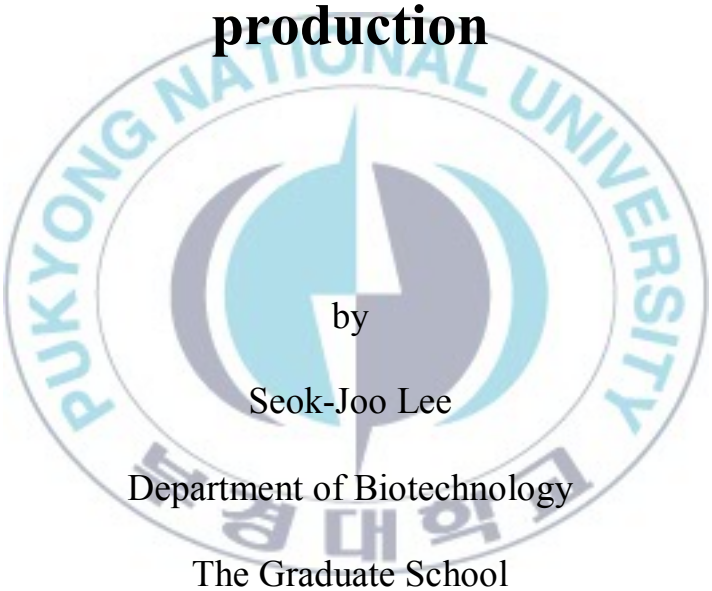
Thesis for the Degree of Master of Engineering

Studies on biomass and oil content of marine microalgae for biodiesel production



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February 2011

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(바이오디젤 생산을 위해 해양미세조류의 바이오매스와 오일 함량에 관한 연구)

Advisor: Prof. Sung-Koo Kim

by

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Studies on biomass and oil content of marine microalgae for biodiesel production

A dissertation

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바이오디젤 생산을 위해 해양 미세조류의 바이오매스와 오일 함량에 관한 연구

이 석 주

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요 약

화석연료로부터의 디젤의 소비와 이로부터 배출되는 NOx, SOx 그리고 CO와 같은 대기 오염물질은 해마다 증가하고 있으므로 해양 미세조류로부터의 바이오디젤을 생산하기 위한 관심이 증가하고 있다. 해양 미세조류는 높은 성장속도와 오일을 체내에 축적할 수 있으며, 배양을 위해 많은 면적의 땅을 필요로 하지 않는다는 장점이 있다. 하지만, 바이오매스 함량, oil 함량을 최대로 생산하기 위한 연구는 세계적으로 꾸준히 진행되고 있지만 아직은 미비한 상태이다. 따라서, 본 연구에서는 *Tetraselmis suecica*, *Phaeodactylum tricornutum*, *Chaetoceros calcitrans*, *Isochrysis galbana* 그리고 *Nannochloropsis oculata*로 부터 높은 바이오매스 함량과 oil 함량을 나타내는 종을 선정하기 위한 실험이 수행되었다. 이와 더불어 oil내에 바이오디젤의 특성에 적합하다고 알려진 C16 (palmitic acid) 와 C18 (stearic acid, oleic acid, linoleic acid and linolenic acid) 의 지방산 함량을 분석하였다.

바이오디젤 생산을 위한 적합한 후보종을 선정하기 위하여 5종의 해양 미세조류는 배양기 내에 f/2 배지와 15-L 의 멸균해수와 f/2 배지를 첨가하고 12:12의 light and dark cycle (L:D cycle), 36.3 μ mol/m^2/s의 조도, 2.5 L/min의 일정한 air를 공급해 주는 동일한 배양조건으로 20±1℃의 실내에서 배양되었다. *T. suecica*가 배양 후 9일째에 정지기에 접어들었으며, 이 때의 바이오매스 함량은 0.58 g/L 였다. *I. galbana*와 *N. oculata*도 0.57 g/L 로 높은 바이오매스 함량을 나타내었지만, 접종 후 정지기까지의 배양기간은 각각 24일, 28일이었다. 하지만, *P. tricoenutum*과 *C. calcitrans*의 바이오매스 함량은 각각 0.26과 0.47 g/L로 낮았다.

Oil 함량은 정지기로부터 9일째로 갈수록 증가하였으며, *P. tricornutum*과 *I. galbana*에서 각각 25.31% 와 23.15% dry cell weight 로 5종 중에서 높은 함량을 나타내었다. 높은 바이오매스 함량을 나타내었던 *T. suecica*와 *N. oculata*는 각각 7.55와 8.23% dry cell weight로 낮은 함량을 정지기로부터 9일째에 나타내었다.

5종의 미세조류는 oil 내의 C16과 C18의 지방산 함량도 oil 함량의 증가와 같은 경향을 나타내었으며 특히, palmitic acid와 oleic acid의 함량이 대부분을 차지하고 있었다. *T. suecica*가 561.22 mg/g oil으로 5종류의 총 지방산 함량을 나타내었다. *I. galbana*도 472.66 mg/g oil의 높은 함량을 나타내었다. *I. galbana*와 *C. calcitrans*는 5종류의 지방산 중 palmitic acid의 함량이 두드러지게 높았으며, *P. tricornutum*의 5종류의 지방산 함량이 실험종 중에서 가장 낮은 299.60 mg/g oil을 나타내었다. 따라서, *T. suecica*와 *I. galbana*가 바이오디젤을 생산하기 위해서 oil을 형성할 수 있는 적합한 종으로 판단되었다.

본 연구에서는 *T. suecica*를 이용하여 배양기간을 단축시키면서 바이오매스 확보와 더불어 높은 oil 함량을 나타낼 수 있는 연구를 수행하기 위해 *T. suecica*의 바이오매스 함량과 배양기간을 단축 시키기 위해 최적의 L:D cycle, light intensity, nitrate concentration 그리고 aeration rate 확립 및 oil 함량을 높이기 위한 2단계 배양을 수행하였다.

다양한 L:D cycles 하에서 *T. suecica*를 배양한 결과, 다른 L:D cycles에서 보다는 24:0의 L:D cycle 하에서 0.78 g/L 로 바이오매스 함량이 나타났으며 정지기에 도달하는 배양기간이 종 선정 실험에서보다 2일 줄어든 6일로 단축되었다. 이 조건을 이용하여 light intensities 조건실험을 한 결과, 145.2 μmol/m^2/s의 light intensity에서 0.89 g/L의 바이오매스 함량과 함께 접종일로부터 5일째에 정지기에 접어든 것을 확인할 수 있었으며, 확립된 L:D cycle과 light intensity를 nitrate concentrations 조건실험에 적용한 결과 18.55 mg/L의 nitrate concentration 하에서 1.07 g/L 의 바이오매스 함량을 확인할 수 있었다. 최적 aeration rate를 확립하고자 다양한 aeration rates 실험을 통해서는 nitrate concentration 실험과 비슷한 1.04 g/L의 바이오매스 함량을 나타내어 2.5 L/min의 air를 공급함이 최적임을 확인할 수 있었다.

각각의 성장 환경 조건하에서의 oil 함량은 light cycle을 줄여줄수록, light intensity를 높여줄수록, nitrate concentration이 낮고, aeration rate가 높아질수록 높은 함량을 나타내었으나, 유의적인 차이를 나타내지는 않았다(P>0.05). 최적의 바이오매스 함량 조건을 토대로 하여 배양된 *T. suecica*의 oil 함량을 더욱 높이기 위하여 정지기에 도달한 *T. suecica*를 다양한 농도의 nitrate가 포함된 배지에 재접종을을 하여 2단계 배양을 수행한 결과, oil 함량은 nitrate를 포함하지 않는 조건에서 접종 후 4일째에 17.28% of dry cell weight로 가장 높게 나타났으며, oil 내의 다섯가지 총 지방산 함량도 720.54 mg/g oil로 증가된 것을 확인할 수 있었다.

따라서, 본 실험들로부터 해양 미세조류인 5종 *T. suecica*, *I. galbana*, *N. oculata*, *P. tricornutum* and *C.*

*calcitrans*를 이용하여 바이오디젤 생산을 위한 바이오매스 및 oil 함량에 대한 기초결과를 도출 할 수 있었고, *T. suecica*를 이용한 바이오매스 함량과 oil 함량을 증대시킬 수 있는 조건들을 확립하였다. 더불어, 향후 바이오디젤 생산을 위한 여러 미세조류의 탐색이 지속적으로 이루어지고, oil 함량의 극대화를 위한 연구가 지속적으로 이루어져야 한다고 판단된다.

Ⅰ. INTRODUCTION

Microalgae have received attentions for the bioenergy production because microalgae can make oil in the cell body as well as carbohydrate and protein (Kim *et al*., 2005; Tran *et al*., 2010). Although the growth of the microalgae depends on characteristics of species, microalgae can grow fast and no land is required for the cultivation in comparison to land-based crop (Widjaja *et al*., 2009). Therefore, the production of biodiesel from microalgae can replace the present diesel production from petroleum in near future (Tran *et al*., 2010).

Biodiesel is one of the liquid-fuels with the sustainability. It has advantages of non-toxic and biodegradable bio-fuel with low pollutant emission (Widjaja *et al*., 2009). Recently, some countries have enforced biodiesel blending to diesel fuel such as BD5 and BD10 using 5% and 10% of biodiesel, respectively. Animal fats, soap-stocks, and fish oils have been already tried to produce biodiesel, however, the total amount of those are insufficient for the world demands (Mata *et al*., 2010). Most of the biodiesel are currently produced from the plant oils such as soybean, rapeseed and palm. However, the price and supply of those are unstable. Thus, inexpensive and secure energy source for the oil production is necessary, and, the potential resource of the future renewable bioenergy has been focused on microalgae.

In this study, five species of microalgae, *Tetraselmis suecica*, *Phaeodactylum tricornutum*, *Chaetoceros calcitrans*, *Isochrysis galbana* and *Nannochloropsis oculata* were evaluated to select microalgae for the production of biodiesel. Those microalgae with the oil content ranging from 15 to 68% are already known as potential species for the oil production (Chisti, 2007). Those microalgae contain large portion of C18 fatty

acids. All of microalgae species used in this study are commonly used as live feeds for larvae culture in aquaculture (Lin *et al*., 2007) with stable cultivation and high nutritional value.

To procure high quantity of oil content, biomass production from microalgae should be enhanced which can be optimized by L:D cycle, light intensity, nitrate concentration and aeration rate (Jacob-Lopes *et al.,* 2009; Kaixian and Borowitzka, 1993; Imamoglu *et al*., 2007; Hsieh *et al*., 2009; Chen and Johns, 1991). Oil from microalgae is chemically similar to that of the land-based crop (Chiu *et al*., 2009). In particularly, oil accumulation in the cell body of microalgae occurs when microalgae are placed in environmental stress such as nitrogen deficiency (Hsieh *et al*., 2009). Several studies have been focused on the growth and oil accumulation of microalgae through cultivation in photobioreactors (Hsieh and Wu, 2009; Fernández *et al*., 2003).

Oil is generally consisted of saturated and unsaturated fatty acids. Palmitic acid ($C_{16:0}$), stearic acid ($C_{18:0}$), oleic acid ($C_{18:1}$), linoleic acid ($C_{18:2}$) and linolenic acid ($C_{18:3}$) are known as common fatty acids for biodiesel production, especially, those of fatty acids are strong candidates for improving the quality of biodiesel (Gerhard, 2008). However, few studies on fatty acid composition of the oil from microalgae have been carried out for biodiesel production (Lin *et al*., 2007).

The aim of this study is to evaluate cell growth, specific growth rate, biomass productivity, oil content and fatty acid composition of oil from five microalgae candidates for biodiesel production in 15-L monospecific batch culture. The microalgal species chosen among five microalgae was also determined optimize growth conditions on various L:D cycles, light intensities, nitrate concentrations and

aeration rates to increase biomass production. After optimizing culture conditions, 2-stage culture with various nitrate concentrations was further carried out for increasing oil content.

Ⅱ. MATERIALS AND METHODS

1. Microalgae & Culture medium

Five species of microalgae, *Tetraselmis suecica*, *Phaeodactylum tricornutum*, *Chaetoceros calcitrans*, *Isochrysis galbana* and *Nannochloropsis oculata* were obtained from NLP corp. (Busan, Korea). All microalgae were cultured in the f/2 media (Guillard and Ryther, 1962).

2. Culture conditions

After the pre-cultivation in 5-L beaker, microalgae were cultivated at 20±1℃ with the illumination of 36.3 μmol/m^2/s in 20-L circular cylindrical photobioreactor with a working volume of 15-L sterilized sea water. Air was provided through air-stone at the rate of 2.5 L/min. All cultures were performed with 12:12 L:D cycle.

For the optimization of culture conditions on the selected species, growth experiments were done at different L:D cycle, light intensity, nitrate concentration and aeration rate. Various light and dark cycles (L:D cycles), light intensities, nitrate concentrations and aeration rates of the culture were varied as 24:0, 20:4, 16:8, 12:12, 8:16, 4:20 and 0:24, 36.3, 60.5, 84.7, 108.9 and 133.1 μmol/m^2/s, N-free, 6.18, 12.37, 18.55 and 24.74 mg/L and 0, 0.61, 1.25, 2.5 and 5.0 L/min, respectively.

Two-stage culture of the species at the stationary phase by the optimized growth conditions from the studies of L:D cycle, light intensity and aeration rate was carried out with artificial sea water including various nitrate concentrations which were N-

free, 4.61, 9.23 and 18.55 mg/L. All of the cultures were harvested every day for 4 days and the content and five total fatty acids were analyzed

3. Analytical methods

3.1. Measurement of cell growth

Samples were obtained daily from the culture and 3% chloroform was added for the fixation of cell mobility. Microalgae were counted in a haemocytometer with a light microscope (Olympus CK40-SLP, Tokyo, JAPAN). For dry cell weight determination, 30 ml of culture samples in triplicate were filtered through pre-weighted 1.2 μm glass-fiber filters (Whatman GF/C; 47mm) to remove water. The retained cells were washed twice with distilled water and dried at 105℃ to constant weight.

3.2. Estimation of nitrate concentration

Nitrate concentration was determined by spectrometry using UV-Vis spectrophotometer (Ultrospec 6300 pro, Biochrom Ltd., England) at 220 nm (Collos *et al*., 1999). The sample was centrifuged at 10,000 rpm for 10 min. The supernatant was used for the measurement of nitrate concentration. A standard curve was constructed and used for the determination of nitrate concentration.

3.3. Cell harvest and oil extraction

The microalgae were harvested by the centrifugation at 8000 rpm, 4℃ for 5 min on 0, 3rd, 6th and 9th day from the stationary phase. The pellet was washed using

distilled water three times. The sample was dried at 105℃ to constant weight. Dried cells were ground using a mortar. The oil from 1.0 g of dried cell was extracted with a modified Soxhlet method (Li *et al*., 2008). Extracted oils from the culture sample were properly dried until reaching constant weight. Oil content was calculated by following equation below:

Where W and Wo are weights of Soxhlet extractor flask after and before extracting oil. DCW is dry cell weight.

3.4. Measurement of specific growth rate

Specific growth rate of microalgae was calculated by following equation below:

Where X_2 is final dcw and X_1 is initial dcw.

3.5. Fatty acid analysis

In order to analyze fatty acids, crude oil extracted by Soxhlet extractor was methylated. Extracted oil was weighed and added to 0.5N NaOH-methanol. The oil with 0.5N NaOH-methanol was heated at 75℃ for 30 min for the saponification of the oil and cooled at room temperature. A solution of 14% Boron trifluorid methanol (SIGMA, St. Louis, MO, USA) was added and heated at 75℃ for 15 min.

Fatty acid methyl ester was made by the addition of hexane. The methyl ester was analyzed by modified method (Liu and Lin, 2001) in gas chromatography (Thermo

Finnigan Trace GC, San Jose, CA, USA) equipped with silica capillary column (007-CW-30-0.25F) and a flame ionization detector (FID). The initial temperature was 100°C, followed by a temperature program of 5℃ min^{-1} to reach a final oven temperature of 200℃. The injector and detector temperature were 200℃ and 250℃, respectively. Carrier gas was N_2 and gas pressure was 65 psi. The quantity of fatty acid was calculated by comparing peak areas with standard curves of palmitic acid (SIGMA, St. Louis, MO, USA), stearic acid (SIGMA, St. Louis, MO, USA), oleic acid (SIGMA, Bangalore, India), linoeic acid (SIGMA, St. Louis, MO, USA) and linolenic acid (SIGMA, St. Louis, MO, USA).

3.6. Statistical analysis

The statistical analyses were performed using the software SAS 9.1 (SAS Institute, Cary, NC, USA). Biomass production, specific growth rate, biomass productivity and oil content were analyzed by one-way analysis of variance (ANOVA). Values are means of triplicates. The data were analyzed statistically by the analysis of variance (ANOVA) and difference between means of the samples was analyzed by the least significant difference (LSD) at probability level of 0.05.

Ⅲ. RESULTS AND DISCUSSION

1. Five microalgae experiment

1.1. Growth and oil content

Biomass (g/L), nitrate concentration (mg/L) and oil content (% of dry cell weight) of five marine microalgae are shown in Fig. 1. The highest biomass production of 0.58 g/L was observed in *T. suecica* on 9th day after the inoculation as shown in Fig. 1(a) and Table 1. The lowest biomass production of 0.26 g/L was observed in *P. tricornutum* on 14th day after the inoculation as shown in Fig. 1(d). However, *P. tricornutum* have been shown the biomass production of 3.0 g/L (Fernández *et al*., 2003). It was considered that *P. tricornutum* needs higher light intensity and aeration than these conditions in this study. Biomass production of *I. galbana* and *N. oculata* were showed 0.57 g/L on 23th and 28th day as shown in Fig. 1(b) and (c), respectively. *I. galbana* showed that maximum dry cell weight with 200 μmol/m^2/s at 15℃ and 30℃ were 0.55 and 0.52 g/L, respectively (Zhu *et al*., 1997). The growth of *I. galbana* in this study showed similar result to those of previous study regardless of light intensity (Zhu *et al*., 1997).

Thirteen mg/L of nitrate were added to the medium and nitrate concentration was decreased until the microalgae reached to the stationary phase as shown in Fig. 1. *T. suecica* culture showed the residual nitrate concentration of 0.51 mg/L on 7th day of the culture as shown in Fig. 1(a). This indicates that the cell growth of *T. suecica* was

fast comparing to that of other microalgae in this study. On 13th day, all microalgae except for *N. oculata* showed residual nitrate of 0.40-1.05 mg/L, however *N. oculata*

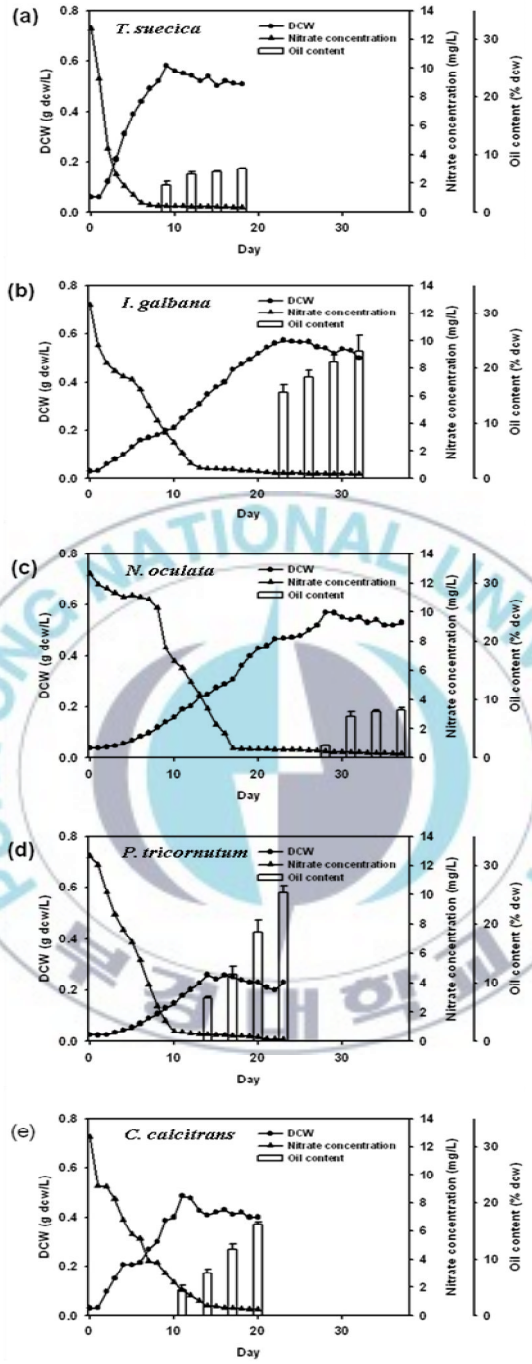

Fig. 1. Culture profiles of oil content (% dcw), nitrate concentration (mg/L) and biomass (g dcw/L) of (a) *T. suecica*, (b) *I. galbana*, (c) *N. oculata*, (d) *P. tricornutum* and (e) *C. calcitrans*.

Table 1. Total biomass, specific growth rate and biomass productivity of five marine microalgae; *T. suecica*, *I. galbana*, *N. oculata*, *P. tricornutum* and *C. calcitrans*

	Biomass production (g/L dcw, AVG±SD)	Specific growth rate (day^{-1}, AVG±SD)	Biomass productivity (mg/L/day, AVG±SD)
T. suecica	0.58 ± 0.02^{a}	0.28 ± 0.02^{a}	64.57 ± 0.55^{a}
I. galbana	0.57 ± 0.01^{a}	0.13 ± 0.02^{c}	24.93 ± 1.70^{c}
N. oculata	0.57 ± 0.03^{a}	0.10 ± 0.02^{c}	20.36 ± 0.93^{d}
P. tricornutum	0.26 ± 0.04^{c}	0.18 ± 0.02^{b}	18.58 ± 1.39^{d}
C. calcitrans	0.48 ± 0.01^{b}	0.27 ± 0.01^{a}	44.09 ± 2.72^{b}

Means in the same column with the same superscript letter are significantly different ($P<0.05$) by least significant difference (LSD) test.

cultures remained 4.37 mg/L nitrate as shown in Fig. 1(c) indicating the slow growth of *N. oculata* as shown in low specific growth rate (Table 1). The growth of *N. oculata* showed the longest culture period of 28 days to reach the stationary phase even after the deficiency of nitrate on 17th day from the inoculation as shown in Fig. 1(c). This indicates that *N. oculata* stores nitrogen source in the cell for the cell growth (Toron *et al*., 2002). A study reported that the nitrate depletion was observed at 7th day of the culture with the stationary phase regardless of initial nitrate concentrations of 0.3, 1.05, 2.1, 3.5 and 6.3 mg/L for the culture of *C. calcitrans* (Corzo *et al*., 2000). However, the nitrate remained at the concentration of 1.79 mg/L with *C. calcitrans* at the stationary phase as shown in Fig. 1(e). The reason for the low uptake of nitrate by *C. calcitrans* was low energy input, therefore, in order to increase the cell growth and uptake of nitrate, high light intensity and high L:D cycle condition should be provided.

T. suecica showed a similar specific growth rate with *C. calcitrans* as shown in Table 1. However, *T. suecica* showed higher biomass productivity than *C. calcitrans*. Therefore, the biomass production of *T. suecica* was higher than that of *C. calcitrans*. *I. galbana* showed slightly higher biomass productivity than those of *P. tricornutum* and *N. oculata* as shown in Table 1. *I. galbana* and *N. oculata* showed higher biomass production of 0.57 g/L. Considering the specific growth rates and biomass productivities of *I. galbana* and *N. oculata*, the growth of both microalgae was slow for the biomass production. However, the *P. tricornutum* showed low biomass production probably due to the low light intensity in this study (Kaixian and

Borowitzka, 1993).

The oil contents for five marine microalgae at 0, 3rd, 6th and 9th day after the stationary phase were shown in Fig. 1. Oil content was increased to 9th day of the stationary phase. *P. tricornutum* showed the highest oil content of 25.31% of dry cell weight on 9th day of the stationary phase among five marine microalgae as shown in Fig. 1(d). Various light intensities of 18, 36 and 72 μmol/m^2/s for oil production have been applied to the culture of *P. tricornutum* at 22℃, 12:12 L:D cycle in 2-L air-lift glass cylinder for 9 days (Kaixian and Borowitzka, 1993). The oil contents by light intensities of 18, 36 and 72 μmol/m^2/s were 9.20, 15.90 and 29.52% of dry cell weight with biomass productions of 0.18, 0.32 and 0.46 g/L, respectively (Kaixian and Borowitzka, 1993). Therefore, it suggests that high light intensity and low temperature could increase the oil accumulation in *P. tricornutum.*

The oil content of 23.15% of dry cell weight from *I. galbana* was obtained using f/2 medium with $NaNO_3$ as N source as shown in Fig. 1(b). *I. galbana* showed that oil content reached to 24.65% of dry cell weight with modified Walne medium containing NH_4NO_3 as N source (Lin *et al*., 2007). The oil content in this study with *I. galbana* was consistent (Lin *et al*., 2007). It suggested that $NaNO_3$ in f/2 medium and NH_4NO_3 in Walne medium produced similar oil content to *I. galbana*. *C. calcitrans* and *N. oculata* showed the oil contents of 16.25% and 8.23% of dry cell weight on 9th day after the stationary phase as shown in Fig. 1(e) and (c), respectively. *T. suecica* showed the shortest culture period with the oil content of 7.55% of dry cell weight on 9th day of the stationary phase as shown in Fig. 1(a). Oil content of *T. suecica* is in the range of 15 to 23% of dry cell weight (Chisti, 2007). High oil content of 26.8% of dry

cell weight in *T. suecica* was obtained with continuous addition of nitrogen deficient medium for 38 days of cultivation (Thomas *et al*., 1984).

1.2. Fatty acids composition

Changes in five fatty acids composition and oil content after the stationary phases of five microalgae cultures were shown in Fig. 2. Palmitic acid contents of all microalgae were increased at early stationary phase as shown in Fig. 2. As shown in Fig. 2(b), high palmitic acid content of 417.33 mg/g oil was observed in *I. galbana* on 9th day of the stationary phase. High oleic acid content of 235.61 mg/g oil was observed in *T. suecica* on 3rd day of the stationary phase as shown in Fig. 2(a). Stearic acid, linoleic acid and linolenic acid contents were lower than 30 mg/g oil in the five microalgae as shown in Fig. 2. *N. oculata* showed the increase of 1.3-fold on palmitic acid and oleic acid contents from early stationary phase to the late stationary phase (Toron *et al*., 2002). The same tendency was observed in this study with increases of 1.4-fold on palmitic acid content and 1.3-fold on oleic acid content as shown in Fig. 2(c). The palmitic acid content of *P. tricornutum* increased by 1.9-fold at early stationary phase to late stationary phase, however, oleic acid content of *P. tricornutum* decreased by 0.8-fold (Toron *et al*., 2002). Same tendency of *P. tricornutum* was observed in this study as shown in Fig. 2(d).

Oil contents of *I. galbana* and *C. calcitrans* increased as palmitic acid contents increased from 0 to 9th day of the stationary phase as shown in Fig. 2(b) and (e). However, the other fatty acids contents were not increased as oil content increased as shown in Fig. 2. Palmitic acid content of *T. suecica* increased to 297.55 mg/g oil from 0 to 3rd day of the stationary phase as shown in Fig. 2(a). However, oil content of *T.*

suecica remained at low level of 7.55% of dry cell weight. Oil content of *N. oculata* increased from 0 to 3rd day of the stationary phase. However, five fatty acids contents

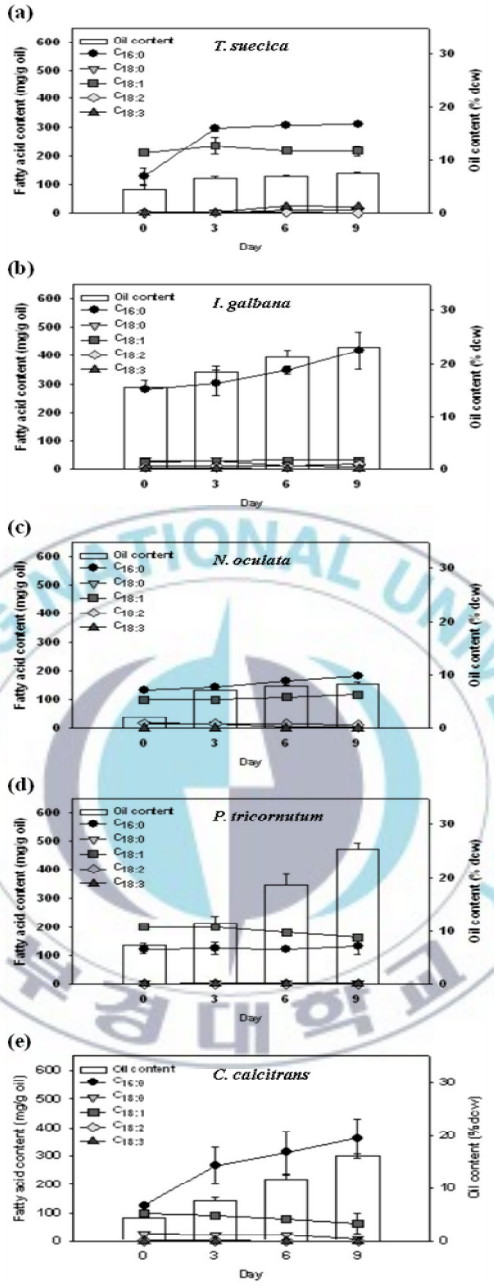

Fig. 2. The change of oil content and fatty acid composition of palmitic acid ($C_{16:0}$), stearic acid ($C_{18:0}$), oleic acid ($C_{18:1}$), linoleic acid ($C_{18:2}$) and

linolenic acid ($C_{18:3}$) at 0, 3rd, 6th and 9th day of the stationary phase on (a) *T. suecica*, (b) *I. galbana*, (c) *N. oculata*, (d) *P. tricornutum* and (e) *C. calcitrans.*

did not change in *N. oculata* culture as shown in Fig. 2(c). Oil content of *P. tricornutum* increased to 25.31% of dry cell weight from 0 to 9th day of the stationary phase as shown in Fig. 2(d). However, five fatty acids content of *P. tricornutum* decreased from 3 to 9th day of the stationary phase as shown in Fig. 2(d). The fatty acid composition of microalgae could be easily changed by controlling the culture condition and period (Lin *et al*., 2007; Zhu *et al.,* 1997; Toron *et al*., 2002). The C16 and C18 fatty acids content of *Chlorella* sp. was changed significantly by the culture conditions during the 5 days of cultivation period (Johnson *et al.,* 2009). It suggests that changes on specific fatty acid contents in total fatty acids are depended on species characteristics, culture condition and period.

In the previous study, the C16 and C18 fatty acids, particularly oleic and palmitic acid, were recognized as the most common components of biodiesel (Gerhard, 2008). Palmitic acid contents were increased and unsaturated fatty acids were decreased during post-exponential phase to stationary phase in *Schizochytrium limacinum* SR21 and *Nannochloropsis oculata* cultivation (Morita *et al*., 2006; Hodgson *et al*., 1991). Five microalgae in this study also showed the increment of palmitic acid content during the early stationary phase. It presumed that microalgae uses unsaturated fatty acids as energy sources and accumulate saturated fatty acids when nutrients deficiency occurs in the culture medium (Jeh *et al*., 2007).

From the present study, *T. suecica* with high biomass production and *I. galbana* with high oil content were chosen for the production of biodiesel. However, *T. suecica*

with short culture period was further studied to optimize L:D cycle, light intensity, nitrate concentration and aeration rate for the production of biomass, and the increase of oil content through 2-stage cultivation system.

2. Optimization of growth condition

2.1. Effect of light and dark cycle

T. suecica was cultured on various L:D cycles to increase biomass production and reduce culture period as shown in Fig. 3. *T. suecica* with 24:0 L:D cycle was faster to reach the stationary phase on 6th day. Moreover, nitrate was almost absorbed by *T. suecica* with 24:0 L:D cycle in 4 days from the inoculation. As shown in Fig. 3(b), nitrate was almost consumed by *T. suecica* on various L:D cycles except for the condition of 4:20 and 0:24 L:D cycle which remained the nitrate concentration up to 4 mg/L on 14th day. *T. suecica* could consume nitrate faster and showed high biomass production at the conditions of high L:D cycles.

The results of biomass production, specific growth rate, biomass productivity and oil content of *T. suecica* with various L:D cycles were shown in Table 2. *T. suecica* at 24:0 L:D cycle showed high biomass production of 0.78 g/L and high specific growth rate and biomass productivity of 0.48 /day and 129.87 mg/L/day, respectively. The tendency of biomass production of 0.78 g/L on various L:D cycles from the present study showed similar trends to those of previous study (Jacob-Lopes *et al.,* 2009). It indicates that the biomass production could be increased by high L:D cycles.

Oil contents of *T. suecica* at various L:D cycles were increased by long dark cycle. High oil content of *T. suecica* was shown 11.14% of dry cell weight on 4:20 L:D cycle as shown in Table 2. It indicates that *T. suecica* could accumulate oil by long dark cycle. However, there were no significant differences among 12:12, 8:16 and

4:20 L:D cycles as shown in Table 2 (P>0.05).

Therefore, the condition of 24:0 L:D cycle was used for the further study to increase biomass production.

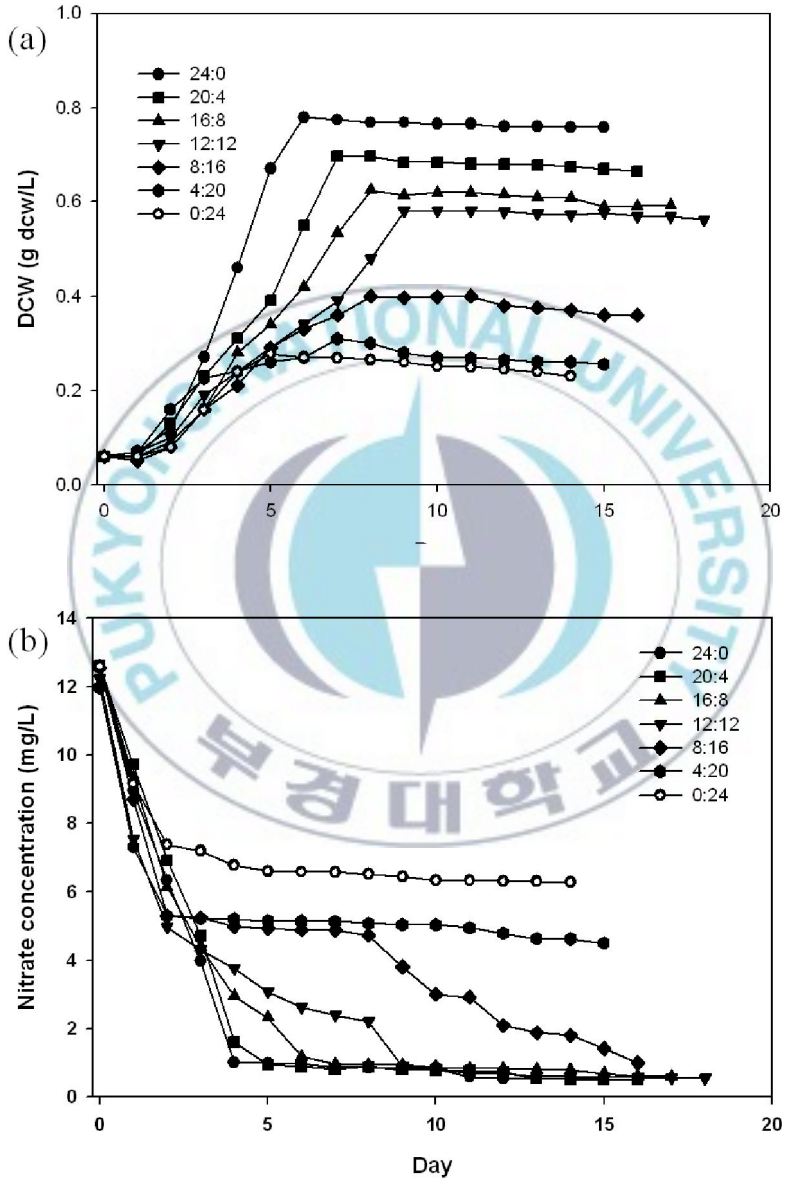
(a)
DCW (g dcw/L)
24:0
20:4
16:8
12:12
8:16
4:20
0:24
(b)
Nitrate concentration (mg/L)
Day

Fig. 3. Changes of (a) growth and (b) nitrate concentration from *T. suecica* on various L:D cycles.

Table 2. Total biomass, specific growth rate, biomass productivity and oil content of *T. suecica* on various L:D cycles

L:D cycle	Biomass production (g/L, AVG±SD)	Specific growth rate (day^{-1}, AVG±SD)	Biomass productivity (mg/L/day, AVG±SD)	Oil content (% of dry cell weight, AVG±SD)
24:0	0.78 ± 0.05^{a}	0.48 ± 0.01^{a}	129.87 ± 9.42^{a}	7.17 ± 0.12^{bd}
20:4	0.70 ± 0.11^{ab}	0.41 ± 0.02^{b}	99.63 ± 16.16^{ab}	7.25 ± 0.07^{bc}
16:8	0.62 ± 0.09^{ab}	0.33 ± 0.02^{bd}	78.05 ± 12.37^{bc}	8.48 ± 0.48^{be}
12:12	0.58 ± 0.09^{abc}	0.26 ± 0.02^{cd}	64.56 ± 10.21^{cd}	9.48 ± 0.62^{ab}
8:16	0.40 ± 0.09^{c}	0.30 ± 0.03^{d}	50.00 ± 12.37^{cf}	11.03 ± 1.47^{a}
4:20	0.31 ± 0.07^{cd}	0.21 ± 0.03^{d}	44.29 ± 10.10^{def}	11.14 ± 1.58^{a}
0:24	0.28 ± 0.09^{cd}	0.38 ± 0.08^{bc}	55.56 ± 18.38^{ce}	6.36 ± 1.88^{cde}

Means in the same column with the same superscript letter are significantly different ($P<0.05$) by least significant difference (LSD) test.

2.2. Effect of light intensity

Various light intensities were used with the optimized condition of 24:0 L:D cycle on biomass production. Culture period of *T. suecica* to reach the stationary phase was 5 days with the light intensities of 108.9 and 133.1 μmol/m^2/s, however, other groups with low light intensities showed 6 days to reach the stationary phase. Nitrate was almost consumed by *T. suecica* of all group of experiment within 5 days after the inoculation as shown in Fig. 4(b).

Total biomass, specific growth rate, biomass productivity and oil content of *T. suecica* on various light intensities were shown in Table 2. *T. suecica* on the light intensity of 108.9 μmol/m^2/s showed high biomass production of 0.89 g/L and specific growth rate and biomass productivity of 0.67 /day and 178.00 mg/L/day, respectively. However, light intensity of 133.1 μmol/m^2/s did not increase the high biomass production comparing to the light intensity of 108.9 μmol/m^2/s, as photoinhibition on photosynthetic system would be occurred with the conditions of light intensity over 108.9 μmol/m^2/s (Imamoglu *et al*., 2007). As shown in Fig. 4 and Table 3, light intensity is essential energy source to enhance the biomass production (Kaixian and Borowitzka, 1993).

High oil content of *T. suecica* was shown on high light intensity of 133.1 μmol/m^2/s as shown in Table 3. It indicates that the conditions of long light cycle and high light intensity on *T. suecica* could accumulate high oil content (Kaixian and Borowitzka, 1993).

Therefore, optimized light intensity of 108.9 μmol/m^2/s could be used for increasing biomass production in further study.

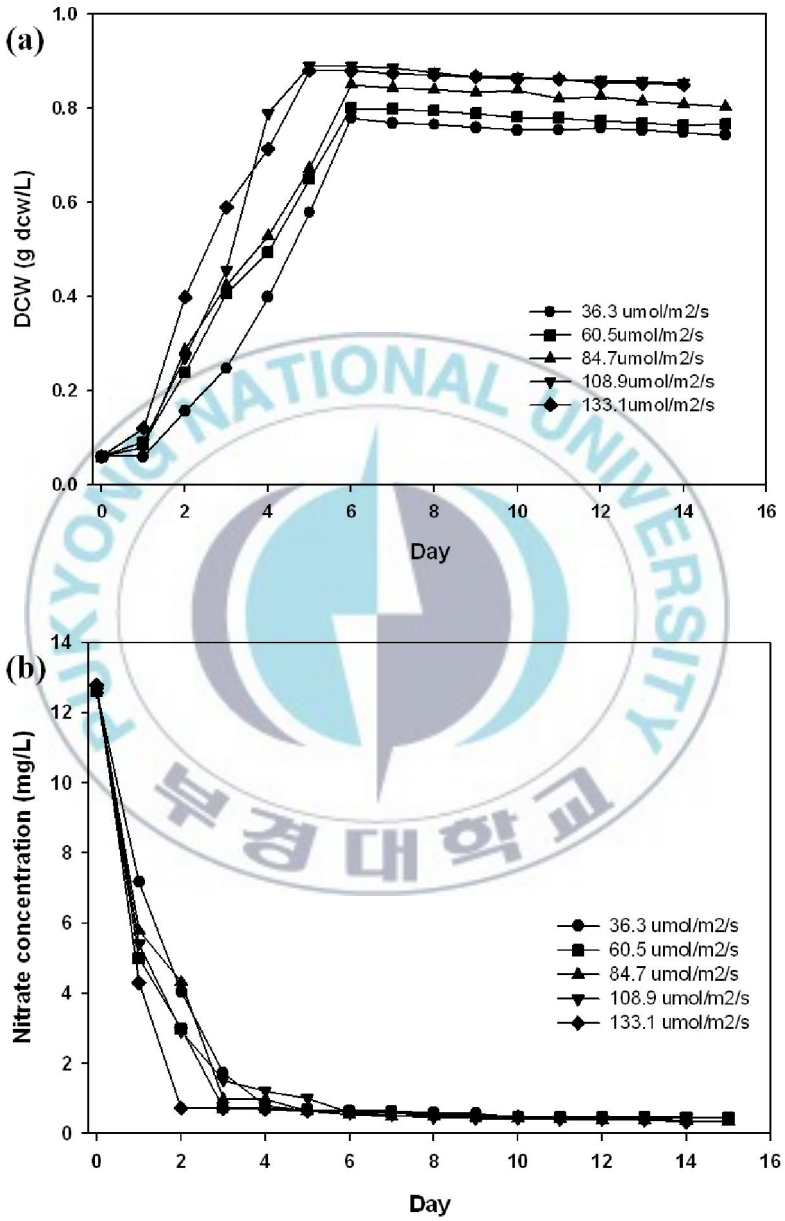

Fig. 4. Changes of (a) growth and (b) nitrate concentration from *T. suecica* on

various light intensities.

Table 3. Total biomass, specific growth rate, biomass productivity and oil content of *T. suecica* on various light intensities

μmol/m^2/s	Biomass production (g/L, AVG±SD)	Specific growth rate (day^{-1}, AVG±SD)	Biomass productivity (mg/L/day, AVG±SD)	Oil content (% of dry cell weight, AVG±SD)
36.3	0.78 ± 0.11^{e}	0.51 ± 0.02^{b}	129.87 ± 18.85^{b}	7.83 ± 0.31^{bc}
60.5	0.80 ± 0.05^{d}	0.52 ± 0.01^{b}	133.33 ± 9.42^{b}	10.23 ± 1.15^{ac}
84.7	0.85 ± 0.05^{c}	0.53 ± 0.01^{b}	141.67 ± 10.28^{b}	10.25 ± 0.94^{ab}
108.9	0.89 ± 0.07^{a}	0.67 ± 0.02^{a}	178.00 ± 14.14^{a}	10.97 ± 1.40^{a}
133.1	0.88 ± 0.05^{b}	0.67 ± 0.02^{a}	176.00 ± 11.31^{a}	12.22 ± 1.15^{a}

Means in the same column with the same superscript letter are significantly different ($P<0.05$) by least significant difference (LSD) test.

2.3. Effect of nitrate concentration

Various nitrate concentrations were used with the optimized conditions of 24:0 L:D cycle and light intensity of 108.9 μmol/m^2/s on the previous study for the production of high biomass. To optimize nitrate concentration for the growth, various nitrate concentrations of N-free, 6.18, 12.37, 18.55 and 24.74 mg/L were applied to the culture as shown in Fig. 5. *T. suecica* with nitrate concentrations of 12.37, 18.55 and 24.74 mg/L reached to the stationary phase on 5th day as shown in Fig. 5. Nitrate was consumed early phase of the culture by *T. suecica* at the condition with low nitrate concentrations as shown in Fig. 5(b). As shown in Table 4, high biomass production of 1.07 and 1.00 g/L of *T. suecica* was observed with the nitrate concentration of 18.55 and 24.74 mg/L similar to result with Hsieh *et al.* (2009), Kaixian and Borowitzka (1993). However, nitrate concentration of 24.74 mg/L did not produce higher biomass comparing to that of 18.55 mg/L. *T. suecica* showed the biomass production of 0.36 g/L with N-free. It would be due to stored the nitrate in the cell body during *T. suecica* was cultured in pre-cultivation (Toron *et al*., 2002).

Comparing the condition of N-free to the nitrate added conditions for the culture, high oil content of 11.60% of dry cell weight was observed with the N-free condition which was significant differences with 18.55 and 24.75 mg/L of nitrate concentration (P<0.05). Consequently, the condition of N-free was considered to be appropriate to accumulate oil (Hsieh *et al*., 2009). The results imply that various nitrate concentrations influence culture period, biomass production and oil content (Hsieh *et*

al., 2009)

Therefore, nitrate concentration of 18.55 mg/L was applied to the culture for high biomass production in further study.


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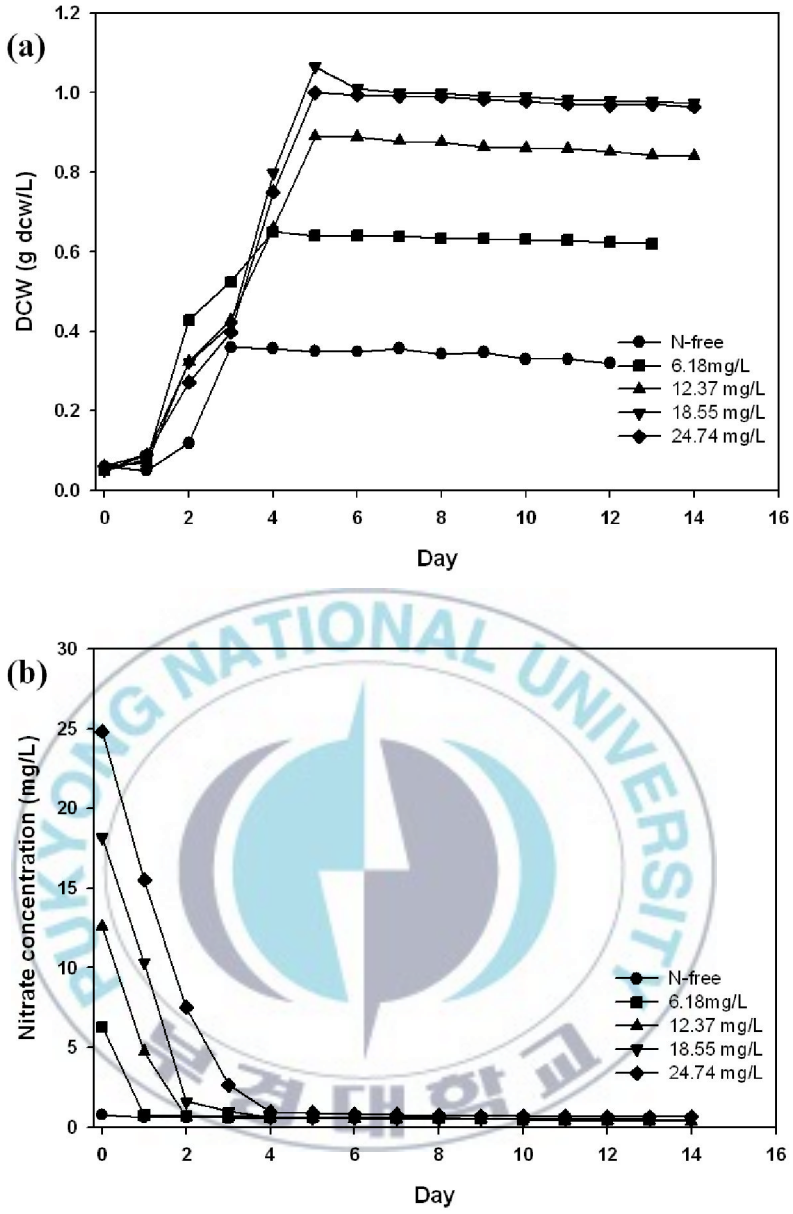

Fig. 5. Changes of (a) growth and (b) nitrate concentration from *T. suecica* on various nitrate concentrations.

Table 4. Total biomass, specific growth rate, biomass productivity and oil content of *T. suecica* on various nitrate concentrations

mg/L	Biomass production (g/L, AVG±SD)	Specific growth rate (day^{-1}, AVG±SD)	Biomass productivity (mg/L/day, AVG±SD)	Oil content (% of dry cell weight, AVG±SD)
N-free	0.36 ± 0.11^{d}	0.89 ± 0.15^{a}	120.00 ± 37.71^{d}	11.60 ± 1.13^{a}
6.18	0.72 ± 0.04^{c}	0.67 ± 0.02^{ab}	143.84 ± 12.37^{bcd}	11.27 ± 0.90^{ab}
12.37	0.89 ± 0.07^{b}	0.54 ± 0.02^{bcd}	148.22 ± 14.14^{ac}	10.71 ± 0.97^{ac}
18.55	1.07 ± 0.04^{a}	0.76 ± 0.03^{ac}	213.00 ± 8.48^{a}	11.25 ± 0.35^{bc}
24.74	1.00 ± 0.02^{ab}	0.70 ± 0.02^{ad}	200.00 ± 5.65^{ab}	8.23 ± 0.53^{c}

Means in the same column with the same superscript letter are significantly different ($P<0.05$) by least significant difference (LSD) test.

2.4. Effect of aeration rate

As shown in Fig. 6, the cell growth and nitrate concentrations of *T. suecica* with various aeration rates were observed. Culture periods of *T. suecica* on aeration rates of 2.5 and 5.0 L/min were same to reach the stationary phase as shown in Fig. 6(a). *T. suecica* with low aeration rates of 1.25, 0.61 and 0 L/min showed long culture period over 10 days to reach the stationary phase. Most nitrates were consumed within 7 days after the inoculation except for group without aeration.

Total biomass, specific growth rate, biomass productivity and oil content of *T. suecica* with various aeration rates were listed in Table 5. High biomass production of 1.04 and 1.05 g/L from *T. suecica* was observed on aeration rates of 2.5 and 5.0 L/min. However, aeration rates of 2.5 and 5.0 L/min showed significant difference for biomass production as well as specific growth rate and biomass productivity ($P<0.05$).

High oil content of *T. suecica* was observed with the aeration rate of 2.5 L/min as shown in Table 5. However, oil content did not increase over the aeration rate of 2.5 L/min. In the results, aeration rate of 2.5 L/min was chosen to increase biomass production of *T. suecica*.

From the studies of L:D cycle, light intensity, nitrate concentration and aeration rate, the biomass production of *T. suecica* could be enhanced from 0.58 g/L to 1.07 g/L and culture period was reduced by the optimized all growth conditions from 9th day to 5th day to reach the stationary phase.

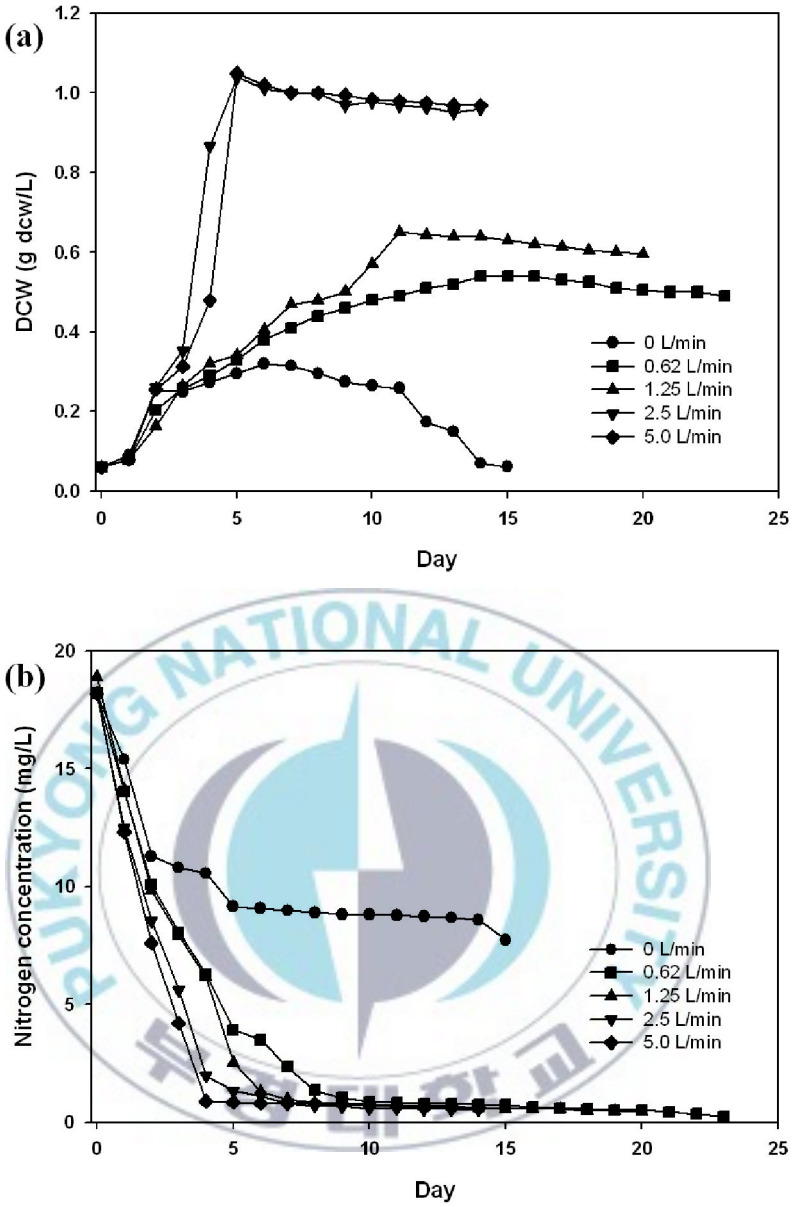

Fig. 6. Changes of (a) growth and (b) nitrate concentration from *T. suecica* on various aeration rates.

Table 5. Total biomass, specific growth rate, biomass productivity and oil content of *T. suecica* on various aeration rates

L/min	Biomass production (g/L, AVG±SD)	Specific growth rate (day^{-1}, AVG±SD)	Biomass productivity (mg/L/day, AVG±SD)	Oil content (% of dry cell weight, AVG±SD)
0	0.32 ± 0.11^{c}	0.33 ± 0.07^{b}	53.33 ± 18.85^{b}	2.72 ± 0.19^{c}
0.61	0.54 ± 0.02^{b}	0.17 ± 0.04^{c}	38.57 ± 2.02^{b}	5.96 ± 1.18^{b}
1.25	0.65 ± 0.06^{b}	0.24 ± 0.01^{bc}	59.09 ± 5.78^{b}	6.38 ± 0.43^{b}
2.5	1.04 ± 0.02^{a}	0.71 ± 0.04^{a}	207.80 ± 5.65^{a}	11.04 ± 1.38^{a}
5.0	1.05 ± 0.04^{a}	0.72 ± 0.01^{a}	209.86 ± 8.48^{a}	10.85 ± 0.77^{a}

Means in the same column with the same superscript letter are significantly different ($P<0.05$) by least significant difference (LSD) test.

2.5. Two-stage culture for increasing oil content

T. suecica grown until the stationary phase by optimized growth culture conditions

was reinoculated with various nitrate concentrations of N-free, 4.18, 9.27 and 18.55 mg/L.

Su *et al*. (2010) showed the results of oil and fatty acid content increased by nitrate limitation condition at 2-stage culture. In this study, oil content of *T. suecica* was increased to 17.28% of dry cell weight on 4th day in the N-free group that showed similar tendency to previous study (Widjaja *et al*., 2009; Su *et al*., 2010). It considers that the conditions of nitrate depletion increased oil content of *T. suecica* and the other groups of nitrate concentrations did not increase in oil content for 2-stage culture. As oil content of *T. suecica* was increased, the contents of five total fatty acids were also increased to 720.21 mg/g oil on 4th day in N-free group as shown in Fig. 7(d). C16 and C18 series fatty acids were accumulated by increasing oil content. In contrast, nitrate concentrations of 4.18, 9.27 and 18.55 mg/L decreased oil and five fatty acid total fatty acid contents as shown in Fig. 7(a), (b) and (c).

In the results of 2-stage culture, oil content of *T. suecica* was increased from 7.55% of dry cell weight to 17.28% of dry cell weight, and the contents of five total fatty acids were increased from 540.21 mg/g oil to 720.54 mg/g oil.

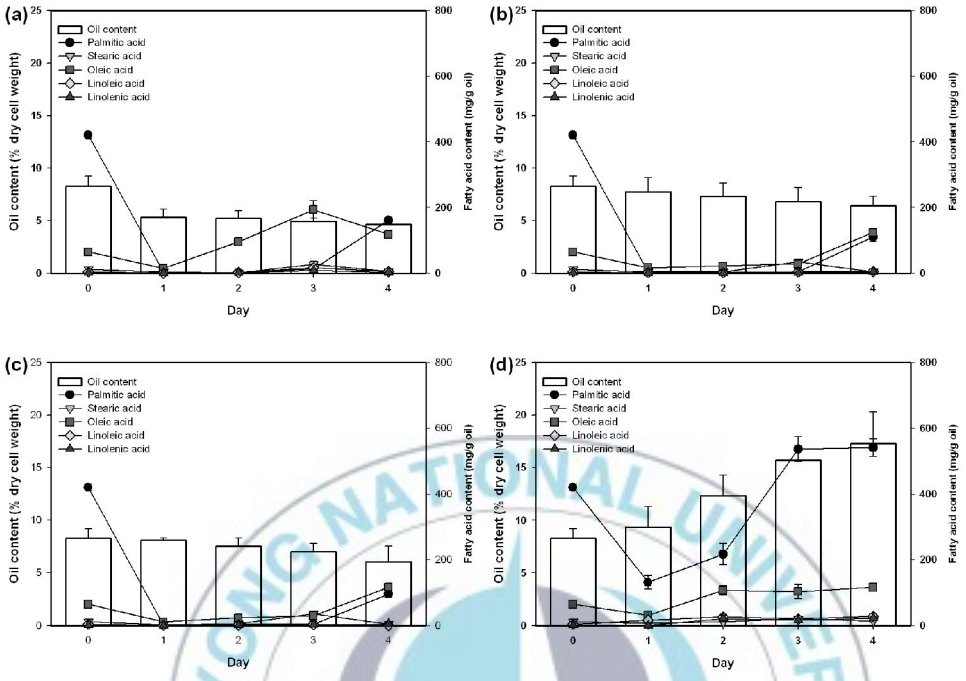

Fig. 7. Results of 2-stage culture for oil accumulation on *T. suecica*. Nitrate concentrations were (a) 18.55 mg/L, (b) 9.23 mg/L, (c) 4.61 mg/L and (d) N-free.

Ⅳ. CONCLUSION

Five marine microalgae showed distinct characteristics of growth, oil and fatty acids contents under culture conditions of 12:12 L:D cycle with 36.3 μmol/m^2/s and 20±1℃. *T. suecica* showed rapid growth and remarkable nutrient uptake rate for the biomass production of 0.58 g/L. *I. galbana* produced high biomass of 0.57 g/L next to *T. suecica*, however, culture period to reach the stationary phase was 23 days which is longer than 9 days of *T. suecica*. *P. tricornutum* and *C. calcitrans* showed low biomass production of 0.26 g/L and 0.47 g/L, however, oil contents of 25.13% and 16.25% of dry cell weight were obtained, respectively. *N. oculata* showed the longest culture period and low oil content of 8.23% of dry cell weight.

Even though oil content of *T. suecica* was low, total quantity of C16 and C18 fatty acid contents reached to the highest value of 561.22 mg/g oil. *I. galbana* also showed high oil and total C16 and C18 fatty acids contents of 23.15% of dry cell weight, and 472.66 mg/g oil, respectively. Therefore, *T. suecica* and *I. galbana* can be suitable candidates as oil forming microalgae for the biodiesel production.

To optimize biomass production of *T. suecica*, various L:D cycles, light intensities, nitrate concentrations and aeration rates were applied to the culture. From the results of various environmental factors, *T. suecica* showed high biomass production of 1.04 g/L from optimized growth conditions of 24:0 L:D cycle, 145.2 μmol/m^2/s, 18.55 mg/L and 2.5 L/min. In addition, the optimized growth conditions reduced the culture period of 5 days to reach the stationary phase.

Two-stage culture was carried out for the increment of oil content with various nitrate concentrations with *T. suecica*. Nitrate concentration changed oil content and five total fatty acid content of *T. suecica* grown from the optimized growth condition

until the stationary phase by nitrate stress. The condition of N-free led to high oil content and five total fatty acid content of 17.28% of dry cell weight and 720.54 mg/g oil at day-4 after the reinoculation on *T. suecica* as second stage culture. However, the oil content and five total fatty acid content of *T. suecica* with the conditions of 24:0 L:D cycle, light intensity of 108.9 μmol/m^2/s, nitrate concentration of 18.55 mg/L and aeration rate of 2.5 L/min were decreased due to the addition of nitrate.

In the results of this study, the biomass production and oil content of *T. suecica* was enhanced to 1.04 g/L and 17.28% of dry cell weight by the optimized growth conditions and 2-stage culture with controlling nitrate, respectively.

V. ACKNOWLEDGMENT

학부 3학년 때에 생물고분자공학 실험실에 들어오면서부터 현재까지 끊임없는 지도를 해주신 저의 지도교수님이신 김성구 교수님께 깊은 감사를 드립니다. 그리고 항상 실험실원들을 걱정해 주시며 챙겨주셨던 사모님께도 감사의 말씀을 드립니다.

학부생활과 대학원 생활을 하면서 많은 도움을 주신 김중균 교수님, 공인수 교수님, 이형호 교수님, 박남규 교수님, 홍용기 교수님 그리고 바이오 에너지에 대해서 많은 가르침을 주셨던 정귀택 교수님께 깊이 감사드립니다.

해양 미세조류 실험을 하면서 이것저것 세세한 부분까지 신경써주고, 항상 웃음으로 대해주셨던 수근선배에게도 깊이 감사드립니다. 그리고 실험실에서 항상 즐겁게 생활을 할 수 있게 웃음을 주었던 승준이, 미란이, 지숙이에게도 고마움을 전하며, S 클럽의 일원이면서 웃음을 잃지 않게 해주었던 유경이와 혜진이에게도 고마움을 전합니다. 편입을 하여 별 어려움 없이 잘 생활할 수 있도록 많은 도움을 준 채훈이 형, 학부생과 대학원생 수업에서 많은 도움을 주신 고혜진 박사님에게도 고마움을 전합니다.

대학원을 들어오며 2년간 서로 의지하며 지낼 수 있었던 아람이, 유리, 혜림이, 경은이에게도 끝까지 잘 마무리한 것에 대해 고마움과 함께 축하의 말을 전합니다.

2년 동안 대학원 생활을 하며 말없이 뒤에서 지켜봐주시며, 마음속으로 고생만 하신 저의 사랑하는 아버지와 어머니께 깊이 감사드립니다. 해외에서 일하느라 고생하시는 매형과 저를 자신보다도 더 걱정해 주는 우리 누나 그리고 작년에 세상에 태어나 갖은 애교와 웃음을 선사하는 조카 재민이에게도 고마움을 전합니다.

항상 모임에 잘 못나가고 했지만 무한한 믿음으로 뒤에서 친구라는 이름으로 저를 지지해 주던 개똘의 경덕이 형, 성욱이 형, 동준이 형, 상엽이, 무찬이, 우곤이, 문호 그리고 홍근이에게도 고마움을 전하며,

오랜만에 만나도 서먹함을 느낄 수 없으며 따뜻한 우정을 느낄 수 있었던 고등학교 친구인 상준이, 도훈이, 현재, 얼마 전 결혼한 영민이, 인석이, 곧 결혼할 진재 그리고 곧 회계사가 될 동준이에게도 고마움을 전합니다. 그리고 뉴질랜드에서 사업을 하며 좋은 성과를 거두고 있는 선학이, 전화하면 항상 즐겁게 받아주며, 재미를 주던 미정이에게도 고마움을 전합니다.

이외에도 제가 대학원 생활을 하며 많은 도움을 주며 지지를 해주신 분들께도 깊은 감사의 말씀을 드리며 이 논문을 바칩니다.

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