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γ-Linolenic Acid Production by Microalgae

MORIO HIRANO,*[,1] HIROFUMI MORI,[1] YUUKI MIURA,[2] NAOKI MATSUNAGA,[2] NORIYUKI NAKAMURA,[2] AND TADASHI MATSUNAGA[2]

[1]*Research Laboratory of Biotechnology, Onoda Cement Co., Ltd., 1-Chome, 1-7, Toyosu, Koto-ku, Tokyo 135, Japan; and* [2]*Department of Biotechnology, Tokyo University of Agriculture and Technology, Koganei, Tokyo 184, Japan*

ABSTRACT

The production of γ-linolenic acid from algae in fresh and marine water was investigated. When *Spirulina platensis* was left in the dark condition, it contained about one and a half times γ-linolenic acid compared with conventional *Spirulina platensis*. Marine microalga, *Chlorella sp.* NKG 042401 contained about 10% of γ-linolenic acid. The highest γ-linolenic acid content was obtained when this alga was cultured under the radiation of around 100 μEinstein/m^2/s.

Index Entries: γ-linolenic acid; *Spirulina platensis;* green algae; lipid; marine microalgae.

INTRODUCTION

Unsaturated fatty acids in foods are assimilated in the organisms and play important roles in them (*1–4*). Unsaturated fatty acids in the organisms are elongated or further unsaturated to physiologically-active substances, such as prostaglandins or leukotrienes via linoleic acid, α-linolenic acid, γ-linolenic acid, arachidonic acid, eicosapentaenoic acid, and so on. The rate-determining step in these transformations is the enzyme reaction from linoleic acid to γ-linolenic acid. Therefore, it is recommended to take γ-linolenic acid through appropriate foods to smoothly proceed the formation of prostaglandins, leukotrienes, and so on.

The seed of evening primrose, some kind of fungi and blue-green algae *Spirulina platensis* are already known as the microorganisms contain-

*Author to whom all correspondence and reprint requests should be addressed.

ing γ-linolenic acid, and their contents of γ-linolenic acid are about 2% for evening primrose seed (*5*), 3% for dried *Mortierela* (*6*), and 1% for dried *Spirulina platensis* (*7*), respectively. The ratios of γ-linolenic acid vs total fatty acid are 7% for evening primose and 8% for *Mortierela*, whereas the ratio for *Spirulina platensis* reaches about 25%. Therefore, the effective production of highly pure γ-linolenic acid from *Spirulina* oil would be most promising.

Microalgae require only light energy, air, and minerals for their growth and contain protein, lipid, and carbohydrate, as well as vitamins, carotenoid, chlorophyll, and so on (*8*). Microalgae have been used for food (*9*), animal feeds (*10*), a source of chemicals (*11*), and energy production (*12,13*). Only few have attempted to employ microalgae for the production of bioactive substances. In this study, we selected *Spirulina platensis* as a fresh water microalga for the production of γ-linolenic acid and investigated its culture in the dark to enhance the content of γ-linolenic acid. Besides, we found *Chlorella sp.* as the marine microalga containing γ-linolenic acid and investigated its culture conditions for γ-linolenic acid production.

MATERIALS AND METHODS

Reagents

All solvents and reagents were commercially available analytical or laboratory grade materials. Deionized water was used in all procedures.

Strains

Spirulina platensis M-135 was donated by the National Institute for Environmental Studies (Tsukuba, Japan). *Chlorella sp.* NKG 042401 and other marine microalgae were isolated in our laboratory at the Tokyo University of Agriculture and Technology. Samples were collected from coastal areas of Japan, enriched, and purified by selecting isolated colonies on agar plate.

Culture of *Spirulina platensis* M-135

Spirulina platensis M-135 was cultured in SOT medium (*14*) containing 16.8 g $NaHCO_3$, 0.5 g K_2HPO_4, 2.5 g $NaNO_3$, 1.0 g K_2SO_4, 1.0 g NaCl, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.04 g $CaCl_2 \cdot 2H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.08 g ethylene diamine tetraacetate, and A-5 trace metals 1.0 mL, in 1 L of water. The A-5 trace metal solution contained 2.86 g H_3BO_3, 1.81 g $MnCl_2 \cdot 4H_2O$, 0.222 g $ZnSO_4 \cdot 7H_2O$, 0.039 g $Na_2MoO_4 \cdot 2H_2O$, 0.079 g $CuSO_4 \cdot 5H_2O$, 0.049 g $Co(NO_3) \cdot 6H_2O$ in 1 L of water. *Spirulina platensis* M-135 was inoculated into 200 L SOT medium in a cylindrical water tank (ϕ 50 cm × h 150 cm) and bubbled with air at 30°C, under a light intensity of about 200

μEinsteins/m²/s from solar and cool-white fluorescent lights for 19 d. And the *Spirulina platensis* M-135 was further cultured in the following conditions: (1) in the dark at 30°C, (c) in the dark at 20°C and, (3) under cool-white fluorescent light (200 μEinsteins/m²/s) at 30°C for 1 wk. The cells were collected by filtration and washed twice with water.

Culture of *Chlorella sp.* NKG 042401

Chlorella sp. NKG 042401 was cultured in the modified BG-11 medium, in which 10 g NaCl and 1 μg vitamin B_{12} were added to the BG-11 medium (*15*). The BG-11 medium was adjusted to pH 7.8 using 0.1*N* NaOH. *Chlorella sp.* NKG 042401 was inoculated into 50 mL in the modified BG-11 medium in a 100 mL Erlenmyer flask and bubbled with air at 30°C under various light intensities in the range of 0 to ~400 μEinsteins/m²/s from cool-white fluorescent light. The cells were centrifuged at 6000*g* for 10 min and washed twice with 3% NaCl solution.

Fatty Acid Analysis

Fatty acid methyl esters were prepared from the 20 mg of dry cells directly by transmethylation with 5 mL of 5% HCl · CH_3OH for 3 h at 100°C, extracted with hexane, and analyzed by gas chromatography (Shimadzu, GC-9A) using a DEGS-column(ϕ 3 mm × 2 m) at 180°C. Components of fatty acids were identified by comparing their retention time to methyl pentadecanoate as standard. Quantitative determinations were made using the internal standard method.

Concentration of γ-Linolenic Acid by the Urea Adduct Method

Methyl-γ-linolenate was concentrated by the following urea adduct method (*16*). The sample of fatty acid methyl esters (1 mg) was dissolved in methyl isobutyl ketone (1 μL) and urea saturated methanol (0.2 mL) and heated under a nitrogen atmosphere. They were left overnight at room temperature and the resulting crystals of urea were filtered off and the filtrate was evaporated. The residue was dissolved with *n*-hexane and washed with water. *n*-Hexane was evaporated and an obtained sample was analyzed as described above.

RESULTS AND DISCUSSION

Culture of *Spirulina platensis* Containing Higher Amounts of γ-Linolenic Acid

Spirulina platensis M-135 was cultured for 19 d under solar and fluorescent light at 30°C and then left in the dark at 30°C, in the dark at 20°C, and under fluorescent light at 30°C. Figure 1 shows the growth of *Spirulina*

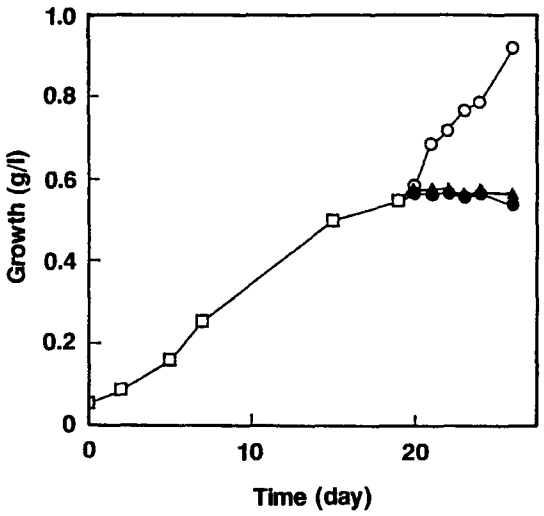

Fig. 1. Growth of *Spirulina platensis*. *Spirulina platensis* was cultured in 200 L SOT medium and bubbled with air for 19 d at 30°C under light irradiation (200 μEinstein/m²/s, □) and further cultured for 7 d at 30°C under light irradiation (○), at 30°C in the dark (●) and 20°C in the dark (▲).

Table 1
Total Fatty Acid and γ-Linolenic Acid Content
in *Spirulina platensis* Cells Under Various Conditions

	30°C		20°C	
	TFA[a]	γ-LA[a]	TFA	γ-LA
Light	33.1	7.9	-	-
Dark (168 h)	46.1	12.1	39.4	10.4

[a]TFA: total fatty acid; γ-LA: γ-linolenic acid. TFA and γ-LA are expressed in mg/g dry weight cells.

platensis in various experimental conditions. The growth was not observed in both cases left at 20 and 30°C in the dark conditions.

As shown in Table 1, the total amount of fatty acid contained in *Spirulina platensis* under solar and fluorescent light, before dark treatment, was 33.1 mg/g dried alga, and the content of γ-linolenic acid was 7.9 mg/g dried alga. When this *Spirulina platensis* was left for 168 h in the dark with aeration, the total fatty acid contents in *Spirulina platensis* increased by 39%, as shown in Fig. 2. With these increases of the total fatty acid, γ-linolenic acid has also increased by 35% after 16 h culture in the dark and 53% after 168 h culture in the dark (Fig. 3). Thus, the *Spirulina platensis* containing total fatty acid of 46.1 mg/g dried alga and γ-linolenic acid of 12.1 mg/g dried alga was obtained after culturing 168 h in the dark, and the accumulation of total fatty acid, and especially of γ-linolenic acid, were ascertained in the alga cultured in the dark, as shown in Fig. 3. On

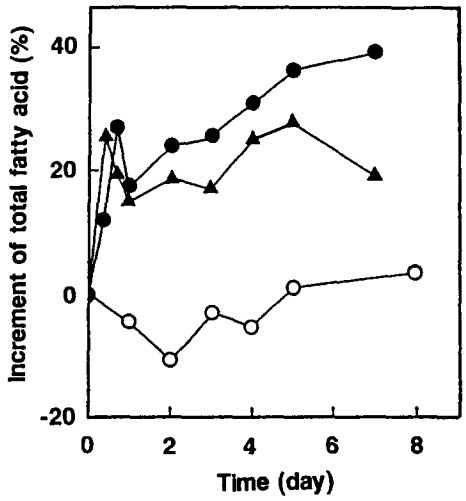

Fig. 2. Time course of total fatty acid increment in *Spirulina platensis*. *Spirulina platensis* was cultured in 200 L SOT medium for 19 d at 30°C aerobically under light irradiation (200 μEinstein/m²/s). Collected cells were used for experiments. Experiments were performed at 30°C under the light irradiation (○), 30°C in the dark (●), and 20°C in the dark (▲).

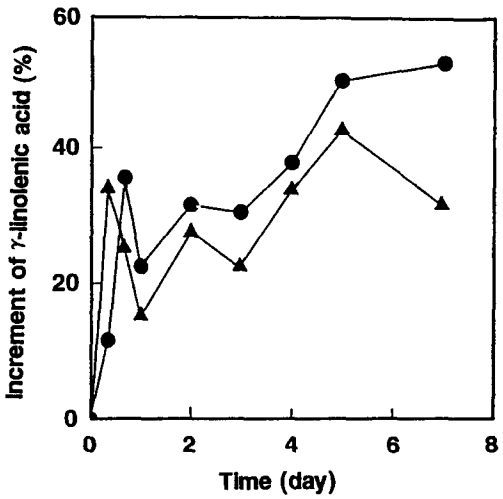

Fig. 3. Time course of γ-linolenic acid increment in *Spirulina platensis*. Experimental conditions and symbols are the same as in Fig. 2.

the other hand, we tried to enhance the synthesis of fatty acid and increase the γ-linolenic acid by drastically decreasing the culture temperature from 30 to 20°C. As shown in Figs. 2 and 3, no significant effect of decreasing culture temperature was observed.

When *Spirulina platensis* was left in the dark, consumption of intracellular fatty acids and sugars occurred. Sugars were preferentially consumed under the dark condition in comparison with the fatty acids.

As a result, the relative content of fatty acids increased. Thus, it was assured from these results that the *Spirulina platensis* having higher γ-linolenic acid could be obtained effectively by culturing well under light and then leaving in the dark for a week.

Concentration of γ-Linolenic Acid in *Spirulina platensis* by Urea Adduct Method

When the urea was crystalized in the presence of the long-chain compound, the urea formed the hexagonal needles and incorporated the long-chain compound. It is required for the linear structure for incorporating materials. Using this property, γ-linolenic acid appeared in the filtrate. γ-Linolenic acid was concentrated by the urea adduct method for the purpose of obtaining fatty acid containing higher γ-linolenic acid as a main component from the higher fatty acid in *Spirulina platensis*. During urea addition, the higher fatty acids are excluded successively from the system in the order from the saturated straight-chain fatty acid (*16*). Thus, γ-linolenic acid, having the highest unsaturation degree among the principal higher fatty acids, that are contained in *Spirulina platensis* or *Mortierela*, will be concentrated at each urea addition step. Therefore, this urea addition step for obtaining highly pure γ-linolenic acid is effective when the ratio of γ-linolenic acid in total fatty acid is considerably high, but the ratio of oleic or linoleic acid, which may give unfavorable effect on the concentration of γ-linolenic acid, is relatively low.

The ratios of γ-linolenic acid/total fatty acid are 25 and 8%, respectively, for *Spirulina platensis* and *Mortierela*, and the ratios of oleic + linoleic acid/total fatty acid are 15 and 57%, respectively. Therefore, the *Spirulina platensis* is more advantageous than *Mortierela* as the material to obtain the fatty acid containing highly pure γ-linolenic acid by the urea adduct method. The γ-linolenic acid content increased to 63% with the first urea addition. Fatty acid containing 82% γ-linolenic acid was obtained by repeating the urea addition twice, and the total yield of γ-linolenic acid of 73% was obtained. This result is superior to the previous result with *Mortierela* (*17*), and the *Spirulina platensis* could be the excellent material for the production of highly pure γ-linolenic acid.

Production of γ-Linolenic Acid with Marine Microalgae

Recently, we have isolated about 300 strains of marine algae from the samples collected in the sea around Japan (*18*), and then 30 strains, which had grown rapidly, were selected and cultured with aeration. The fatty acid compositions of these cultured algae were investigated (Table 2), and the result showed the presence of γ-linolenic acid in *Chlorella sp.* NKG 042401. The content of γ-linolenic acid was about 10% of that total fatty

Table 2
Fatty Acid Composition of Marine Green Algae (*Chlorella sp.*)[a]

Fatty acids wt%	Strains			
	0001	040501	042401	060401
14:0	3.7	0.3	0.5	2.0
16:0	18.8	18.4	21.8	28.6
16:1	5.0	1.6	3.4	tr
16:2	14.2	6.4	2.8	1.6
18:0	4.9	16.4	6.0	16.3
18:1	5.0	0.2	8.2	12.2
18:2	36.4	9.2	27.5	0.5
18:3 (α)	0	44.0	13.5	27.4
18:3 (γ)	0	0	10.5	0
others	12.0	3.5	6.2	11.4
TFA[b]	7.6	5.8	8.2	6.6

[a]Each fatty acid values are expressed in wt% per TFA.
[b]TFA: Total fatty acid. Values of TFA are expressed in wt% per dry weight cells.

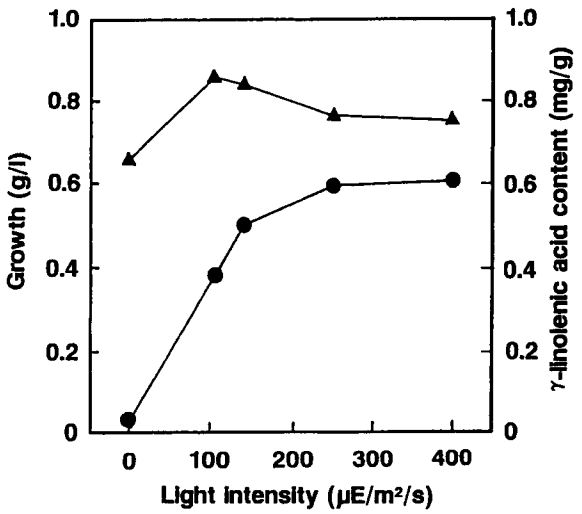

Fig. 4. Effect of light intensity growth (●) and γ-linolenic acid content (▲) of *Chlorella sp.* NKG 042401. *Chlorella sp.* NKG 042401 was cultured in 50 mL modified BG-11 medium for 7 d at 30°C.

acid. α-Linolenic acid, which was hardly detected in *Spirulina platensis,* represented about 10% of the total fatty acid. The effect of the light intensity on the growth of *Chlorella sp.* NKG 042401 and the content of γ-linolenic acid were investigated. As shown in Fig. 4, their growth rate increased with increasing light intensity and reached the maximum rate at 100 to ~140 μEinsteins/m²/s. When *Chlorella sp.* NKG 042401 was cultured for

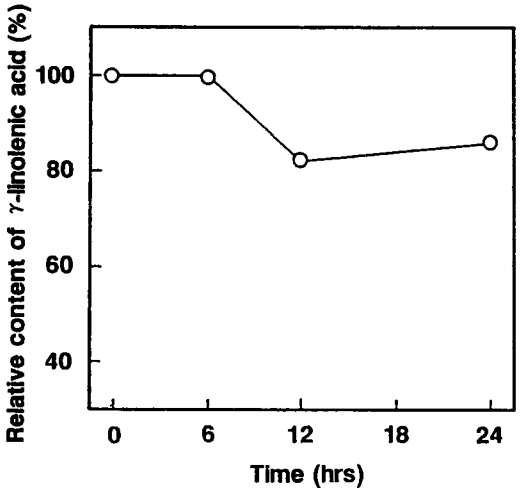

Fig. 5. Time course of relative γ-linolenic acid content in *Chlorella sp.* NKG 042401 left under dark condition. *Chlorella sp.* NKG 042401 was cultured in 50 mL modified BG-11 medium for 7 d at 30°C under light radiation (100 μEinstein/m²/s). Experiment was performed at 30°C in the dark.

7 d under the light intensity around 100 μEinsteins/m²/s, the highest γ-linolenic acid content was obtained. *Chlorella sp.* NKG 042401 was also cultured under the light and then left in the dark in order to increase the content of γ-linolenic acid, as observed in *Spirulina platensis.* But both total fatty acid and γ-linolenic acid decreased continuously just after the beginning of culture in the dark (Fig. 5). Therefore, *Chlorella sp.* NKG 042401 was cultured under the light intensity of about 100 μEinsteins/m²/s without further treatment in the dark for obtaining a higher γ-linolenic acid content.

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