation with LEDs with a mixture of wavelengths.

2.2.3. Lipid extraction and transesterification

After cell growth, suspended cells were harvested by centrifugation (994 × *g*, 10 min) and lyophilized (SFDSM-24L; SamWon Industry, Seoul, Korea). Total lipids were extracted with chloroform/methanol (2:1, v/v) and quantified gravimetrically (Bligh and Dyer, 1959). The lipid content of dry weight was calculated using Eq. (1):

$$\text{Lipid content (\% of DCW)} = \frac{(W_2 - W_1)}{\text{DCW}} \times 100 \qquad \text{Eq. (1)}$$

where lipid content is the cellular lipid content of the microalgae (% of DCW), W_1 (g) is the weight of the empty 20 mL glass tube, W_2 (g) is the weight of the lipid extracting 20 mL glass tube, and DCW (g) is the dry cell weight of the microalgae.

The lipid composition was determined as fatty acid methyl esters (FAMEs) via the direct transesterification method reported by Dhup et al. (2014). FAME profiling was performed by gas chromatography (GC; YL 6100; YoungLin Inc., Anyang, Korea) with a flame ionization detector (FID) and a silica capillary column (HP-INNOWAX; 30 m × 0.32 mm × 0.5 μm; Agilent Technologies, Santa Clara, CA, USA). The column temperature adjustments were carried out as follows: 140°C for 5 min, followed by a temperature increase to 240°C at a rate of 5°C/min; this temperature was maintained for 5 min. Both injector and FID detector temperatures were set at 250°C. The FAMEs were identified by comparing their retention times against those of authentic standards.

2.2.4. Analyses

The numbers of inoculated microalgal cells were determined by counting using a hemocytometer and a microscope (CK40-SLP; Olympus, Tokyo, Japan). The cultures were sampled at 24 h intervals. Based on the standard curve of OD_{680} and DCW, one unit of OD_{680} corresponded to 0.42, 0.42, 0.27, 0.22, and 0.28 g DCW/L dry biomass weights of *P. tricornutum*, *I. galbana*, *N. salina*, *N. oceanica*, and *P. atomus*, respectively. The specific growth rate (μ) of microalgae was calculated using Eq. (2):

$$\mu = \frac{(DCW_2 - DCW_1)}{(DCW_1 \times (T_2 - T_1))} \quad \text{Eq. (2)}$$

where μ is the specific growth rate (h^{-1}), and DCW_1 and DCW_2 are the initial and final dry cell weights at times T_1 and T_2, respectively. Nitrate concentration was determined according to standard methods using an ultraviolet-visible (UV-Vis) spectrophotometer (Ultrospec™ 6300 Pro; Biochrom Ltd.) (Collos et al., 1999). Chl a, Chl b, and total carotenoid contents have been well established (Lichtenthaler et al., 1987). The determination of their levels in whole pigment extract of green plant tissue using a UV-Vis spectrophotometer was performed using Eqs. (3–5).

Chlorophyll a (Chl a, μg/mL) = Eq. (3)

11.24 × Absorbance (661.6 nm) – 2.04 × Absorbance (644.8 nm)

Chlorophyll b (Chl b, μg/mL) = Eq. (4)

20.13 × Absorbance (644.8 nm) – 4.19 × Absorbance (661.6 nm)

Total carotenoids (TC, μg/mL) = Eq. (5)

[1,000 × Absorbance (470 nm) – 1.90 × Chl a (μg/mL) – 6.314 × Chl b (μg/mL)]/214

2.2.5. Statistical analysis

Each experiment was carried out in triplicate. The statistical significance of differences in biomass and lipid contents was evaluated by one way analysis of variance and Duncan's multiple range test ($P < 0.05$), using SPSS software (ver. 23; SPSS Inc., Chicago, IL, USA).

2.3. Results and Discussion

2.3.1. Effects of LED light with a mixture of wavelengths on the growth of microalgae

Five microalgae, *P. tricornutum*, *I. galbana*, *N. salina*, *N. oceanica*, and *P. atomus*, were cultured in 2 L flasks with a working volume of 1.5 L at 20°C ± 1°C, an aeration rate of 2.5 L/min, a nitrate concentration of 16 mg/L, and a 12/12 h light/dark cycle under a light intensity of 100 μmol/m^2/s, for either LED light with a mixture of red and blue wavelengths or fluorescent light for 13 days. Several studies have applied LED light with a mixture of wavelengths to promote microalgal photosynthesis, in which the DCW upon treatment with red:blue light at a ratio of 1:1 was found to be higher than those of the other treatments, such as at red:blue light ratios of 1:9, 3:7, 7:3, and 9:1 (Kim et al., 2013; Yan and Zheng, 2014). Therefore, in this study, culture under light of red:blue wavelengths at a ratio of 1:1 was carried out for the five microalgae and their growth was compared to that upon culture using conventional fluorescent light for 13 days, as shown in Fig. 2. The five microalgae reached the stationary phase of growth at 10 days and reached the stage of a depleted nitrate concentration at 6–8 days (data not shown). Among them, *I. galbana* produced the highest biomass of the five microalgae under the LED light with a mixture of wavelengths, achieving 1.03 g DCW/L, followed by *P. tricornutum* (0.95 g DCW/L),

N. salina (0.85 g DCW/L), *P. atomus* (0.67 g DCW/L), and *N. oceanica* (0.62 g DCW/L). In addition, the mixed LED values of specific growth rate (μ) of *P. tricornutum*, *I. galbana*, *N. salina*, *N. oceanica*, and *P. atomus* were 0.034, 0.038, 0.030, 0.020, and 0.023 h^{-1}, respectively. Similar results were obtained previously for the culture of *Chlorella* sp., which showed maximum biomass production of 0.49 g DCW/L using a mixture of red (660 nm) and blue (460 nm) LEDs at a 1:1 ratio (Yan and Zheng, 2014). These results indicate that light of red and blue wavelengths is sufficient for microalgal photosynthesis, because microalgal plastids are closely related to those of terrestrial plants in terms of structure, metabolism, and biochemical composition (Schulze et al., 2014). Therefore, treatment using LED light with a mixture of wavelengths is more suitable for promoting microalgal growth than treatment under conventional fluorescent light.

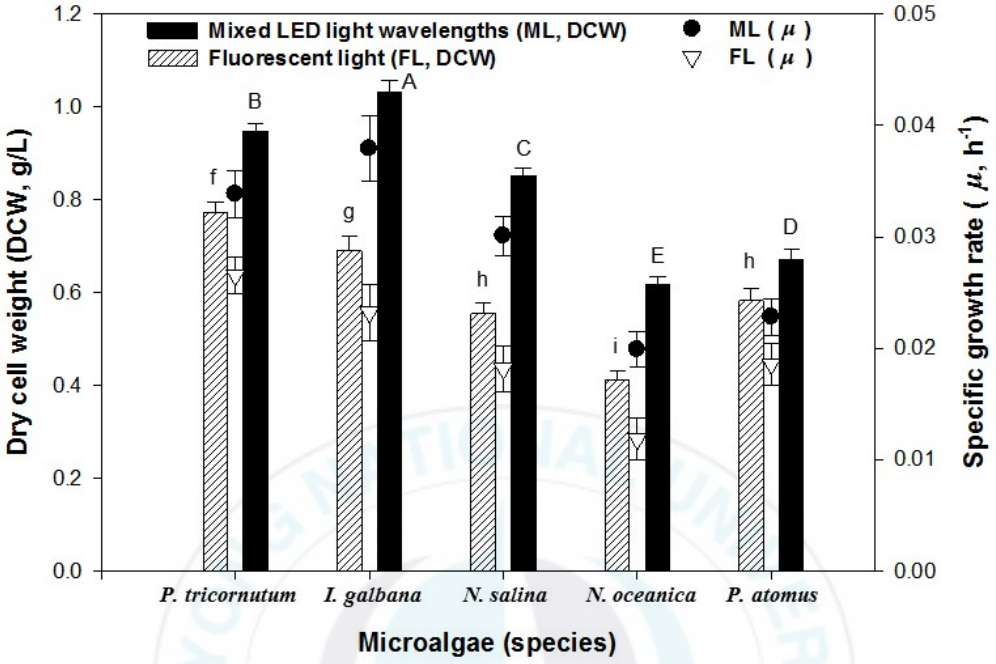

Fig. 2. Effects of LED light with a mixture of red and blue wavelengths and fluorescent light on the growth of five microalgae.

Different capital and small letters indicate significant differences ($P<0.05$, Duncan's test).

2.3.2. Effects of LED light with a mixture of wavelengths on Chl a, Chl b, and carotenoids

As shown in Figs. 2 and 3, the maximum biomass for the five microalgae corresponded to increments in the pigments (Chl a, Chl b, and carotenoids) after 13 days in culture. Figures 3(A) and (B) show that the pigment concentrations upon exposure to LED light with a mixture of wavelengths were as follows: *P. tricornutum* (Chl a: 9.91 μg/mL; Chl b: 0.92 μg/mL), *I. galbana* (Chl a: 11.38 μg/mL; Chl b: 1.48 μg/mL), *N. salina* (Chl a: 9.95 μg/mL; Chl b: 2.06 μg/mL), *N. oceanica* (Chl a: 5.64 μg/mL; Chl b: 0.66 μg/mL), and *P. atomus* (Chl a: 2.97 μg/mL; Chl b: 0.35 μg/mL). These results indicate that a suitable light composition is critical for the growth of microalgae. Figure 3(C) shows that the highest carotenoid content of 6.30 μg/mL was obtained with *I. galbana* exposed to LED light with a mixture of wavelengths. *P. tricornutum, N. salina, N. oceanica,* and *P. atomus* showed carotenoid contents of 4.70, 3.12, 2.54, and 1.19 μg/mL, respectively. Thus, the treatment with LED light with a mixture of wavelengths achieved higher levels of pigments (Chl a, Chl b, and carotenoids) than treatment with fluorescent light in this study. These results show that the photosynthesis based production of valuable carotenoids such as β-carotene and lutein was carried out using microalgae. Therefore, the production of two essential chlorophylls and carotenoids was sufficiently stimulated to achieve the highest growth rate of microalgae, especially upon exposure to LED light with a mixture of red (660 nm) and blue (465 nm) wavelengths.

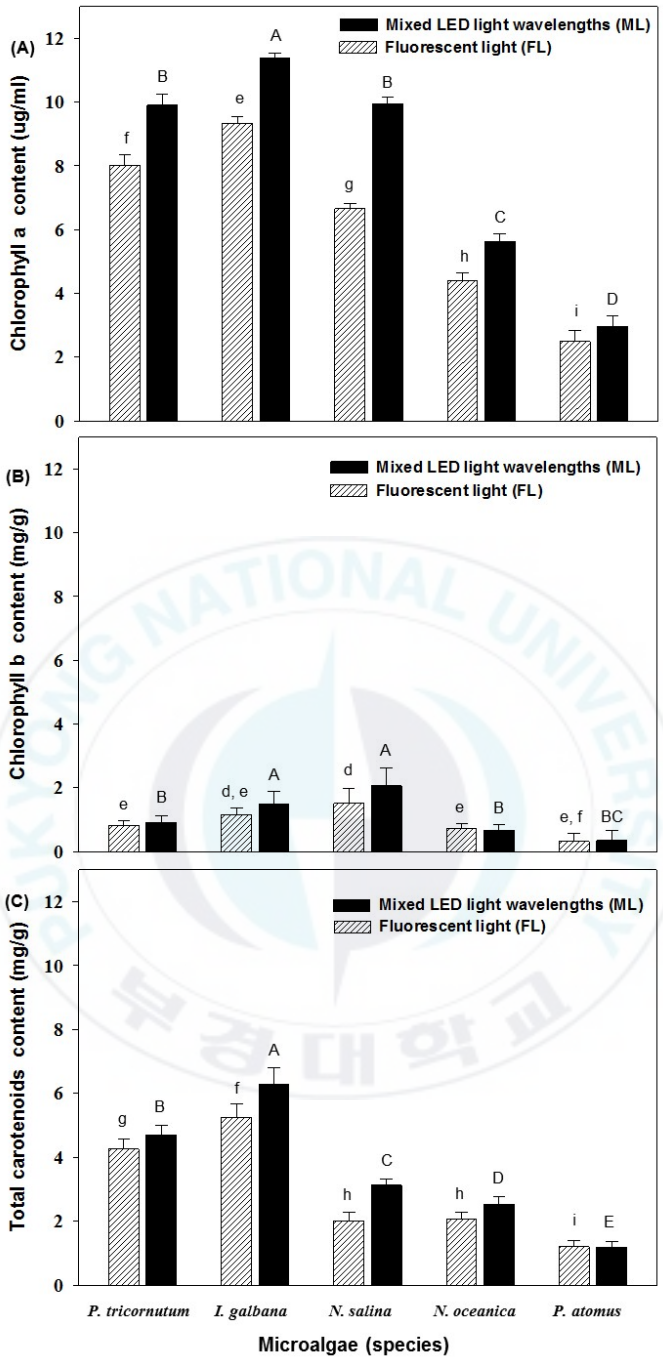

Fig. 3. Effects of LED light with a mixture of wavelengths on the production of (A) chlorophyll a, (B) chlorophyll b, and (C) carotenoids in five microalgae during 13 days of culture.

Different capital and small letters indicate significant differences ($P<0.05$, Duncan's test).

2.3.3. Lipid productivity and consumption of light energy

Five microalgae for biomass production were cultured until the early stationary phase, at 10 days; then, lipid accumulation was carried out for the following 3 days. During the stationary phase, it is assumed that the level of nutrients also decreases in line with the cessation of cell growth, causing nutrient stress (starvation period) that prompts the cell to activate its secondary metabolism, such as lipid accumulation (Rodolfi et al., 2008). As shown in Fig. 4, the highest lipid productivity of microalgae during 3 days of exposure to LED light with a mixture of wavelengths was 47.3 mg/L/day for *P. tricornutum*, 47.8 mg/L/day for *I. galbana*, 45.7 mg/L/day for *N. salina*, 29.3 mg/L/day for *N. oceanica*, and 34.3 mg/L/day for *P. atomus*. Upon comparison with the results on exposure to fluorescent light, the five microalgae in this study revealed notable abilities to accumulate lipids, surpassing the average total lipid productivity (mg/L/day) of 16 to 37 mg/L/day reported for algal species (Illman et al., 2000; Li et al., 2011). However, the lipid productivities of *N. oceanica* and *P. atomus* were not high due to the low microalgal biomass productivity, as shown in Figs. 2 and 4. Thus, *P. tricornutum* and *I. galbana* were the best species in this study with regard to biomass production and lipid accumulation. The total electricity consumption yield upon exposure to LED light with a mixture of wavelengths and to fluorescent light sources was as follows, in descending order: *I. galbana* (23.7 kw-h/g lipid and 70.9 kw-h/g lipid), *P. tricornutum* (24.0 kw-h/g lipid and 65.8 kw-h/g lipid), *N. salina* (24.8 kw-h/g lipid and 80.2 kw-h/g lipid), *P. atomus* (33.1 kw-h/g lipid and 79.3 kw-h/g lipid), and *N. oceanica* (38.7 kw-h/g lipid and 118.8 kw-h/g lipid). These results show that a mixture of red and blue LED lights enhances biomass and lipid production in an economical manner and provides a total electricity consumption yield that is approximately 31.0% to 41.7% higher than for fluorescent light.

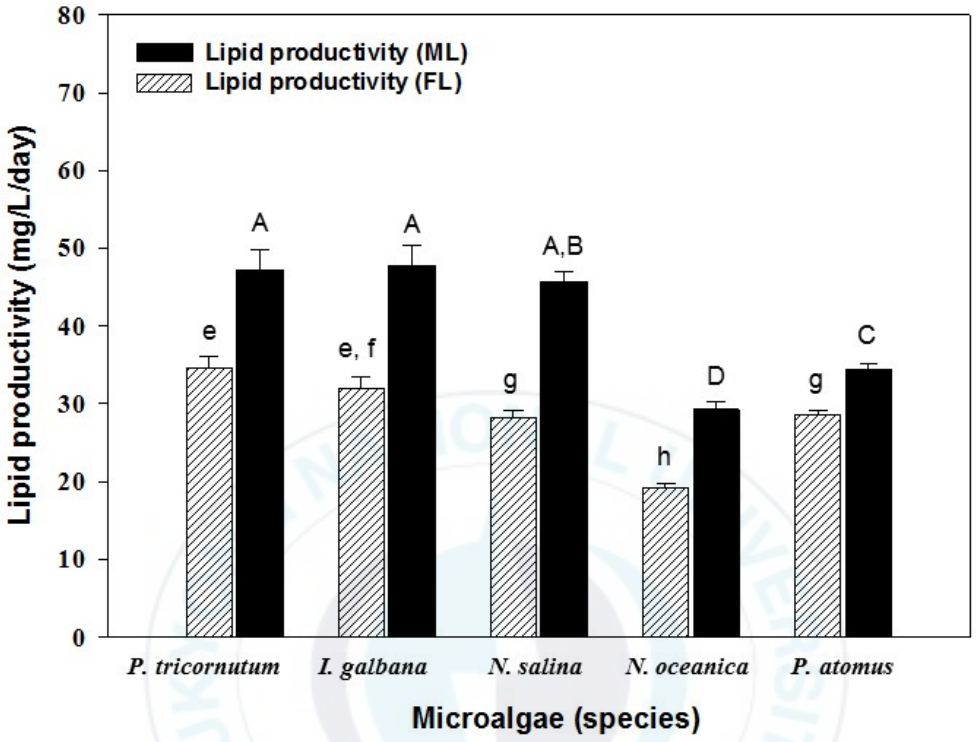

Fig. 4. The profiles of lipid productivity (mg/L/day) of five microalgae using LED light with a mixture of red and blue wavelengths and fluorescent light during 3 days of nutrient stress (starvation).

Different capital and small letters indicate significant differences ($P<0.05$, Duncan's test).

2.3.4. Fatty acid composition

The lipids extracted from five microalgae, *P. tricornutum*, *I. galbana*, *N. salina*, *N. oceanica*, and *P. atomus*, were converted to FAMEs; their compositions are depicted in Fig. 5. Five species of microalgae were enriched in the seven most common C14–C20 fatty acids, namely, myristic (C14:0), palmitic (C16:0), stearic (C18:0), oleic (C18:1), linoleic (C18:2), linolenic (C18:3), and arachidic (C20:0) acids. The most abundant fatty acid among the five microalgae was palmitic acid (C16:0), accounting for 33.8%–56.0% (w/w) of the total fatty acid content. These results are similar to those reported by Converti et al. (2009). They reported that the palmitic acid (C16:0) found in *C. vulgaris* and *N. oculata* represented 47%–66% (w/w) and 58%–62% (w/w) of the total fatty acids, respectively. A similar reported that *N. salina*, *N. oceanica*, and *N. oculata* contained palmitic acid (C16:0) and oleic acid (C18:1), primarily (Ra et al., 2016). This indicates that the earliest precursor for fatty acid synthesis is palmitic acid (C16:0).

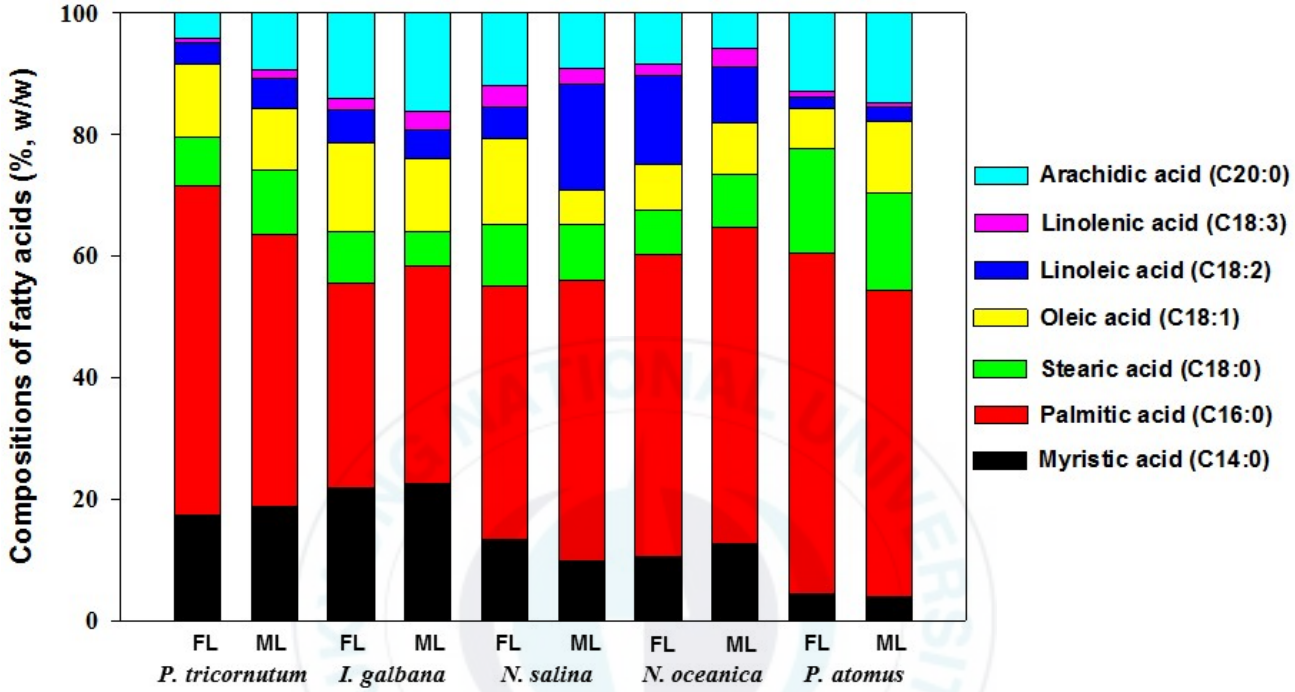

Fig. 5. Fatty acid compositions of five microalgae under LED light with a mixture of red and blue wavelengths and fluorescent light.

2.4. Conclusions

Among the five strains with high rates of growth and lipid productivity, the three species *P. tricornutum*, *I. galbana*, and *N. salina* were selected for biodiesel production due to their high biomass and lipid productivity upon exposure to LED light with a mixture of wavelengths compared with those under ordinary fluorescent light. The best strains were *P. tricornutum* and *I. galbana*, with lipid productivity of 47.3 mg/L/day and 47.8 mg/L/day, and palmitic acid levels of 44.90% (w/w) and 35.9% (w/w), respectively. The electricity consumption yield of lipids using LED light with a mixture of wavelengths was one third of that using fluorescent light.

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