Culture Conditions Affect Eicosapentaenoic Acid Content of *Chlorella minutissima*

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ABSTRACT

Marine chlorella known to contain high amount of eicosapentaenoic acid (20:5ω3) are a potential natural source of this fatty acid for health foods and pharmaceuticals. The effect of culture conditions on lipid content and fatty acid composition of *Chlorella minutissima*, therefore, was investigated. Lipid content of cells grown at 25 C was 60% higher than that of the cells grown at 20 C. Fatty acid composition was affected by culture temperature and supplementation of NaCl. Eicosapentaenoic acid 20:5ω3 content was 45% (w/w) in the cells grown at 20 C, whereas that of the cells grown at 25 C was only 20% (w/w). Supplementation with NaCl also increased the percentage of 20:5ω3 acid to the same extent.

INTRODUCTION

The lipids of some marine microorganisms and fishes are known to contain a relatively high amount of polyunsaturated fatty acids (PUFA), especially 20:5ω3 and 22:6ω3 fatty acids. Several investigators (1,2) have reported that synthesis of ω3 PUFA in marine fishes is very low. Recently, Watanabe and his coworkers (3) found that these ω3 PUFA are essential fatty acids for marine fishes and speculated that their high levels in marine fishes are derived from marine microorganisms, such as diatoms, chlorella and dinoflagellates. Such organisms are known to contain high amounts of ω3 PUFA (4). In fact, marine chlorella are frequently used as feed for marine fish farming in Japan.

Eicosapentaenoic acid (20:5ω3) has been claimed as one of the precursors for prostaglandin. Furthermore, Dyerberg and his coworkers (5) have reported that 20:5ω3 may play an important role for mammals as an agent to prevent blood platelet aggregation. The high content of 20:5ω3 acid in marine chlorella has led us to speculate that marine chlorella might be used as a source of this fatty acid for health foods and pharmaceuticals. In this paper, we examine the effect of light intensity, culture temperature and concentration of sea water concentrate (SWC) or NaCl on the growth rate, total lipids content and total fatty acid composition of *C. minutissima*.

MATERIALS AND METHODS

Growth of Cells

A culture of *Chlorella minutissima* was obtained from Dr. C. Kitajima, Nagasaki Prefectural Institute of Fish, Japan. The basic medium used was Mann and Myers medium (6) supplemented with 0.2% (w/v) SWC (natural sea water=40.9 g SWC/1) and 0.05% (v/v) soil extract.

Autoclave-sterilized medium was inoculated at the rate of 1.0×10^7 cells/mL with inoculum that was grown in basic medium aerated with 5% (by volume) CO_2 in air under 10 joules/m^2·sec fluorescent light at 20 C for 6 days. The inoculum cells were collected by centrifugation and washed 3 times with salt-free basic medium.

To study the effect of light intensity on growth rate, lipids content and fatty acid composition, the inoculated flasks were incubated at 20 C for 4 days under 12 joules/m^2·sec light intensity. The flasks were aerated with filter-sterilized 5% CO_2 in air at the rate of about 50 mL/min. At the end of incubation, cells were harvested by centrifugation, washed and lyophilized.

Extraction of Lipids

Ca. 200 mg of dry cells were first homogenized with 5 mL of water, then extracted (7) twice with 25 mL of hexane-isopropanol (3:2 v/v) and filtered. The combined filtrate was washed with ½ volume of 6% (w/v) Na_2SO_4 solution. Hexane layers were collected and evaporated under reduced pressure and used for the measurement of total lipids content.

Analysis of Fatty Acid Composition

Ca. 10 mg of lipids was transesterified by adding 1 mL of BF_3-methanol (Applied Science, State College, PA) and holding the mixture at 85 C for 1 hr under N_2 gas. After cooling, 1 mL of water was added and fatty acid methyl esters were extracted with petroleum ether. After drying by Na_2SO_4, the solvent was evaporated under N_2 gas. The methyl esters were dissolved in 0.5 mL of CS_2 and analyzed by gas-liquid chromatography (GLC) using an instrument with a flame ionization detector (FID) (Shimadzu GC-6AM). A 7 ft × 3 mm i.d. glass column was packed with 15% (w/w) EGSS-Y (Applied Science, State College, PA) on Chromosorb-W (100-120 mesh). The analysis was conducted at a temperature of 194 C and hydrogen was used as a carrier gas. The fatty acids were identified by comparing their retention times with those of known fatty acids (Applied Science, State College, PA). Fatty acid contents were determined by comparing their peak areas with a 17:0 fatty acid internal standard.

RESULTS

Data in Table I indicate the effect of light intensity. The growth rate of *C. minutissima* in LI (1.5 joules/m^2·sec) was only 1/5 that in HI (12 joules/m^2·sec). Light intensity had no marked effect on the percentage of total lipids content and total fatty acid content, although slight changes in fatty acid composition of *C. minutissima* grown in different light intensity were noted. Fatty acid in the highest concentration of *C. minutissima* grown in LI and HI was 20:5ω3 (39.1 and 43.5% (w/w), respectively), followed by 16:1ω9 (20.5 and 22.2% (w/w)), 16:0 (16.2 and 14.0% (w/w)), and short-chain acids.

The effect of temperature on total lipids content and fatty acid composition is shown in Table II. The organism grew as well at 20 C as at 25 C. The total lipids content and fatty acid composition, however, were greatly affected by growth temperature. As the temperature increased from 20 C as at 25 C, total lipids content increased ca. 60%, from 14.5 to 23.2% (w/w). While fatty acids 16:1ω9 and 20:5ω3 decreased 47% and 55%, other unsaturated fatty acids increased significantly as temperature increased.

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TABLE I

Effect of Light Intensity on Growth Rate, Total Lipids Content and Fatty Acids Composition of *Chlorella minutissima*[a]

	Light intensity	
	LI[b]	HI[c]
Culture days	4	4
Wet cell concentration (g/L)	0.47 ± 0.001	2.15 ± 0.10
Dry cell concentration (mg/L)	66 ± 6	386 ± 20
Total lipids content (% by weight)	11.3 ± 2.8	12.4 ± 0.0
Total fatty acids content (% by weight in total lipids)	38.9 ± 3.7	42.2 ± 3.1
Fatty acids composition[d]		
C-14[e]	10.4 ± 2.0	11.1 ± 0.7
16:0	16.2 ± 0.7	14.0 ± 1.3
16:1ω9	20.5 ± 1.0	22.2 ± 1.3
16:2ω6	1.3 ± 0.4	0.9 ± 0.0
18:0	0.5 ± 0.1	–
18:1ω9	3.1 ± 0.4	1.4 ± 0.3
18:2ω6	2.9 ± 0.7	2.0 ± 0.1
18:3ω6	–	–
18:4ω3+20:1 ω9	–	–
20:3ω6	–	–
20:4ω6+22:0	5.6 ± 0.4	4.3 ± 0.0
20:4ω3	–	–
20:5ω3	39.1 ± 0.9	43.5 ± 2.1

[a]Average of 2 experiments ± SD.
[b]LI = low light (1.5 joules/m² ·sec).
[c]HI = high light (12 joules/m² ·sec).
[d]Data expressed as percentage of total fatty acids by weight.
[e]Total contents of short-chain fatty acids.

TABLE II

Effect of Temperature on Growth Rate, Total Lipids Content and Fatty Acids Composition of *Chlorella minutissima*[a]

	Temperature	
	20 C	25 C
Culture days	6	6
Wet cell concentration (g/L)	1.16 ± 0.04	1.20 ± 0.03
Dry cell concentration (mg/L)	239 ± 17	268 ± 4
Total lipids content (% by weight)	14.5 ± 0.1	23.2 ± 2.7
Total fatty acids content (% by weight in total lipids)	41.2 ± 1.4	45.4 ± 5.7
Fatty acids composition[b]		
C-14[c]	11.9 ± 2.0	9.4 ± 1.8
16:0	13.4 ± 0.4	14.5 ± 0.1
16:1ω9	21.2 ± 0.4	11.2 ± 1.3
16:2ω6	2.1 ± 0.0	6.6 ± 0.9
18:0	–	0.4 ± 0.3
18:1ω9	1.4 ± 0.3	8.6 ± 1.0
18:2ω6	1.7 ± 0.0	2.2 ± 0.3
18:3ω6	–	7.9 ± 1.3
18:4ω3+20:1 ω9	–	7.7 ± 1.1
20:3ω6	–	4.6 ± 0.9
20:4ω6+22:0	3.4 ± 1.1	3.2 ± 0.6
20:4ω3	–	2.5 ± 0.6
20:5ω3	44.7 ± 2.3	20.3 ± 1.8

[a]Average of 2 experiments ± SD.
[b]Data expressed as percentage of total fatty acids by weight.
[c]Total contents of short-chain fatty acids.

The effect of salt on growth rate, total lipids content and fatty acid composition was investigated by adding various amounts of SWC or NaCl to the salt-free basic medium (Table III). Maximum cell growth was observed in media containing 0.2% (w/v) of SWC or NaCl. The cells grown in SWC- or NaCl-enriched media contained slightly lower total extractable lipids, but a higher percentage of total fatty acids. The fatty acid composition, however, was significantly affected by the concentration of SWC or NaCl. As the concentration of SWC or NaCl in the medium increased, the percentage of 20:5ω3 acid of the cells increased, whereas that of 16:0, 18:1ω9 and 18:2ω6 decreased.

DISCUSSION

The relationship between light intensity and fatty acid compositions of diatoms and *Euglena* has been studied by several investigators. Erwin and Block (8,9) found that C_{20} and C_{22} PUFA contents of *Euglena gracilis* Z. grown in organic medium in the dark were greater than those of cells grown in light. Orcutt and Patterson (10) investigated the fatty acid composition of marine diatoms (*Nitzchia closterium*) grown in synthetic sea water and found that the percentage of 20:5ω3 in cells increased under low light intensity. However, slight decrease in the percentage of 20:5ω3 was observed in the cells of *C. minutissima* grown under the low light intensity investigated in this study.

However, the cellular fatty acid composition was found to be extremely sensitive to growth temperature. Other investigators have found increased synthesis of unsaturated fatty acids at lower temperatures in blue-green algae (11, 12), bacteria (13), yeast (14) and mold (15). The reasons for these temperature-associated changes have not been fully explained. Researchers have suggested (16) that unsaturated fatty acids increase the thermal stability of cells, thus the increase in unsaturated fatty acids at a lower growth temperature is a way of adapting to the environment. Our data (Table II) seem to be the result of such adaptation to environmental temperature change. Brown and Rose (14) postulated that because of increased solubility at lower temperatures, a greater amount of intracellular molecular oxygen is available, which is required by oxygen-dependent enzymes that catalyze the desaturation of long-chain fatty acids. The activity of the desaturating enzymes and the enzymes that are involved in elongation of the fatty acid may be extremely sensitive to temperature. We also may speculate that those fatty acids that increased at 25 C are the intermediates in the biosynthetic route of 20:5ω3 acid (17).

Some researchers investigated the use of artificial sea water as the growth medium for marine algae. McLachlan (18) found that most marine algae grew well in the medium composed of 50% (v/v) natural sea water and 50% (v/v) artificial sea water. However, when the concentration of natural sea water was reduced to 25% (v/v), growth rate of some of those marine algae decreased. Allen (19) found that marine diatoms did not grow in 100% artificial sea water, which contains 4% NaCl (w/v). However, they grew well in artificial sea water supplemented with 1% (v/v) natural sea water. Hirata (20) found that *Chlorella saccharophila* grew well in the medium containing from 0.5-4.5% (w/v) sea salt, but it did not grow in the medium without sea salt. Our study shows that the growth rate of *C. minutissima* in a salt-free basic medium can be improved when supplemented with NaCl or SWC, indicating that the salt-free basic medium used in this study contains adequate trace elements and that NaCl is the limiting factor for growth. Furthermore, the addition of NaCl to the salt-free basic medium greatly increases the percentage of 20:5ω3 acid of the cells and decreases that of 16:0 acid, suggesting that the enzymes that carry out the elongation and desaturation may require NaCl. But the effect of NaCl or culture temperature on those enzymes may not be the same, especially on the enzymes that catalyze 16:10 acid transformation.

TABLE III

Effect of Sea Water Concentrate (SWC) and NaCl Concentration on Growth Rate, Total Lipids Content and Fatty Acids Composition of *Chlorella minutissima*[a]

	Salt supplementation concentration					
		SWC			NaCl	
	0	0.05	0.2	1.0	0.2	1.0
Culture days	4	4	4	4	4	4
Wet cell concentration (g/L)	0.82 ± 0.03[a]	1.64 ± 0.13	1.91 ± 0.01	1.77 ± 0.10	2.42 ± 0.17	2.20 ± 0.14
Dry cell concentration (mg/L)	110 ± 7	325 ± 21	410 ± 3	379 ± 9	495 ± 64	392 ± 14
Total lipids content (% by weight)	17.0 ± 0.1	15.4 ± 5.8	12.6 ± 3.5	15.3 ± 1.7	7.8 ± 4.0	12.8 ± 1.1
Total fatty acids content (% by weight in total lipids)	40.0 ± 1.4	53.1 ± 5.8	46.1 ± 1.7	52.1 ± 5.4	45.3 ± 7.8	40.4 ± 3.0
Fatty acids composition[b]						
C-14[c]	6.9 ± 0.6	7.6 ± 0.4	10.3 ± 4.0	8.6 ± 0.3	7.2 ± 1.3	9.2 ± 0.4
16:0	32.8 ± 9.1	22.6 ± 0.3	17.9 ± 3.4	15.5 ± 0.0	18.8 ± 1.0	16.1 ± 2.8
16:1ω9	23.4 ± 2.3	23.0 ± 3.7	21.1 ± 1.6	21.2 ± 0.7	21.7 ± 0.4	24.5 ± 2.8
16:2ω6	1.8 ± 0.3	2.9 ± 2.7	0.8 ± 0.1	2.9 ± 0.3	0.3 ± 0.1	0.7 ± 1.4
18:0	1.7 ± 0.3	0.5 ± 0.1	0.5 ± 0.0	0.7 ± 0.7	0.5 ± 0.3	—
18:1ω9	6.1 ± 0.6	5.4 ± 3.1	2.5 ± 2.1	2.3 ± 1.8	2.7 ± 0.0	2.9 ± 0.4
18:2ω6	4.4 ± 0.4	2.3 ± 1.1	2.8 ± 1.1	3.0 ± 1.8	2.3 ± 0.1	1.9 ± 0.1
18:3ω6	—	2.4 ± 3.4	—	1.0 ± 1.4	—	—
18:4ω3+20:1ω9	2.3 ± 0.9	2.0 ± 2.8	—	1.1 ± 1.6	—	—
20:3ω6	0.8 ± 1.1	1.2 ± 1.7	—	0.6 ± 0.9	—	—
20:4ω6+22:0	3.9 ± 0.9	4.3 ± 1.7	6.2 ± 1.0	3.7 ± 0.3	5.7 ± 0.7	4.6 ± 0.1
20:4ω3	—	0.9 ± 1.3	—	—	—	—
20:5ω3	16.1 ± 1.1	26.0 ± 6.5	38.2 ± 1.0	39.9 ± 2.0	40.8 ± 2.0	40.0 ± 0.6

[a]Average of 2 experiments ± SD.
[b]Data expressed as percentage of total fatty acids by weight.
[c]Total contents of short-chain fatty acids.

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