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Abstract	In recent years, oil-rich microalgae have been recognized as a renewable energy for the future. Lipid production of microalgae is said to be influenced by environmental factors such as light, temperature and nutrients. Above all, light is known as a principal factor that affects overall biomass productivity. When microalgae are grown under various light conditions, they exhibit remarkable changes in their chemical composition, pigment content and photosynthetic activities. It is also known that many microalgae have the ability to produce substantial amounts of triacylglycerol (TAG), the main neutral lipid said to be useful for bio fuel source, when put under stressful environment. In this thesis, we aimed to clarify the relationship between light and lipid production in oil-rich microalgae using <i>Pseudochlorisystis ellipsoidea</i> and <i>Nannochloropsis oceanica</i> . In the first half, we observed <i>P.ellipsoidea</i> with the addition of photosynthesis inhibitor DCMU and discussed the influence of photosynthesis on lipid production. In the second half, we studied the change of useful lipids under different light conditions with <i>N. oceanica</i> . In this digest we cover part of the later half, and clarify how the lipids of <i>N. oceanica</i> change under different light conditions. Using liquid chromatography-time of flight hybrid mass spectrometer (LC-TOFMS), we measured samples under five different light conditions (51, 94, 213, 465, 538 μ mol/m ² /sec) and analyzed the data using Master-Hands. We discussed how different light conditions affect useful lipids, focusing especially on TAG and eicosapentaenoic acid (EPA).
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The effect of light on lipid production of oil-rich microalgae

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ABSTRACT

In recent years, oil-rich microalgae have been recognized as a renewable energy for the future. Lipid production of microalgae is said to be influenced by environmental factors such as light, temperature and nutrients. Above all, light is known as a principal factor that affects overall biomass productivity. When microalgae are grown under various light conditions, they exhibit remarkable changes in their chemical composition, pigment content and photosynthetic activities. It is also known that many microalgae have the ability to produce substantial amounts of triacylglycerol (TAG), the main neutral lipid said to be useful for bio fuel source, when put under stressful environment. In this thesis, we aimed to clarify the relationship between light and lipid production in oil-rich microalgae using *Pseudochlorisystis ellipsoidea* and *Nannochloropsis oceanica*. In the first half, we observed *P. ellipsoidea* with the addition of photosynthesis inhibitor DCMU and discussed the influence of photosynthesis on lipid production. In the second half, we studied the change of useful lipids under different light conditions with *N. oceanica*. In this digest we cover part of the later half, and clarify how the lipids of *N. oceanica* change under different light conditions. Using liquid chromatography-time of flight hybrid mass spectrometer (LC-TOFMS), we measured samples under five different light conditions (51, 94, 213, 465, 538 μ mol/m²/sec) and analyzed the data using Master-Hands. We discussed how different light conditions affect useful lipids, focusing especially on TAG and eicosapentaenoic acid (EPA).

Keywords: *Nannochloropsis oceanica*, Lipid accumulation, Triacylglycerol (TAG), Eicosapentaenoic acid (EPA), LC-TOFMS

1. Introduction

Algae are coming to front for their ability to make oil as a renewable energy for the future. Microalgae have been said to be a sustainable energy source since they absorb CO₂ by photosynthesis and microalgal oil does not increase CO₂ in the atmosphere. Furthermore, unlike the bio-fuels made from food, they can be cultivated throughout the year, and also is not competitive with food crops, which is an important point since the world is said to face the problem of maintaining a secure food supply. Since these advantages, microalgae are recently noticed with increased attention (Yusuf, 2007). However, with microalgae, it is not known about their specific pathways of photosynthesis and lipid accumulation under metabolite levels (Hu *et al.*, 2008).

Species of the genus *Nannochloropsis*, which is a marine micro algae, is known to accumulate relatively large amount of lipids, and it has the ability to produce more than 68% per dry weight (Yusuf, 2007). Especially, *Nannochloropsis oceanica* have been recently recognized as high lipid-content microalgae. Since microalgae with high lipid contents have high energy values, it has been considered as a potential source of renewable energy (Renaud *et al.*, 1991; Scragg *et al.*, 2002; Su *et al.*, 2007, 2008; Takagi *et al.*, 2006). There are many benefits on using marine algae, such as *Nannochloropsis oceanica*, as our new energy source. When thinking about putting it into practical use, unlike other green algae which they need to be put under nutrition deficiency conditions in order to accumulate oil, *Nannochloropsis oceanica* can accumulate oil under natural cultivation conditions. Moreover, the biggest benefit of marine micro algae is that it can be cultivated using sea water. Considering the fact that the world will need to face severe problems with water resource depletion, this can be a very important benefit for us humans to survive.

Environmental factors such as light, temperature, and nutrients are known to affect the lipid of algae. (Harwood, 1984; Thompson, 1996). Also, above all, light is a principal factor that affects overall biomass productivity and therefore light is an essential factor when growing algae as a new source of energy. When micro algae are grown under various light conditions, they exhibit remarkable changes in their chemical composition, pigment content, and photosynthetic activities (Falkowski and Owens, 1980; Post *et al.*, 1985; Richardson *et al.*, 1983; Sukenik *et al.*, 1987). Generally, it is known that the formation of polar lipids, especially the membrane polar lipids such as phospholipids and glycolipids, are induced under light limitation conditions (low light intensity). Galactolipids, which is a lipid said to be contained mainly in chloroplast membranes, are classified as glycolipids. On the contrary, under light inhibition conditions (high light intensity) the amount of neutral storage lipids, mainly triacylglycerol (TAG), increases while the total polar lipid content decreases (Brown *et al.*, 1996; Khotimchenko and Yakovleva, 2005; Napolitano, 1994; Orcutt and Patterson, 1974; Spoehr and Milner, 1949; Sukenik *et al.*, 1989).

In this study, we aim to clarify how light affects the production of useful lipids especially TAG and EPA in *Nannochloropsis oceanica*. Triacylglycerol (TAG) is known as a useful lipid for bio-fuels. Unlike the glycerolipids found in membranes, TAG does not perform a structural role but instead serve primarily as a storage form of carbon and energy. It is said that this lipid is most efficient when converting it to biodiesel, but little is known about the regulation of TAG formation at the molecular and cellular level (Hu *et al.*, 2008). Eicosapentaenoic acid (EPA, FA20:5) is an important polyunsaturated fatty acid (PUFA). It has numerous neutraceutical and pharmaceutical applications (Shahidi and Wanasundara, 1998). EPA is important in the treatment of atherosclerosis, cancer, rheumatoid arthritis, psoriasis and diseases of old age, such as Alzheimer's and age-related macular degeneration (Drevon *et al.*, 1993; Simopoulos 1999). In order to put these lipids to practical use, evaluation of each light condition is needed. Therefore, this study will also focus to consider under which condition *Nannochloropsis oceanica* accumulates useful lipids the most.

2. Materials and methods

Nannochloropsis oceanica CS-246 was obtained from the Australian National Algae Culture Collection at the Commonwealth Scientific and Industrial Research Organization (CSIRO, Australia). It was grown in f/2 medium (Guillard and Ryther, 1962) (Table 1) under 25°C, with 32‰ salinity. The cultures were grown in flat flasks (working volume: 400ml) with a light to dark cycle of 14:10 hour at nine different light intensities shown in figure 2. For this study, five light intensities which had a division rate over one were used. The light conditions are grouped as light limitation conditions (51, 94μmol/m²/sec), light saturation conditions (213, 465μmol/m²/sec), and light inhibition condition (538μmol /m²/sec).

Cell density was measured by a particle counter, CDA-1000 (Sysmex, Japan). Approximately 1.0×10⁷ cells were collected from three individual cultures of the five different light conditions. These

cultures were centrifuged at 3000g for 5 min, and washed once with 50 mL of 0.5% w/v mannitol. The cells were centrifuged at 5000g for 2 min, and the pellet was resuspended in 510 μ L methanol, and 10 μ L of the suspension was used for peaks normalization. The rest of the suspension was stored at -80°C. Lipids were extracted as described by Bligh and Dyer (1959).

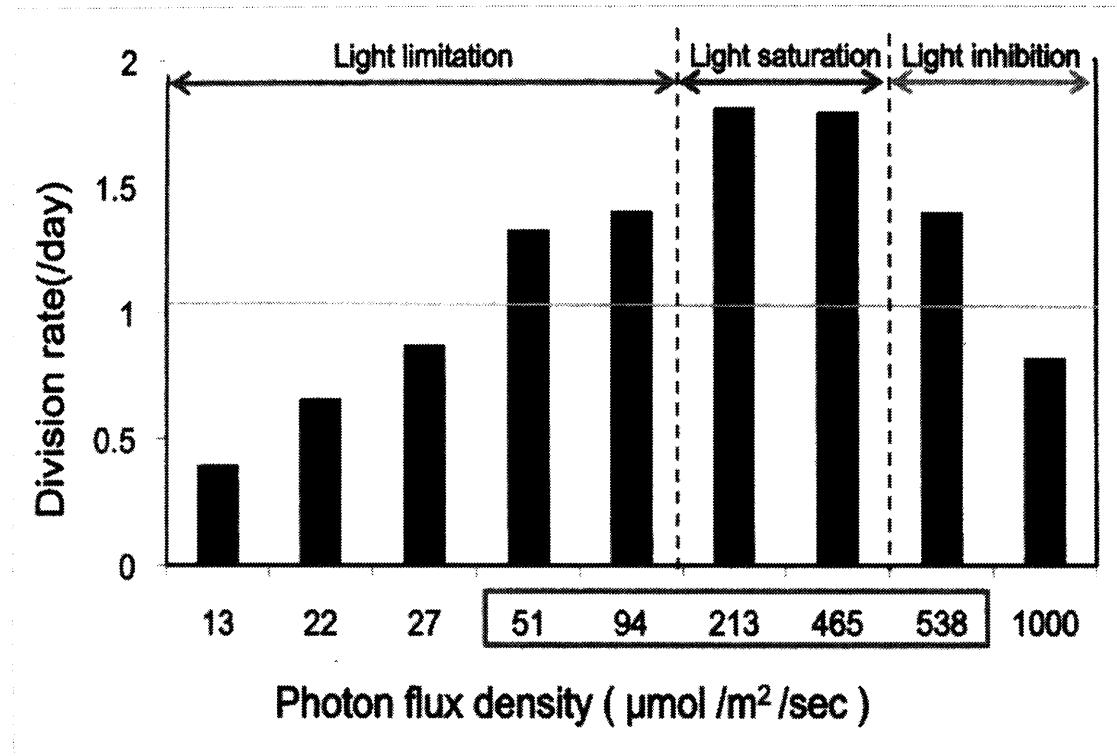


Figure 2 Division rate of *Nannochloropsis oceanica* under different light conditions

Division rate of *N.oceanica* under nine different light intensities, light limitation condition (13, 22, 27, 51, 94 $\mu\text{mol}/\text{m}^2/\text{sec}$), light saturation condition (213, 465 $\mu\text{mol}/\text{m}^2/\text{sec}$) and light inhibition condition (538, 1000 $\mu\text{mol}/\text{m}^2/\text{sec}$). Five light intensities, which had a division rate over one, shown in red (51, 94, 213, 465, 538 $\mu\text{mol}/\text{m}^2/\text{sec}$) were used in this study.

The samples were homogenized with approximately 2 g of ø0.5 mm zirconia beads using Micro Smash MS-100R (Tomy Seiko, Japan) at 4000 rpm for twice of 90 sec. These samples were mixed with 500 μ L chloroform containing 10 μ M 1,2-dihexanoyl(d22)-sn-glycero-3-phosphocholine (PC6:0 d22 : Avanti Polar Lipids, USA) and 10 μ M 1,2-dimyristoyl(d54)-sn-glycero-3-phosphocholine (PC14:0 d54 : Avanti Polar Lipids, USA) as internal standards, and 450 μ L water was added to each samples. The mixtures were centrifuged at 2000 g for 15 min. The organic phase was reserved in a glass vial and stored at -80°C until measurement by LC-TOFMS. Lipids were measured by a LC-TOFMS system, Agilent 1200 HPLC (Agilent Technologies, USA) and Agilent 6210 Time of Flight mass spectrometer (Agilent Technologies, USA). The measuring condition used the same conditions described by Ito (2012). Peak extraction was carried out with Master-Hands-2.13.0.8 software (Sugimoto *et al.*, 2010). The alignment of the peaks was performed according to the *m/z* value and normalized retention time. TAG was relatively quantified using the internal standard, and normalized by cell numbers used for lipid preparation. Identification of peaks was performed based on the *m/z* value.

Table 1. f/2 culture medium composition per liter

NaNO ₃	75.0	g
NaH ₂ PO ₄ ·H ₂ O	5.0	g
Na ₂ SiO ₃ ·9H ₂ O	3.0	g
Trace metals solution	1.0	mL
Vitamins Solution	0.5	mL

3. Results

As a result, we were able to quantitatively analyze triacylglycerol (TAG) 48:0 and eicosapentaenoic acid (EPA). The amount of TAG48:0 (48 being the number of carbon chain length and

0 being the number of saturation) is shown in figure 3. TAG48:0 increased as the light intensity became higher and it was most accumulated under light inhibition condition (538 μ mol /m²/sec).

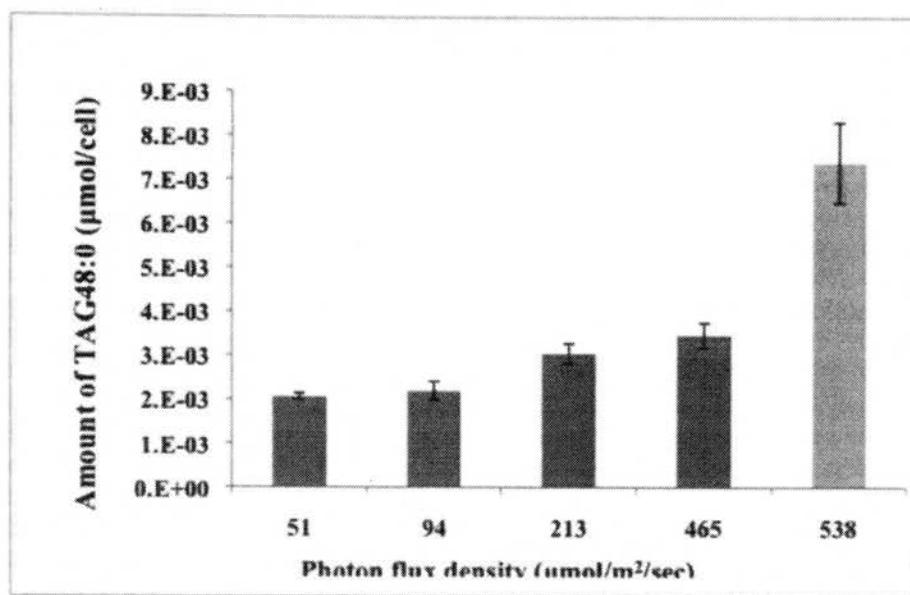


Figure 3. Amount of triacylglycerol 48:0 under different light intensity

Amount of TAG48:0 per cell under light limitation condition (51, 94 μ mol/m²/sec), light saturation condition (213, 465 μ mol/m²/sec) and light inhibition condition (538 μ mol/m²/sec)

Amount of eicosapentaenoic acid (EPA) is shown in figure 4. There was no significant difference between light limitation condition (51, 94 μ mol/m²/sec) and light saturation condition (213, 465 μ mol/m²/sec). However, looking at the light inhibition condition (538 μ mol/m²/sec), the amount of EPA increased significantly.

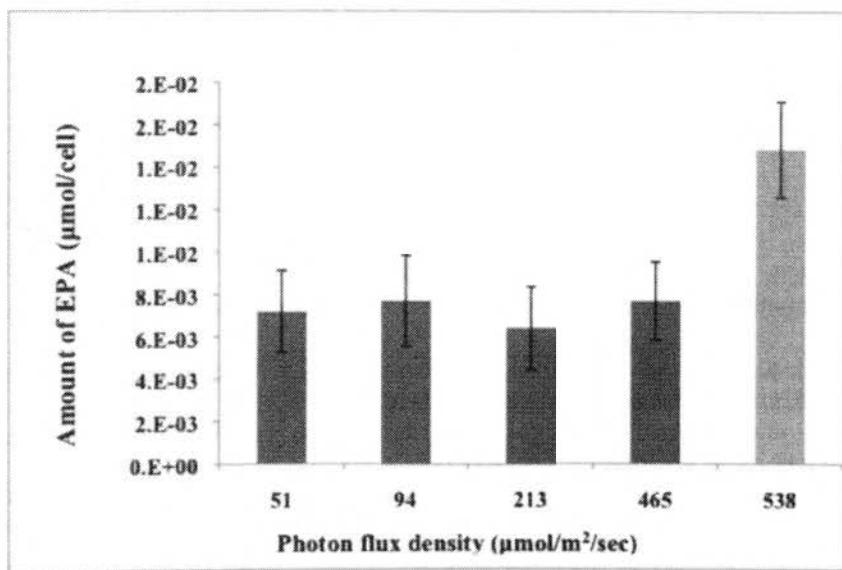


Figure 4. Amount of eicosapentaenoic acid (EPA) under different light intensity

Concentration of eicosapentaenoic acid (EPA, FA20:5) per cell under light limitation condition (51, 94 μ mol/m²/sec), light saturation condition (213, 465 μ mol/m²/sec) and light inhibition condition (538 μ mol/m²/sec)

4. Discussion

The amount of TAG48:0 increased as the light intensity became higher and *N.oceanica* accumulated TAG48:0 the most under light inhibition condition. As described in the background, it is generally known that neutral storage lipids, mainly TAG, increases under light inhibition conditions. This is a phenomenon known in many of the other algae such as *Thalassiosira pseudonana* (Brown *et al.*, 1996), *Cladophora* sp. (Napolitano, 1994), and *Chlorella* (Spoehr and Milner, 1949). Therefore, it can be said that *N.oceanica* is not an exception. It can be said that *N.oceanica* accumulates TAG under high light intensity as a stress response.

There was no significant difference in the amount of EPA between light limitation condition and light saturation condition. A significant increase of EPA was seen under light inhibition condition (538 μ mol /m²/sec). This is an interesting result since there is a previous study which shows an opposite result saying that an exponential reduction of EPA was seen in response to high light intensity (Sukenik, 1989). However the amount of EPA in figure 4 shows only the free EPA in the cells and the previous study showed the amount of EPA of total lipids. EPA is known to be a fatty acid that is mainly associated with galactolipids which are diagnostic membrane lipids in chloroplast membranes. Therefore, it can be presumed that under low light conditions, EPA exists in the cells as a component of galactolipids thus it has to activate photosynthesis to its best ability. Moreover, as the light intensity gets higher and *N.oceanica* is put under light inhibition condition, it is predictable that galactolipids are degraded. In consequence, the amount of free EPA increased under light inhibition condition. This is an agreeable result since the efficiency of photosynthesis is significantly reduced when algae are exposed to high light levels.

In conclusion, the best way to make *N.oceanica* accumulate a large amount of TAG, it should be cultivated by two stages. First, cultivating it under light saturation condition and making the biomass productivity maximize, move on to the next stage under light inhibition condition to accumulate TAG to their best. For EPA, a best condition cannot be suggested from these results hence the actual total amount of EPA is not measured.

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