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Article

Growth and Nutritional Composition of Eustigmatophyceae *Monodus subterraneus* and *Nannochloropsis oceanica* in Autotrophic and Mixotrophic Culture

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Abstract : Eicosapentaenoic acid (EPA) produced from marine organisms is widely used in nutraceuticals. *Monodus subterraneus* and *Nannochloropsis oceanica*, which are representative freshwater and marine Eustigmatophyceae, respectively, are known to have a high content of protein and lipid, particularly, EPA. In this study, to compare the growth and nutritional composition of *M. subterraneus* and *N. oceanica*, they were cultured in autotrophic and mixotrophic conditions with JM and f/2 medium, respectively, at 25°C. In addition, 80 μmol photons m^{-2} s^{-1} with 24-hour and 12-hour light was provided, with the addition of 2% glucose to the medium for the mixotrophic culture. With regard to growth, *M. subterraneus* showed 10 times higher biomass in a mixotrophic culture than in an autotrophic one. However, no significant difference was observed for *N. oceanica* between the two culture methods. With respect to nutritional composition, *M. subterraneus* cultured autotrophically had a higher protein and lipid content, particularly EPA, than that cultured mixotrophically, but no significant difference was found in the two cultures of *N. oceanica.* Furthermore, *M. subterraneus* cultured autotrophically with continuous light showed higher nutritional composition, particularly EPA, than *N. oceanica.* In conclusion, the mass culture of freshwater *M. subterraneus* is much easier and more economical than marine *N. oceanica*. In addition, production of EPA will be economically improved if mixotrophic culturing of *M. subterraneus* is first conducted to maximize the biomass, and then secondary autotrophic culturing is performed.

Key words : autotrophic, EPA, mixotrophic, *Monodus subterraneus*, *Nannochloropsis oceanica*

1. Introduction

Polyunsaturated fatty acids (PUFA) have been used as a material in aquaculture feeds or in functional health supplement foods. Among them, n3 highly unsaturated fatty acids (HUFA), such as EPA (20:5n3, eicosapentaenoic acid) and DHA (22:6n3, docosahexanoic acid), are useful for the treatment of various illnesses (Swanson et al. 2012; Sears et al. 2013). HUFA are generally produced from marine pelagic fish, such as salmon and mackerel, and therefore the supply can vary according to the price and quality of the fish, while pollution caused by pesticides and heavy metals has become a problem (Yamada et al. 1989). In addition, the amount of fish caught globally is decreasing, whereas the demand for HUFA is increasing explosively (FAO 2013). Thus, mass production of HUFA from other marine organisms which can be substituted for fish is required (Cohen and Cohen 1991; Elbert 2011). In this regard, suitable microalgae for HUFA production, particularly for the production of EPA, needs to be explored for mass culture, to allow for the mass production of economical and stable HUFA (El et al. 2000).

Microalgae produce PUFA, but freshwater microalgae have been shown to produce less HUFA than marine microalgae (Bajpai and Bajpai 1993). Therefore, HUFA production efforts have been aimed at using marine microalgae, such as *Isochrysis galbana*, *Nannochloropsis*

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oceanica and *Phaeodactylum tricornutum* (Hemaiswarya et al. 2011; Priyadarshani et al. 2012; Arndt and Sommer 2014). Marine microalgae are generally difficult to culture compared to freshwater microalgae, and the culture location is limited to coastal areas. In addition, the culture density is relatively low. Thus, the unit cost of production of marine microalgae is higher compared to that of freshwater microalgae.

Nannochloropsis oceanica, a representative species of marine Eustigmatophyceae, has a high lipid content, and therefore has been widely cultured and examined for live food in aquaculture (Bruneel et al. 2013; Yin et al. 2013), bioenergy (Giorno et al. 2013; Su 2013) and functional medicine (Kuňo et al. 2013). *Monodus subterraneus* is a representative freshwater Eustigmatophyceae and has a higher content of both protein and lipid compared to *N. oceanica* (Jo 2014), including higher amounts of EPA than *Isochrysis* sp. (Řezanka et al. 2010; Borowitzka 2013). In addition, mass culturing of *M. subterraneus* is possible by using mixotrophic as well as autotrophic culture methods. Consequently, it has received attention as a species to obtain high biomass for EPA production (Lam 2000; Kim and Hur 2013). Therefore, in this study, the growth and nutritional composition of amino acids and fatty acids in *M. subterraneus* and *N. oceanica* were compared, between the autotrophic and mixotrophic culture methods. In addition, growth and nutritional composition of *M. subterraneus* depending on the changeover culture method were examined. Mixotrophic culture of *M. subterraneus* was first conducted to maximize the biomass and then secondary autotrophic culture was performed to obtain high nutrient.

2. Materials and Methods

The growth and nutritional composition of *M. subterraneus* and *N. oceanica* according to trophic culture method

Culture of algae

M. subterraneus (KMMCC-1348, 6.76 ± 0.61 μm (major axis), 3.66 ± 0.55 μm (minor axis)) and *N. oceanica* (KMMCC-359, diameter: 2.64 ± 0.41 μm), received from the Korea Marine Microalgae Culture Center (KMMCC), Pukyong National University, were cultured for this study. Jaworski's medium (JM) (Thompson et al. 1988) for *M. subterraneus* and f/2 medium (Guillard and Ryther 1962) for *N. oceanica* were used, and the two species were cultured at 25°C with continuous light of 80 μmol photons m^{-2} s^{-1} (Bae and Hur 2011a; Jo 2014).

Experiments for each culture type were classified into autotrophic cultures and mixotrophic cultures, with glucose used as the carbon source for mixotrophic cultures. Glucose was dissolved in distilled water to make a 50% solution, which was autoclaved. It was then added to sterilized media in a clean bench. 100 mL of media was transferred into a 250 mL flask, and 4 mL of glucose was added to each flask to make a medium concentration of 2% (Kim and Hur 2013). The same medium mentioned above was used for culturing, and standing culture was conducted in a low temperature incubator with an L:D cycle of 24:0 and 12:12 using 80 μmol photons m^{-2} s^{-1} at 25°C. All experimental groups were inoculated with 10% culture stock in the log phase of growth, and cultured for nine days, in triplicate.

Growth rate and dry weight

Cell density was measured in triplicate with a hemacytometer at the same time every day during the culture period to calculate the average daily specific growth rate (SGR) (Guillard 1973; $SGR = 3.322 \times \log(N_2/N_1)/(t_2-t_1)$, N_2: No. of cells at t_2, N_1: No. of cells at t_1). The dry weight of the cells was measured on the final day of culturing. The 1.2 μm glass microfiber filters (GF/C, Whatman) were dried at 60°C for two hours, and 50 mL of culture medium per sample was filtered, rinsed with the same volume of distilled water and dried at 60°C for an additional two hours. The average dry weight of the cells was measured by dividing total weight of biomass with total number of cells in triplicate (Kim and Hur 2013).

Analysis of amino acids and fatty acids

The microalgae used in the experiments were mass cultured on a 3 L scale using the same culture method described above. The cells in the log phase of growth were harvested by centrifugation (3,000 rpm, 25 min) on the 9th day of culturing, and then stored at −80°C until further analysis was performed. Crude proteins in samples were estimated by Kiehldahl method (Strickland and Parsons 1972). Crude lipids were determined by ether extraction (AOAC 1984).

For analysis of amino acids, 20 mg of sample infused with 15 mL of 6 N HCl was heated, sealed, and hydrolyzed at 110°C for 24 h. The sample was then filtered and dried to remove the HCl. Twenty-five mL of the sample was set with sodium dilution buffer (pH 2.2), and a portion of the sample was analyzed with the ninhydrin method using an amino acid analyzer (HSAAA, Hitachi L-8800, Japan). Conditions of the analysis were as follows: column size,

4 × 150 mm; absorbance wavelength, 570 nm and 440 nm; reagent flow rate, 0.25 mL/min; buffer flow rate, 0.45 mL/min; reactor temperature, 120°C; reactor size, 15 m; and analysis time, 65 min (Park et al. 2013).

Also quantities of amino acids in microalgae were conducted from a quantitative mixture of 16 free amino acid standards (Type H; WAKO Pure Chemical Industries. Ltd., Japan) (Kwon et al. 2013a).

For the analysis of fatty acids, lipids were determined as by Folch et al. (1957). Twenty milligrams of each sample was put in a 15 mL flask with 2 mL of 10% BF_3-methanol. Nitrogen was added to the sample, and it was heated at 85°C for 90 min to draw methyl ester (Morrison and Smith 1964; Budge 1999). A gas chromatograph (HP 6890N; Agilent, Santa Clara, CA, USA) equipped with an Auto Sampler (Agilent) was used for the analysis, with a w-wax column (30 m long, 0.25 mm I.d., 0.25 μm film thickness; Supelco, Bellefonte, PA, USA) for separation. Nitrogen was used as the carried gas, and the flow rate was set at 30 mL min^{-1}. The column temperature profile was as follows: temperature stood at 200°C for 3 min, increased from 200 to 230°C at 1°C min^{-1}, and then was held at 230°C for 25 min. The temperature of the injector was 250°C, and the flame ionization detector (FID) was held at 250°C. Fatty acid peaks were integrated using HP-6890. Gas chromatography software was utilized, and identification of fatty acid peaks and quantitation of fatty acids were made with reference to quantitative fatty acid methyl ester (FAME) standards (PUFA 37 component FAME Mix; Supelco, U.S.A.) (Kwon et al. 2013b).

Growth and nutritional composition of *M. subterraneus* according to changeover culture method

M. subterraneus at an initial density of 1×10^6 cells/mL was inoculated in 3 L of JM medium with 2% glucose, and standing culture was conducted in quadruplicate at 25°C with continuous light of 80 μmol photons m^{-2} s^{-1} in mixotrophic culture for seven days. The cells of *M. subterraneus* reaching the log phase (ca. 2×10^7 cells/mL) were harvested by centrifugation (3,000 rpm, 25 min), and all cells were then reinoculated into 3 L of JM medium for autotrophic culturing, and 3 L JM medium with 2% glucose for mixotrophic culturing. The same method mentioned above for autotrophic and mixotrophic culturing was used. The growth rate, dry weight, and composition of amino acids and fatty acids were measured after culturing for seven days in duplicate.

Statistical analysis

The results were analyzed by one-way ANOVA, and Duncan's multiple range test (Duncan 1955) was applied for the significance level ($P < 0.05$). The SPSS program version 17 (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses.

3. Results

Growth and nutritional composition of *M. subterraneus* and *N. oceanica* according to trophic culture method

Growth rate

The SGR of *M. subterraneus* and *N. oceanica* cultured

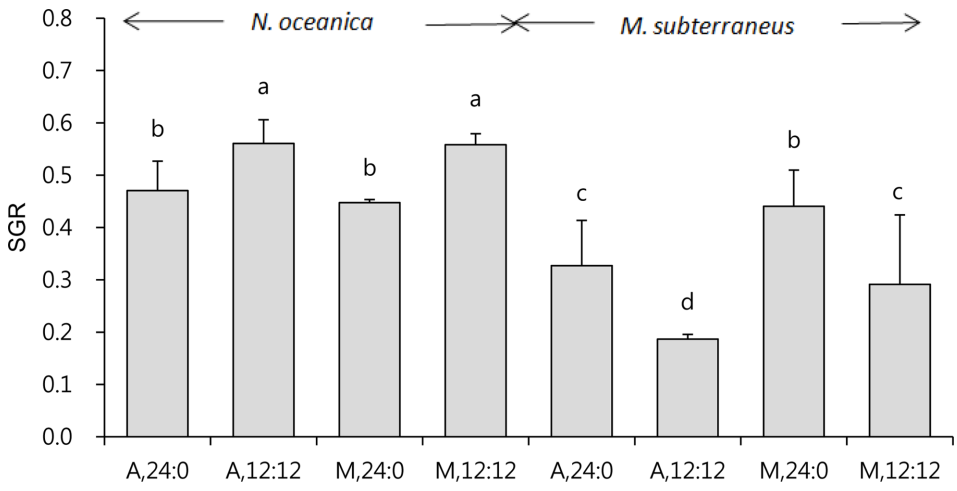

Fig. 1. Specific growth rate (SGR) of *Monodus subterraneus* and *Nannochloropsis oceanica* cultured under different trophic conditions (A: autotrophic, M: mixotrophic, 24:0 and 12:12 = L:D cycle). Different letters above the bars indicate significant differences ($P < 0.05$)

autotrophically and mixotrophically are shown in Fig. 1. The SGR of *M. subterraneus* with 24-hour of continuous light was significantly higher than with 12-hour of light in both autotrophic and mixotrophic cultures. In addition, the SGR of the mixotrophic culture was significantly higher than that of the autotrophic culture, regardless of the photoperiod ($P < 0.05$, $df = 47$, $F = 45.5$). However, no significant difference in the SGR was observed between the autotrophic and mixotrophic cultures of *N. oceanica,* while the SGR of the experimental group with 12-hours of light was higher than that with continuous light. Therefore, the opposite result was observed compared to *M. subterraneus*. The SGR of *M. subterraneus* was 0.441 (maximum density 1.564×10^7 cells/mL) at the highest rate, occurring in the experimental group of the mixotrophic culture with continuous light. The SGR of *N. oceanica* was 0.561 (maximum density 3.311×10^7 cells/mL) and 0.558 (maximum density 3.227×10^7 cells/mL) at the highest rates observed for autotrophic and mixotrophic cultures, respectively, with 12-hours of light. Generally, the growth rate according to cell number was significantly higher in *N. oceanica*, whose cell size is smaller than *M. subterraneus*.

Dry weight of biomass

The results of the dry weight of biomass per 50 mL of culture medium are shown in Fig. 2. The dry weight of *N. oceanica* was 107-129 μg regardless of the culture method or lighting cycle, and therefore no significant difference

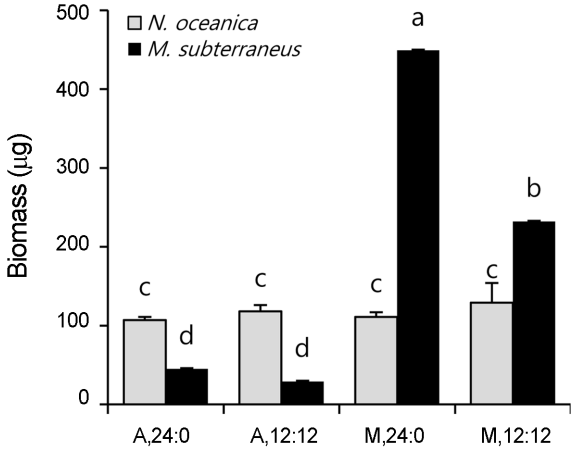

Fig. 2. Biomass in dry weight from 50 mL of *Monodus subterraneus* and *Nannochloropsis oceanica* cultured under different trophic conditions (A: autotrophic, M: mixotrophic, 24:0 and and 12:12 = L:D cycle). Different letters above the bars indicate significant differences ($P < 0.05$)

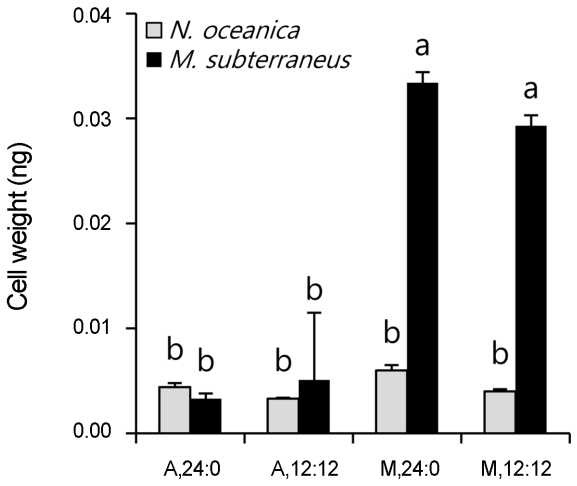

Fig. 3. Cell dry weight of *Monodus subterraneus* and *Nannochloropsis oceanica* cultured with different trophic conditions (A: autotrophic, M: mixotrophic, 24:0 and 12:12 = L:D cycle). Different letters on the bar indicate significant differences ($P < 0.05$)

was observed between the experimental groups. However, the dry weight of the mixotrophic culture of *M. subterraneus* was exceptionally higher than the autotrophic culture ($P < 0.05$, $df = 15$, $F = 41.8$). The dry weight (449 μg) in the mixotrophic culture with 24-hour continuous light was two-fold higher than that with 12-hours of light (232 μg). The dry weight in the mixotrophic cultures was ten-fold higher than the autotrophic cultures. The dry weight per cell was not different between the experimental groups for *N. oceanica,* but the mixotrophic culture was much higher than the autotrophic culture of *M. subterraneus* ($P < 0.05$, $df = 15$, $F = 58.3$). In the experimental group of mixotrophic *M. subterraneus* cultures, those cultured with continuous light had a dry weight per cell of 0.0334 ng, which was similar to those cultured with 12-hours of light (0.0293 ng). However, there was no difference produced by the lighting cycles in the autotrophic cultures (Fig. 3).

Nutritional composition

The amino acid composition of *M. subterraneus* and *N. oceanica* according to culture method is presented in Table 1. *M. subterraneus* had the highest amino acid content of glutamic acid, at 10-13%, while cysteine content was the lowest, at 0.8-0.9%. The total protein content was the highest, measured at 359 μg mg^{-1}, in the autotrophic culture with 24-hour continuous light, and the lowest, measured at 89 μg mg^{-1}, in the autotrophic culture with 12 hours of light. In the group with continuous light, the protein content in the autotrophic culture group was

Table 1. Amino acids contents (%) of *Monodus subterraneus* and *Nannochloropsis oceanica* cultured under different trophic conditions at 25°C with 80 μmol photons m^{-2} s^{-1}

Amino acids (%)	*Monodus subterraneus*				*Nannochloropsis oceanica*			
	Autotrophic		Mixotrophic		Autotrophic		Mixotrophic	
	12:12	24:00	12:12	24:00	12:12	24:00	12:12	24:00
Threonine	5.5 ± 0.1^{b}	5.9 ± 0.1^{ab}	5.9 ± 0.0^{ab}	6.3 ± 0.0^{a}	5.4 ± 0.0^{b}	5.4 ± 0.5^{b}	5.5 ± 0.1^{b}	5.5 ± 0.1^{b}
Valine	4.6 ± 0.1^{c}	5.2 ± 0.2^{b}	6.1 ± 0.1^{a}	6.0 ± 0.2^{ab}	4.7 ± 0.2^{c}	4.9 ± 0.7^{bc}	5.0 ± 0.1^{bc}	4.9 ± 0.1^{bc}
Methionine	1.0 ± 0.1^{c}	0.5 ± 0.1^{d}	2.8 ± 0.0^{a}	2.1 ± 0.1^{b}	0.6 ± 0.0^{d}	0.4 ± 0.6^{e}	1.1 ± 0.1^{c}	0.4 ± 0.3^{de}
Isolencine	3.6 ± 0.1^{c}	4.0 ± 0.1^{a}	4.1 ± 0.1^{a}	4.1 ± 0.0^{a}	3.2 ± 0.2^{d}	3.2 ± 0.4^{d}	3.4 ± 0.1^{cd}	3.3 ± 0.3^{cd}
Leucine	8.8 ± 0.0^{b}	9.4 ± 0.0^{a}	8.2 ± 0.1^{d}	8.1 ± 0.1^{e}	8.6 ± 0.3^{c}	8.3 ± 0.3^{d}	8.4 ± 0.0^{cd}	8.5 ± 0.2^{cd}
Phenylalanine	5.3 ± 0.0^{b}	5.4 ± 0.0^{ab}	4.5 ± 0.1^{c}	4.5 ± 0.1^{c}	4.7 ± 0.4^{c}	4.6 ± 0.2^{c}	4.8 ± 0.0^{c}	4.7 ± 0.4^{cd}
Lysine	7.0 ± 0.0^{b}	7.3 ± 0.1^{ab}	4.9 ± 0.0^{e}	5.6 ± 0.0^{d}	6.9 ± 0.2^{c}	6.9 ± 0.6^{c}	6.8 ± 0.0^{c}	6.8 ± 0.1^{c}
Histidine	2.3 ± 0.0^{a}	2.2 ± 0.1^{b}	1.9 ± 0.1^{c}	2.2 ± 0.2^{b}	2.1 ± 0.1^{c}	2.0 ± 0.3^{c}	2.0 ± 0.0^{c}	2.2 ± 0.2^{b}
Aspartic acid	10.3 ± 0.1^{a}	7.2 ± 0.0^{c}	10.2 ± 0.1^{a}	10.7 ± 0.1^{a}	9.2 ± 0.6^{b}	8.9 ± 1.3^{c}	9.3 ± 0.1^{b}	9.2 ± 0.7^{b}
Serine	5.4 ± 0.0^{ab}	5.6 ± 0.1^{a}	4.6 ± 0.1^{c}	5.4 ± 0.1^{ab}	5.1 ± 0.2^{b}	5.1 ± 0.1^{b}	5.0 ± 0.0^{bc}	5.3 ± 0.2^{ab}
Glutamic acid	13.6 ± 0.1^{b}	13.8 ± 0.2^{a}	10.1 ± 0.2^{c}	10.8 ± 0.2^{c}	13.7 ± 0.4^{b}	14.0 ± 0.8^{a}	12.7 ± 0.0^{c}	13.9 ± 0.1^{a}
Glycine	5.9 ± 0.0^{a}	6.0 ± 0.0^{a}	5.1 ± 0.1^{c}	5.2 ± 0.0^{ab}	5.7 ± 0.1^{b}	5.6 ± 0.5^{b}	5.7 ± 0.0^{b}	5.7 ± 0.1^{b}
Alanine	8.0 ± 0.1^{d}	8.1 ± 0.1^{cd}	11.2 ± 0.1^{a}	10.3 ± 0.1^{b}	8.2 ± 0.4^{cd}	8.0 ± 1.2^{d}	8.6 ± 0.1^{c}	8.2 ± 0.1^{cd}
Cysteine	0.8 ± 0.0^{b}	0.8 ± 0.0^{b}	0.9 ± 0.0^{a}	0.9 ± 0.0^{a}	0.7 ± 0.0^{d}	0.7 ± 0.1^{d}	0.8 ± 0.0^{b}	0.7 ± 0.1^{d}
Tyrosine	3.0 ± 0.0^{ab}	3.1 ± 0.5^{a}	2.8 ± 0.1^{b}	2.6 ± 0.3^{bcd}	2.8 ± 0.1^{ab}	2.6 ± 1.0^{bc}	3.0 ± 0.0^{a}	2.3 ± 0.0^{cd}
HN_3	2.3 ± 0.0^{d}	2.2 ± 0.1^{d}	3.3 ± 0.1^{b}	3.7 ± 0.5^{a}	1.8 ± 0.2^{e}	2.7 ± 1.0^{bc}	1.7 ± 0.0^{e}	2.4 ± 0.2^{cd}
Arginine	7.2 ± 0.0^{b}	7.7 ± 0.0^{a}	7.5 ± 0.1^{ab}	7.1 ± 0.1^{b}	6.3 ± 0.5^{c}	6.4 ± 0.2^{c}	6.4 ± 0.0^{c}	6.5 ± 0.7^{c}
Proline	5.5 ± 0.1^{c}	5.7 ± 0.2^{c}	5.8 ± 0.4^{c}	4.7 ± 0.2^{d}	10.5 ± 2.8^{a}	10.4 ± 4.2^{ab}	9.7 ± 0.1^{b}	9.9 ± 3.4^{ab}
EAA	38.2 ± 2.5^{b}	39.9 ± 2.8^{ab}	38.5 ± 2.0^{b}	38.8 ± 2.1^{b}	36.1 ± 2.6^{c}	35.7 ± 2.6^{c}	36.8 ± 2.4^{c}	36.2 ± 2.6^{c}
NEAA	61.8 ± 3.8^{c}	60.1 ± 3.7^{d}	61.6 ± 3.5^{c}	61.2 ± 3.5^{c}	64.0 ± 4.1^{ab}	64.3 ± 4.0^{a}	63.2 ± 3.8^{b}	63.8 ± 4.0^{ab}
Protein (μg/mg)	89.9 ± 6.2^{d}	359.6 ± 6.9^{a}	169.1 ± 10.5^{bc}	130.0 ± 31.1^{cd}	222.4 ± 7.9^{b}	199.9 ± 10.2^{bc}	244.0 ± 18.4^{b}	205.5 ± 15.8^{b}

12:12 and 24:0 = L:D cycle; EAA, essential amino acids; NEAA, non-essential amino acids
Different letters in the same row indicate significant differences ($P < 0.05$)

significantly higher than in the mixotrophic culture group ($P < 0.05$, $df = 23$, $F = 12.7$), whereas the opposite trend was observed for the 12-hour light cultures. The proportion of essential amino acids was 38-39%, with no differences observed between the experimental groups.

In the case of *N. oceanica*, glutamic acid was present in the highest amounts, at 12-14%, while cysteine content was the lowest, at 0.7-0.8%, demonstrating a comparable pattern to *M. subterraneus*. The total protein content was 199-244 μg mg^{-1}, and no significant differences were observed between the experimental groups. However, it was significantly lower than that of the autotrophic culture of *M. subterraneus* provided with continuous light. The proportion of essential amino acids was 35-36%, with no differences between the experimental groups. However, it was significantly lower than that of *M. subterraneus* ($P < 0.05$, $df = 23$, $F = 16.3$).

The fatty acid composition of *M. subterraneus* and *N. oceanica* according to culture method is presented in Table 2. In all the experimental groups, the lipid content was 205-345 μg mg^{-1}, with significant differences observed only between experimental groups of *M. subterraneus* autotrophically cultured with continuous light (345 μg mg^{-1}) and mixotrophically cultured with continuous light (205 μg mg^{-1}). No significant differences were observed between other experimental groups. The total lipid content in *M. subterraneus* tended to be higher in the autotrophic cultures than in the mixotrophic cultures, but it did not show a clear trend in *N. oceanica* ($P < 0.05$, $df = 23$, $F = 1.5$). Among the saturated fatty acids, palmitic acid (C16:0) content was the highest, at 12-20 μg mg^{-1}, in all experimental groups, presenting no significant differences. There were 35-51 μg mg^{-1} and 20-31 μg mg^{-1} of saturated and monounsaturated fatty acids, respectively, also with no significant differences between experimental groups. The PUFA content in *M. subterraneus* was significantly higher in the autotrophic culture group (33-43 μg mg^{-1}) than the mixotrophic group (15-16 μg mg^{-1}); however, the content in *N. oceanica* was 28-34 μg mg^{-1} in all experimental groups, showing no significant differences. The n3 HUFA

Table 2. Fatty acids and total lipid contents (μg/mg in dry matter) of *Monodus subterraneus* and *Nannochloropsis oceanica* cultured under different trophic conditions at 25°C with 80 μmol photons m^{-2} s^{-1}

Fatty acid	*Monodus subterraneus*				*Nannochloropsis oceanica*			
	Autotrophic		Mixotrophic		Autotrophic		Mixotrophic	
	12:12	24:00	12:12	24:00	12:12	24:00	12:12	24:00
C10:0	6.3 ± 2.5^{a}	6.3 ± 3.9^{a}	6.3 ± 1.6^{a}	6.0 ± 2.8^{a}	3.6 ± 0.6^{a}	2.6 ± 2.3^{a}	2.5 ± 0.4^{a}	4.5 ± 1.8^{a}
C11:0	2.9 ± 1.1^{a}	2.9 ± 1.7^{a}	1.9 ± 1.8^{ab}	-	1.6 ± 0.3^{ab}	1.8 ± 0.2^{ab}	0.8 ± 0.7^{b}	2.0 ± 0.8^{ab}
C12:0	4.6 ± 1.7^{a}	-	2.1 ± 3.7^{ab}	-	1.7 ± 1.5^{ab}	1.1 ± 1.8^{b}	1.4 ± 1.2^{ab}	1.8 ± 1.7^{ab}
C13:0	2.8 ± 0.8^{a}	2.6 ± 1.4^{ab}	1.2 ± 1.1^{bc}	0.4 ± 0.8^{c}	1.6 ± 0.2^{abc}	1.5 ± 0.2^{abc}	1.0 ± 0.1^{c}	1.7 ± 0.6^{abc}
C14:0	5.4 ± 1.7^{a}	4.9 ± 2.4^{a}	4.5 ± 1.1^{a}	1.1 ± 1.8^{b}	3.6 ± 0.8^{ab}	3.5 ± 0.5^{ab}	2.9 ± 0.6^{ab}	4.0 ± 1.3^{a}
C14:1n5	2.5 ± 0.9^{a}	2.3 ± 1.3^{a}	1.3 ± 1.1^{a}	2.1 ± 1.0^{a}	1.5 ± 0.2^{a}	1.7 ± 0.1^{a}	0.9 ± 0.2^{a}	1.7 ± 0.8^{a}
C15:0	2.1 ± 0.7^{ab}	2.3 ± 1.2^{a}	2.3 ± 0.4^{a}	1.9 ± 0.8^{ab}	1.2 ± 0.2^{ab}	1.3 ± 1.7^{ab}	0.9 ± 0.2^{b}	1.5 ± 0.5^{ab}
C15:1n2	2.2 ± 0.8^{a}	1.9 ± 1.2^{ab}	2.0 ± 0.5^{ab}	1.8 ± 0.8^{ab}	1.2 ± 0.2^{ab}	1.2 ± 0.1^{ab}	0.8 ± 0.1^{b}	1.4 ± 0.6^{ab}
C16:0	18.8 ± 3.2^{a}	19.9 ± 5.2^{a}	12.5 ± 2.6^{a}	12.6 ± 2.2^{a}	14.4 ± 3.6^{a}	14.3 ± 2.8^{a}	20.0 ± 3.9^{a}	18.3 ± 7.4^{a}
C16:1n7	14.5 ± 1.1^{a}	13.4 ± 3.0^{a}	6.9 ± 1.2^{b}	6.6 ± 1.2^{b}	9.2 ± 2.5^{ab}	9.3 ± 2.5^{ab}	14.2 ± 3.3^{a}	12.2 ± 5.1^{a}
C17:0	2.2 ± 0.8^{b}	2.3 ± 1.2^{b}	4.1 ± 0.6^{a}	2.2 ± 0.8^{b}	1.2 ± 0.2^{b}	1.3 ± 0.2^{b}	1.1 ± 0.2^{b}	1.5 ± 0.6^{b}
C17:1n2	2.0 ± 0.6^{bc}	1.9 ± 1.1^{bc}	5.3 ± 1.0^{a}	2.4 ± 0.9^{b}	1.1 ± 0.2^{c}	1.2 ± 0.0^{bc}	1.0 ± 0.12^{c}	1.3 ± 0.6^{bc}
C18:0	3.0 ± 1.2^{ab}	3.2 ± 1.6^{ab}	3.4 ± 0.6^{a}	2.9 ± 1.1^{ab}	1.8 ± 0.4^{ab}	1.9 ± 0.2^{ab}	1.5 ± 0.3^{b}	2.1 ± 0.8^{ab}
C18:1n9	8.8 ± 2.7^{ab}	10.4 ± 3.8^{ab}	11.2 ± 2.9^{a}	8.1 ± 2.7^{ab}	6.6 ± 0.9^{ab}	7.2 ± 1.1^{ab}	5.5 ± 0.9^{b}	8.5 ± 3.3^{ab}
C18:2n6	5.1 ± 1.4^{a}	5.6 ± 2.5^{a}	4.0 ± 0.8^{ab}	4.1 ± 1.6^{ab}	3.3 ± 0.4^{ab}	3.8 ± 0.5^{ab}	2.4 ± 0.3^{b}	4.7 ± 1.8^{ab}
C18:3n6	1.1 ± 1.2^{a}	1.9 ± 1.1^{a}	1.8 ± 0.4^{a}	1.7 ± 0.7^{a}	1.1 ± 0.2^{a}	1.2 ± 0.2^{a}	0.8 ± 0.1^{a}	1.4 ± 0.5^{a}
C18:3n3	1.1 ± 1.1^{ab}	1.8 ± 1.1^{a}	1.8 ± 0.4^{a}	-	0.6 ± 0.5^{ab}	0.4 ± 0.7^{b}	0.7 ± 0.2^{ab}	0.7 ± 0.7^{ab}
C20:0	-	1.0 ± 1.8^{bc}	-	1.1 ± 2.0^{bc}	2.6 ± 0.4^{ab}	2.9 ± 0.3^{ab}	1.9 ± 0.3^{abc}	3.2 ± 1.2^{a}
C20:1n9	1.0 ± 1.7^{a}	0.6 ± 1.1^{a}	-	1.1 ± 1.0^{a}	1.0 ± 0.8^{a}	-		0.5 ± 0.9^{a}
C20:2n2	0.9 ± 1.5^{ab}	0.8 ± 0.7^{ab}	-	1.9 ± 1.0^{a}	0.8 ± 0.7^{ab}	1.3 ± 0.1^{ab}	0.5 ± 0.4^{ab}	1.4 ± 0.6^{ab}
C20:3n6	-	1.8 ± 1.6^{ab}	-	-	0.8 ± 1.3^{b}	2.0 ± 1.7^{ab}	-	3.1 ± 1.2^{a}
C21:0	3.4 ± 1.1^{a}	3.4 ± 1.9^{a}	3.1 ± 0.7^{a}	3.2 ± 1.2^{a}	2.2 ± 0.3^{a}	2.4 ± 0.3^{a}	1.9 ± 0.2^{a}	2.9 ± 1.1^{a}
C20:3n3	4.4 ± 0.6^{ab}	5.0 ± 1.5^{a}	2.7 ± 0.3^{b}	2.9 ± 0.8^{b}	3.5 ± 0.3^{ab}	2.7 ± 0.3^{b}	3.8 ± 0.3^{ab}	3.6 ± 1.6^{ab}
C20:4n6	1.1 ± 1.1^{ab}	1.9 ± 1.2^{a}	-	-	-	0.4 ± 0.7^{b}	0.3 ± 0.5^{b}	-
C22:0	-	-	-	4.0 ± 4.2a	0.7 ± 1.1^{b}	0.9 ± 1.6^{ab}	-	1.5 ± 1.6^{ab}
C20:5n3	20.2 ± 1.1^{ab}	24.3 ± 2.5^{a}	5.6 ± 0.7^{c}	5.4 ± 0.9^{c}	19.4 ± 1.2^{ab}	14.0 ± 0.8^{b}	20.3 ± 2.0^{ab}	19.1 ± 11.4^{ab}
C22:1n9	-	0.9 ± 1.5^{a}	-	-	-	2.8 ± 4.9^{a}	-	0.9 ± 1.6^{a}
C22:6n3	-	-	-	-	-	4.8 ± 0.2^{a}	-	-
Saturated	51.4 ± 14.5^{a}	48.8 ± 19.7^{a}	41.5 ± 11.3^{a}	35.35 ± 10.4^{a}	36.0 ± 5.8^{a}	38.5 ± 8.2^{a}	35.8 ± 7.8^{a}	44.9 ± 14.5^{a}
Monounsaturated	30.8 ± 7.4^{a}	31.5 ± 9.6^{a}	26.7 ± 3.8^{a}	22.0 ± 5.2^{a}	20.5 ± 3.7^{a}	20.5 ± 3.7^{a}	22.4 ± 4.7^{a}	26.5 ± 11.4^{a}
Polyunsaturated	33.8 ± 3.4^{ab}	43.0 ± 7.5^{a}	15.9 ± 1.9^{c}	16.1 ± 4.9^{c}	29.4 ± 2.7^{b}	30.7 ± 3.3^{ab}	28.8 ± 2.3^{b}	34.1 ± 16.2^{ab}
n3 HUFA	24.7 ± 1.3^{a}	29.5 ± 5.3^{a}	10.6 ± 1.2^{b}	8.6 ± 2.1^{b}	22.2 ± 1.6^{a}	21.7 ± 1.4^{a}	22.9 ± 2.0^{a}	22.7 ± 12.7^{a}
Total lipid	324.8 ± 70.6^{ab}	345.2 ± 101.7^{a}	235.6 ± 46.6^{ab}	205.6 ± 56.9^{b}	240.4 ± 34.0^{ab}	250.9 ± 39.2^{ab}	243.5 ± 36.4^{ab}	295.2 ± 114.5^{ab}

12:12 and 24:0 = L:D cycle; HUFA, highly unsaturated fatty acid
Different letters in the same row indicate significant differences ($P < 0.05$)

content in *M. subterraneus* was significantly higher in the autotrophic culture group (24-29 μg mg^{-1}) than the mixotrophic culture group (8-10 μg mg^{-1}), whereas the content in *N. oceanica* was 21-22 μg mg^{-1} in all experimental groups, again demonstrating no difference. For *M. subterraneus,* AA (arachidonic acid, C20:4n6) was observed only in the autotrophic culture group (1.1-1.9 μg mg^{-1}), and it tended to be higher than that of *N. oceanica* (0.3-0.4 μg mg^{-1}). DHA was observed only in the *N. oceanica* group autotrophically cultured with continuous light (4.8 μg mg^{-1}). However, EPA was high in all experimental groups, at 5-24 μg mg^{-1}. The EPA content in *M. subterraneus* was much higher in the autotrophic culture group (20-24 μg mg^{-1}) than the mixotrophic culture group (5.4-5.6 μg mg^{-1}). However, the content in *N. oceanica* was 14-20 μg mg^{-1} in all experimental

groups, with no significant differences between the experimental groups.

Growth and nutritional composition of *M. subterraneus* according to changeover culture method

Growth rate and dry weight

In the above experiments, the growth of the biomass and dry weight of cells in the group cultured mixotrophically with continuous light was higher for *M. subterraneus* than for *N. oceanica*. In addition, the protein and lipid content, particularly EPA, were also much higher in *M. subterraneus.* Therefore, *M. subterraneus* was first cultured mixotrophically with continuous light to produce the maximum biomass, after which the cells were harvested and used to reinoculate new JM medium for autotrophic and mixotrophic cultures. The cells were then cultured with continuous light for 7 days. As a result, the SGR between mixotrophic (0.320, maximum density 7.029×10^7 cells ml^{-1}) and autotrophic (0.313, maximum density 6.842×10^7 cells mL^{-1}) culture did not reveal significant differences. However, the dry weight of cells harvested from 500 mL of culture medium cultured mixotrophically (0.783 g) was approximately three-fold higher than that of the autotrophic culture (0.248 g), representing a significant difference ($P < 0.05$, $df = 5$, $F = 427.4$). In addition, although the number of cells in the autotrophic culture increased 3.4 times compared to the initial inoculation density (1.859×10^7 cells mL^{-1}), the dry weight was comparable to that of the control group of mixotrophic culture (0.272 g). The dry weight per cell in mixotrophic culture (0.0235 ng) was significantly higher than that in the autotrophic culture (0.0091 ng). However, it was significantly lower than that in the control group (0.0292 ng) (Fig. 4) ($P < 0.05$, $df = 5$, $F = 115.5$).

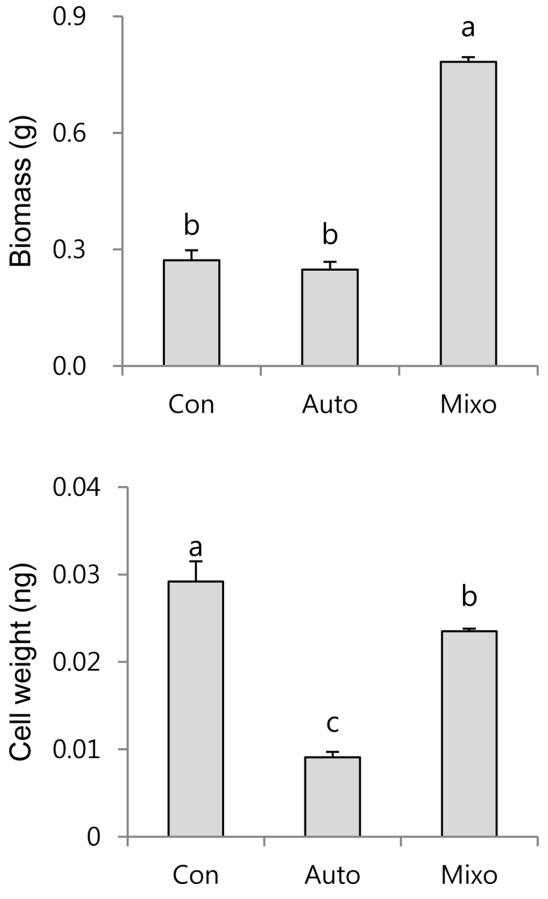

Fig. 4. Biomass in dry weight from 500 mL (up) and cell dry weight (bottom) of *Monodus subterraneus* cultured under different trophic conditions at 25°C and continuous 80 µmol photons m^{-2} s^{-1}. Con, control; Auto, autotrophic; Mixo, mixotrophic. Different letters on the bar indicate significant differences ($P < 0.05$)

Table 3. Amino acids contents (%) of *Monodus subterraneus* recultured autotrophically or mixotrophically from an initial mixotrophic culture at 25°C with continuous provision of 80 µmol photons m^{-2} s^{-1}

Amino acids	Control	Autotrophic	Mixotrophic
Threonine	6.64 ± 0.17^a	6.17 ± 0.06^b	6.66 ± 0.18^a
Valine	6.17 ± 0.61^b	6.21 ± 0.27^b	6.74 ± 0.63^a
Methionine	1.50 ± 0.17^a	1.46 ± 0.08^a	1.30 ± 0.39^a
Isolencine	4.63 ± 0.38^b	4.93 ± 0.24^a	5.04 ± 0.50^a
Leucine	8.86 ± 0.29^b	9.55 ± 0.32^a	9.33 ± 0.33^a
Phenylalanine	4.85 ± 0.14^b	5.38 ± 0.13^a	5.03 ± 0.18^b
Lysine	7.21 ± 0.24^b	7.54 ± 0.18^a	7.61 ± 0.20^a
Aspartic acid	10.68 ± 0.49^a	9.88 ± 0.49^b	9.71 ± 0.66^b
Serine	5.94 ± 0.27^a	4.98 ± 0.41^c	5.70 ± 0.38^b
Glutamic acid	8.71 ± 2.17^a	7.02 ± 1.24^b	7.15 ± 2.95^b
Glycine	5.14 ± 0.40^c	5.78 ± 0.24^a	5.22 ± 0.36^b
Alanine	9.23 ± 0.69^b	9.52 ± 0.44^a	8.02 ± 3.57^c
Cysteine	0.72 ± 0.24^b	0.87 ± 0.06^b	2.01 ± 2.78^a
Tyrosine	2.56 ± 0.31^b	2.98 ± 0.25^a	2.87 ± 0.49^b
HN_3	2.20 ± 0.48^a	2.12 ± 0.20^a	2.27 ± 0.29^a
Histidine	2.88 ± 0.18^a	2.70 ± 0.10^a	2.81 ± 0.20^a
Arginine	6.46 ± 0.40^a	6.49 ± 0.18^a	6.66 ± 0.51^a
Proline	5.62 ± 0.31^b	6.42 ± 0.20^a	5.81 ± 0.28^b
EAA	42.73 ± 2.38^a	43.93 ± 2.56^a	44.52 ± 2.60^a
NEAA	57.27 ± 0.57^a	56.07 ± 0.33^a	55.48 ± 1.31^a
Protein (µg/mg)	129.96 ± 16.43^b	233.67 ± 25.55^a	129.71 ± 23.69^b

Control, initial inocula cultured under mixotrophic condition; EAA, essential amino acids; NEAA, non-essential amino acids
Different letters in the same row indicate significant differences ($P < 0.05$)

Table 4. Fatty acid contents (μg/mg in dry matter) of *Monodus subterraneus* recultured autotrophically or mixotrophically from an initial mixotrophic culture at 25°C with continuous provision of 80 μmol photons m^{-2} s^{-1}

Fatty acids	Control	Autotrophic	Mixotrophic
C10:0	-	-	-
C11:0	5.24±1.94[a]	5.62±1.49[a]	6.69±1.97[a]
C12:0	7.18±3.33[a]	6.27±3.62[a]	6.31±5.35[a]
C13:0	1.99±2.03[b]	4.00±1.24[a]	0.74±1.35[b]
C14:0	7.97±2.92[a]	8.16±2.03[a]	9.30±2.59[a]
C14:1n5	2.93±2.37[b]	4.67±1.27[a]	1.54±1.94[b]
C15:0	2.96±1.51[a]	3.23±0.87[a]	2.83±1.61[a]
C15:1n2	-	-	-
C16:0	20.62±7.13[b]	29.10±7.33[a]	23.16±3.97[b]
C16:1n7	14.60±5.16[b]	22.59±5.58[a]	15.07±2.25[b]
C17:0	2.02±1.84[ab]	3.12±0.83[a]	0.91±1.83[b]
C17:1n2	0.92±1.36[b]	2.58±0.74[a]	0.22±0.75[b]
C18:0	4.32±1.59[a]	3.87±1.08[a]	4.83±1.48[a]
C18:1n9	13.03±4.74[a]	14.00±3.47[a]	13.94±3.74[a]
C18:2n6	7.15±2.60[a]	7.46±1.96[a]	8.07±2.26[a]
C18:3n6	3.22±1.18[a]	-	1.75±1.66[b]
C18:3n3	0.52±1.21[a]	0.63±1.18[a]	-
C20:0	0.07±0.25[a]	-	-
C20:3n3	4.86±1.68[b]	6.17±1.44[a]	5.79±1.25[ab]
C20:4n6	-	-	-
C20:5n3	11.71±4.11[b]	21.85±5.40[a]	13.06±2.21[b]
C22:6n3	-	-	-
Saturated	31.47±11.44[b]	43.85±10.63[a]	30.77±4.71[b]
Monounsaturated	51.92±16.16[a]	63.36±17.07[a]	54.76±13.99[a]
Polyunsaturated	27.46±9.66[b]	36.11±8.85[a]	28.67±5.47[b]
n3 HUFA	12.23±4.55[b]	22.48±5.82[a]	13.06±2.21[b]
Total lipid	310.67±102.61[b]	401.28±100.54[a]	319.75±62.50[b]

Control, initial inocula cultured under mixotrophic condition; HUFA, highly unsaturated fatty acids
Different letters in the same row indicate significant differences ($P < 0.05$)

Nutritional composition

The amino acid composition and protein content of *M. subterraneus* according to changeover culture method is presented in Table 3. The aspartic acid, leucine and glutamic acid content were all high, at 7-10%, while cysteine was the lowest, at 0.7-2%. The total protein content was the highest for the *M. subterraneus* cultured autotrophically, at 233 μg mg^{-1}, while the content in both *M. subterraneus* cultured mixotrophically and the control group was 129 μg mg^{-1}. The essential amino acid content was 42-44%, demonstrating no differences between the experimental groups.

The fatty acid composition of *M. subterraneus* according to changeover culture method is presented in Table 4. The total lipid content in the experimental group in which the method was changed from mixotrophic to autotrophic culturing was significantly higher, at 401 μg mg^{-1} ($P < 0.05$, $df = 35$, $F = 3.7$), while the content in the experimental group of secondary mixotrophic culture was 319 μg mg^{-1}, which was comparable to the 310 μg mg^{-1} observed for the control group of initial inocula cultured mixotrophically. The saturated fatty acid content in the autotrophic culture was significantly higher, at 43 μg mg^{-1} ($P < 0.05$, $df = 35$, $F = 7.3$), but the monounsaturated fatty acid content was 51-63 μg mg^{-1}, representing no significant differences between the experimental groups. However, the amount of C16:1n2 and C17:1n2 was significantly higher in the autotrophic culture. Notably, the PUFA content was significantly higher in the autotrophic culture (36 μg mg^{-1}) than in the mixotrophic culture or the control group. However, C18:3n3 (γ-linolenic acid) was detected only in the mixotrophic culture. Most of the n3 HUFA was EPA, detected at 21 μg mg^{-1} in the autotrophic culture, which was significantly higher than the 13 μg mg^{-1} obtained from the mixotrophic culture. AA and DHA were not detected in any of the experimental groups.

4. Discussion

The freshwater Eustigmatophyceae *M. subterraneus* has a high content of EPA, like the marine Eustigmatophyceae *N. oceanica*, creating the need on efficient culture methods (Borowitzka 2013). The EPA content in *M. subterraneus* varies according to the growth stage, and also depending on external environmental factors such as temperature, media, PH, and intensity of illumination (Cohen 1994, Khozin and Cohen 1996; Khozin and Cohen 2006). BG-11 medium (Liu and Lin 2001) has widely been used to culture *M. subterraneus,* but the growth rate is two- or three-fold higher in JM medium than in BG-11 medium (Jo 2014). It has been shown that the growth rate of autotrophic cultures of *M. subterraneus* is the highest at 25°C with continuous light of 80 μmol photons m^{-2} s^{-1}, and that while heterotrophic culturing is impossible, it can be cultured mixotrophically (Liu and Lin 2001; Jo 2014).

N. oceanica is a representative marine Eustigmatophyceae, and it can be cultured at high-density over ca. 30×10^6 cell/mL (Bae and Hur 2011b) to yield high lipid content (Su 2013). Therefore, it has been widely used to perform mass culturing for the industrial production of n-3 HUFA (Shields and Lupatsch 2012) and biodiesel (Giorno et al.

2013). Although there may be differences between strains of *N. oceanica*, the optimal culture conditions are known to be in f/2 medium, cultured at 25°C with continuous light of 80~100 μmol photons m^{-2} s^{-1} (Bae and Hur 2011b). Therefore, the growth and nutritional composition of the two representative species of Eustigmatophyceae, *M. subterraneus* and *N. oceanica*, should be compared for autotrophic and mixotrophic cultures based on their optimal culture conditions.

Glucose is the most effective organic compound for use as a carbon source, providing better results than acetate or pyruvate in mixotrophic cultures of *M. subterraneus*. When 1% glucose is supplied to the medium, the maximum density can be obtained in 4 or 5 days (Lam 2000). Therefore, 2% glucose was provided in this study, considering the nine-day culturing period (Kim and Hur 2013). The growth rate of *M. subterraneus* and *N. oceanica* according to culture method was much lower in *M. subterraneus* (6.23 ± 0.29 μm), whose cell size is large, than in *N. oceanica* (2.64 ± 0.41 μm), whose cell size is small. In addition, *N. oceanica* did not show any clear differences in the growth rate in relation to culture method, whereas *M. subterraneus* showed a much higher growth rate in the mixotrophic culture with continuous light. In addition, no big differences were observed in the biomass of *N. oceanica* in the different experimental groups, whereas the biomass of *M. subterraneus* was much higher in the mixotrophic culture than the autotrophic culture. Therefore, it was confirmed that mixotrophic culturing is favorable for mass culturing of *M. subterraneus*. *M. subterraneus* was very different from *N. oceanica*, the marine Eustigmatophyceae, in terms of growth by trophic culture method and light period.

Analyzing the protein content and amino acid composition of *M. subterraneus* and *N. oceanica* revealed no clear differences in *N. oceanica* in relation to trophic culture method and light, whereas greatly higher results were obtained for autotrophic cultures of *M. subterraneus* provided with continuous light. In addition, the total protein content in the autotrophic culture of *M. subterraneus* provided with continuous light was 1.5-fold higher than *N. oceanica*. The fatty acid composition, such as total lipid content, n3 HUFA, and especially EPA, revealed similar results. No differences were observed for *N. oceanica* depending on the culture method, whereas higher amounts were observed in the autotrophic culture of *M. subterraneus* cultured with continuous light than in mixotrophic cultures or those cultured with 12-hour light. The PUFA and n3 HUFA content was much lower in the mixotrophic culture of *M. subterraneus* than in *N. oceanica,* but it was higher in the autotrophic culture of *M. subterraneus* than *N. oceanica*. Therefore, it was confirmed that the lipid characteristics of the two species were different and *M. subterraneus* was a more appropriate species than *N. oceanica* with respect to proteins and lipids, particularly EPA.

The growth of *M. subterraneus* was highly increased in the mixotrophic culture, but the nutritional value decreased compared to the autotrophic culture. Therefore, the nutritional value can be increased by conducting autotrophic culturing after culturing mixotrophically to obtain the maximum biomass. In this study, the biomass of *M. subterraneus* was 3.2-fold higher in the group maintaining the mixotrophic culture method compared to the group changing from a mixotrophic to an autotrophic culture. In addition, the total protein, total lipids and PUFA, along with n3 HUFA and EPA of *M. subterraneus* were 1.8-fold, 1.3-fold and 1.7-fold higher, respectively, in the group changing to autotrophic culture. Thus, it was confirmed that if mixotrophic culturing of *M. subterraneus* was first conducted, after which autotrophic culturing was performed, the protein and lipid content, as well as the biomass, can be improved.

Previous studies on the dietary value of *M. subterraneus* and *N. oceanica* for rotifer *Brachionus plicatilis* revealed that the growth rate of the rotifer provided with *M. subterraneus* cultured under autotrophic conditions with continuous light was higher than that of the rotifer provided with *N. oceanica* cultured under autotrophic conditions, while the rotifer provided with *M. subterraneus* cultured in mixotrophic conditions showed the lowest dietary value (Jo 2014). This result may have been caused by the deficiency of essential proteins and lipids, particularly n3 HUFA, in *M. subterraneus* cultured mixotrophically for reproduction of the rotifer (Lubzens and Zmora 2003; Cabrera and Hur 2005; Bae and Hur 2011b).

Based on the results in this study, mass culture of the freshwater Eustigmatophyceae, *M. subterraneus*, is much easier and more economical than marine *N. oceanica.* In addition, the production of protein and n3 HUFA, particularly EPA, will be economically improved if *M. subterraneus* is first cultured mixotrophically to maximize the biomass, followed by secondary autotrophic culturing.

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