0273-2289/92/3435—0273$02.00

Influence of Ammonium Chloride on Growth and Fatty Acid Production by *Spirulina platensis*

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ABSTRACT

Changes in growth and fatty acid content of *Spirulina platensis* were examined after transferring cells into media containing various concentrations of ammonium chloride. Photosynthetic O_2 evolution rate decreased with increasing ammonium chloride concentration. Therefore, the algal growth was interrupted by ammonium chloride addition. On the other hand, total fatty acid content markedly increased after addition of ammonium chloride to a concentration of 15–50 m*M* and was maximized 40–48 h after addition of 25 m*M* ammonium chloride. The increases in palmitic and oleic acid content were especially remarkable. However, this began to decrease 48 h after the addition of 25 m*M* of ammonium chloride. Also, γ-linolenic acid content increased continuously during a 72-h incubation. As a result, *Spirulina platensis* cells containing about 2% γ-linolenic acid were obtained by ammonium chloride treatment, representing an increase of 1.5–2-fold compared to untreated cells.

Index Entries: *Spirulina platensis;* blue-green alga; fatty acid; γ-linolenic acid; ammonium chloride.

Nomenclature: Chl; chlorophyll.

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INTRODUCTION

Polyunsaturated fatty acids are important dietary constituents as precursors of prostaglandins and leukotrienes. The in vivo rate-determining step in the conversion of polyunsaturated fatty acids to such biogenic substances is the enzyme reaction that converts linoleic acid to γ-linolenic acid. Since the activity of the enzyme for this conversion decreases with age in humans (*1*), a greater dietary intake of γ-linolenic acid is recommended. Recently, γ-linolenic acid from some kind of fungi, *Mortierela*, has become commercially available, and is being used for health drinks and cosmetics in Japan.

A blue-green alga, *Spirulina*, is utilized as food in the area around Lake Chad, and is marketed as a healthy aliment in the United States and Japan. *Spirulina* contains 1% γ-linolenic acid in its dry weight. Therefore, it is also an important source of γ-linolenic acid (*2*). Moreover, the γ-linolenic acid ratio to the total fatty acid content of *Spirulina* amounts to approx 25%, which is much higher than in evening primrose seed (7%) (*3*) and *Mortierela* (8%) (*4*), known alternative sources of γ-linolenic acid.

There have been numerous reports describing the relationship between lipid and fatty acid content in microalgae and the culture conditions, e.g., light (*5,6*), temperature (*7,8*), culture age (*7,9*), concentration of nitrogen source (*6,8,10*), and CO_2 concentration (*11*). However, most of the reports showing remarkable changes in the fatty acid content and the level of fatty acid unsaturation are based on green algae or *Euglena*. It has been reported that the fatty acid content of blue-green algae is not as affected by the culture conditions (*10*). For blue-green algae, there are only a few reports, i.e., the fatty acid composition changes according to the incubation temperature, although this change is not very clear compared to those in green algae (*8*). It has also been reported that the fatty acid content and composition in *Spirulina platensis* are slightly affected by culture temperature and nitrogen starvation. However, little change has been observed with culture age and light intensity (*12*).

We have been studying the culture conditions of a blue-green alga, *Spirulina platensis*, to increase the fatty acid content, especially γ-linolenic acid, in the algal cells. Hirano et al. (*13*) previously reported that the fatty acid content of *Spirulina platensis* cells tended to increase when they were incubated in the dark. In this study, we investigated the impact of the addition of ammonium salt to the culture on the fatty acid content of *Spirulina platensis*.

MATERIALS AND METHODS

Strain and Growth Conditions

Spirulina platensis IAM M-135 was obtained from the Institute of Applied Microbiology, University of Tokyo (Tokyo, Japan). *Spirulina platensis*

was grown in SOT medium (pH 9.0) (*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 1.0 mL A-5 trace metals 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 $ZnSO_4 \cdot 7H_2O$, 0.39 g $Na_2MoO_4 \cdot 2H_2O$, 0.079 g $CuSO_4 \cdot 5H_2O$, and 0.049 g $Co(NO_3) \cdot 6H_2O$ in 1 L of water. Algal cells were cultured axenically in flat glass bottles at 30°C with aeration under continuous illumination (2000 lx) from a bank of fluorescent cultivation lamps (Plant Lux, Toshiba Electric Co., Ltd., Japan).

Assay of Photosynthesis

Cells were harvested by centrifugation and resuspended in fresh SOT medium to a density of approx 0.5 g/L. Photosynthetic O_2 evolution of the cell suspension was measured using an oxygen electrode (Rank Brothers, UK) under a projection lamp (20,000 lx, Fuji Electric Lamp Industrial Co., Ltd., Japan) at 30°C. After measuring the photosynthetic O_2 evolution of the cell suspension without ammonium for 4 min, 4*M* ammonium chloride solution was added to obtain the correct concentrations with a microsyringe. O_2 evolution rates in the presence of ammonium chloride were measured 1–3 min after the addition of ammonium chloride solution. After measuring O_2 evolution, cells were collected by centrifugation in order to measure the amount of Chl according to Mackinney (*15*).

Fatty Acid Analysis

Cells were grown to a concentration of approx 1.5 g/mL, harvested with a sterilized stainless steel net (88-μm mesh), and resuspended in fresh SOT medium. The cell suspension was supplemented with a filtered germ-free solution of 4*M* ammonium chloride to the appropriate concentration and incubated under the conditions described above. Samples were removed at fixed intervals, washed with water, and lyophilized.

Fatty acid methyl esters were prepared, as described previously (*13*), from the dried cells directly by transmethylation with HCl-methanol, extracted with hexane, and analyzed by gas chromatography (Hewlett Packard 5890A). They were applied to a Hewlett Packard Ultra 1 capillary column (0.32 mm×25 m) at 180°C. Fatty acid methyl esters were identified by comparing the retention time with known standard reagents. Fatty acid quantity was estimated from the peak area on the chromatogram using pentadecanoic acid as the internal standard.

RESULTS

Inhibition of Photosynthesis by Ammonium Chloride

Figure 1 shows photosynthetic O_2 evolution of *Spirulina platensis* in medium containing ammonium chloride at various concentrations. The

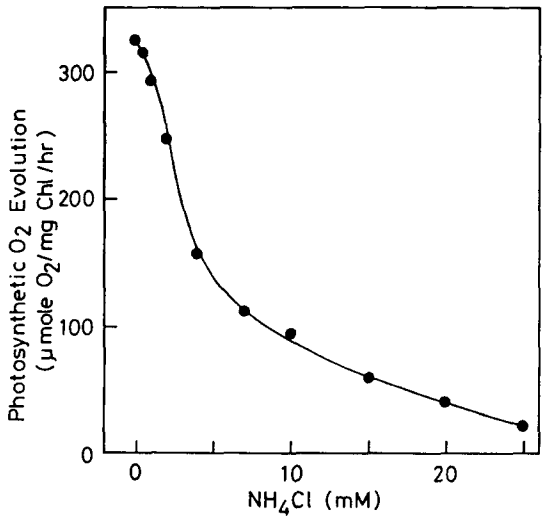

Fig. 1. Effect of ammonium chloride on the photosynthetic O_2 evolution rate of *Spirulina platensis*. O_2 evolution rate was measured 1–3 min after the addition of ammonium chloride under projection lamp (20,000 lx) at 30°C.

photosynthetic activity decreased with increasing ammonium chloride concentration. The photosynthetic O_2 evolution rate was 300–400 μmol O_2/mg Chl/h without ammonium chloride. Above 7 m*M* ammonium chloride, it was <100 μmol O_2/mg Chl/h. This inhibition was reversible. However, when the cell suspension containing ammonium chloride was incubated for over 1 h with irradiation by light, the photosynthetic apparatus was damaged (data not shown).

Effect of Ammonium Chloride Concentration on Growth

Spirulina platensis was transferred to fresh SOT medium, supplemented with various concentrations of ammonium chloride, and incubated at 30°C under flurorescent light (2000 lx). The growth of *Spirulina platensis* after addition of ammonium chloride is shown in Fig. 2. The algal growth was inhibited in the presence of ammonium chloride. *Spirulina platensis* did not grow at ammonium chloride concentrations above 25 m*M*. However, the recovery of algal growth was observed 24 and 40 h after the addition of ammonium chloride at concentrations of 15 and 25 m*M*, respectively.

Changes in Fatty Acid Content and Composition After Addition of Ammonium Chloride

Although the growth of *Spirulina platensis* was inhibited in the presence of ammonium chloride, the total content of fatty acid in the algal cells increased (Fig. 3). The highest content of fatty acid was obtained when

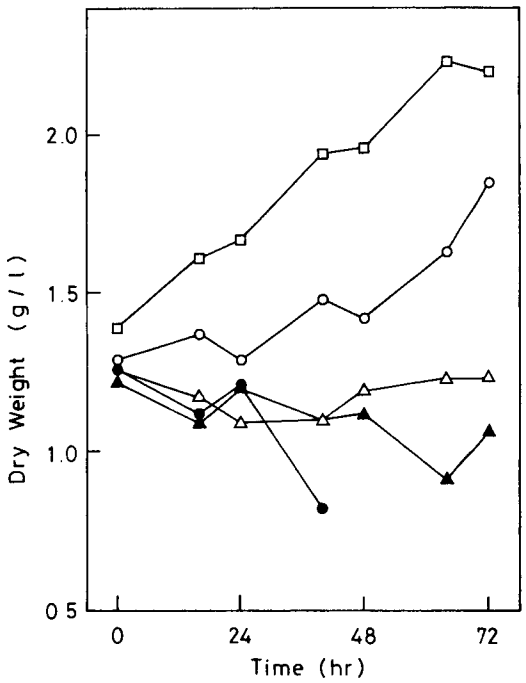

Fig. 2. Growth of *Spirulina platensis*. *Spirulina platensis* cells were incubated under continuous illumination (2000 lx) at 30°C after addition of ammonium chloride at various concentrations; control (□), 15 m*M* (○), 25 m*M* (△), 35 m*M* (▲), 50 m*M* (●).

ammonium chloride was added at 25 m*M*. In the presence of 15 and 25 m*M* ammonium chloride, the total fatty acid content continued to increase during interruption of growth. However, the fatty acid content began to decrease as algal growth began to recover.

Figure 4 shows changes in the major fatty acid content in *Spirulina platensis* when ammonium chloride was added at 25 m*M*. Palmitic acid and oleic acid content remarkably increased when the algal growth was interrupted. The increase of γ-linolenic acid content was also observed, whereas that of linoleic acid was slight until 48 h after the addition of ammonium chloride. When algal growth began to recover, palmitic acid and oleic acid content rapidly decreased, and instead, linoleic acid and γ-linolenic acid content rapidly increased.

Increase of γ-Linolenic Acid Content After Addition of Ammonium Chloride

The statistical data presented in Table 1 show γ-linolenic acid content obtained from a total of 20 separate experiments after ammonium chloride was added at 25 m*M* to the medium at the cell density of 0.5–1.5 g/L. γ-Linolenic acid content notably increased following the addition of ammon-

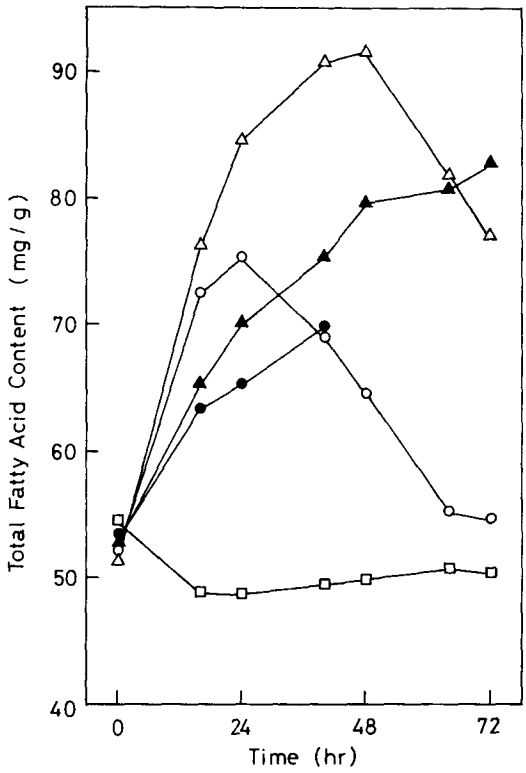

Fig. 3. Time-course of total fatty acid contents in *Spirulina platensis*. Experimental conditions and symbols are the same as in Fig. 2.

ium chloride, and the increase was significant according to the *t*-test at a level of significance of 1% both 40 and 72 h after the addition of ammonium chloride. As shown in Table 2, a marked increase was also observed in the γ-linolenic acid content following addition of 25 m*M* ammonium chloride when the cell suspension was concentrated to give an algal cell density of 5 g/L, in which light could not penetrate to reach individual cells.

DISCUSSION

Photosynthetic activity of *Spirulina platensis* was inhibited dose-dependently when ammonium chloride was added to the culture medium. Though the data were not shown in this article, the damage in the photosynthetic apparatus was observed when cells were incubated in the presence of ammonium chloride under the light irradiation, but little affected in the dark. The inhibition of photosynthesis caused the interruption of the algal growth. However, when the concentration of ammonium ions decreased owing to vaporization of ammonia, which was converted from ammonium ion, because of the alkaline nature of the SOT medium, the

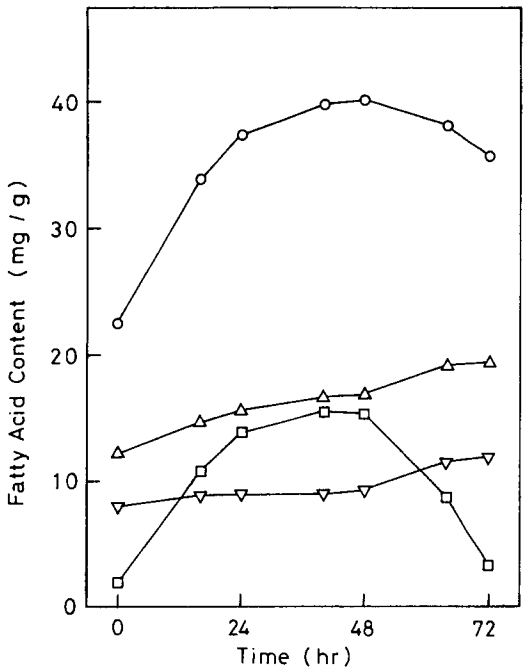

Fig. 4. Time-course of the major fatty acid content in *Spirulina platensis*. *Spirulina platensis* cells were incubated in the light at 30°C after addition of 25 m*M* ammonium chloride. Changes in palmitic acid (○), oleic acid (□), linoleic acid (▽), and γ-linolenic acid (△) content were observed.

Table 1
γ-Linolenic Acid Content in *Spirulina platensis* after Addition of 25 m*M* Ammonium Chloride at Cell Density of 0.5–1.5 g/L

Time, h	0	40	72
X_{max}[a]	14.0	19.9	20.8
X_{min}[a]	11.1	14.8	16.4
X_{AV}[a]	12.49	16.95	18.85
V[a]	0.575	1.332	0.930

[a] X_{max}: The maximum result, X_{min}: The minimal result, X_{AV}: The average, V: Unbiased variance. X_{max}, X_{min}, and X_{AV} are expressed in mg/g dry wt cells. The results are obtained from a total of 20 separate experiments.

photosynthetic activity recovered (data not shown), and after a while, the algal growth recovered. However, the cells burst when the residual ammonium ions remained at a concentration of more than 10 m*M* and after 2 d of incubation when some waste material accumulated in the culture medium. This is why the algal cells were transferred to fresh medium before the addition of ammonium chloride.

Table 2
Total Fatty Acid and γ-Linolenic Acid Content in *Spirulina platensis* after Addition of 25 m*M* Ammonium Chloride at Cell Density of 5.0 g/L

Time, h	0	40	72
TFA[a]	49.9	76.1	78.5
γ-LA[a]	12.2	15.9	17.2

[a]TFA: Total fatty acid content; γ-LA: γ-linolenic acid content. TFA and γ-LA are expressed in mg/g dry wt cells. The results are the average of two separate experiments.

The increase of total fatty acid content was most significant when *Spirulina platensis* cells were incubated with 25 m*M* ammonium chloride. The change in palmitic acid content paralleled that of the total fatty acid. On the other hand, the desaturation of C_{18} fatty acid was limited during interruption of growth. Therefore, oleic acid accumulated. When the algal growth recovered, oleic acid was rapidly converted to linoleic acid and γ-linolenic acid.

It has been reported that relatively large amounts of lipids and fatty acids were observed at low nitrogen concentrations in the case of green algae (*10*). Our present results of ammonium chloride addition to the culture medium of *Spirulina platensis* are in contrast to that report. Total lipid content also increased with increasing total fatty acid content after the addition of ammonium chloride (data not shown). Another report has indicated that the addition of ammonium chloride to *Spirulina platensis* cultures caused glutamate and alanine accumulation resulting from the effects on the glutamine synthetase–glutamate synthetase pathway and alanine dehydrogenase (*16*). It is reasonable to consider that activation of amino acid synthesis can cause decreased acetyl Co-A-mediated fatty acid synthesis. However, in this study, the fatty acid content increased. Kanazawa et al. (*17*) reported that, in their ^{14}C incorporation experiments using *Chlorella pyrenoidosa*, ammonium chloride addition decreased sucrose synthesis, but accelerated ^{14}C incorporation into lipids, amino acids, and carboxylic acids. Similar results have been obtained with several strains of *Chlorella* by Miyachi and Miyachi (*18*). However, these observations have been limited in the short term.

According to a report by Pohl and Wagner (*6*), the level of polyunsaturated fatty acids increases at high nitrogen concentrations, though lipids content decreases. The increase strongly correlates with photosynthesis and requires light. Furthermore, algal cells that have adapted to low levels of CO_2 showed elevated levels of unsaturated fatty acids (*11*). Therefore, there may be a relationship between the interruption of desaturation of C_{18} fatty acid, which stopped at oleic acid, and the inhibition of photosynthetic activity by the addition of ammonium chloride in the light. Conversely, ammonium chloride treatment in the dark, which did not strongly

affect the fatty acid composition, also showed weak damage to the photosynthetic apparatus, whereas total fatty acid content increased as it increased in the light (data not shown).

The statistical data obtained from a total of 20 separate experiments show the notable increase of γ-linolenic acid content in *Spirulina platensis* by the addition of 25 m*M* ammonium chloride. The increase was also observed following the addition of ammonium chloride when the algal cells were concentrated so that light could not penetrate to individual cells. This suggests that secondary incubation to increase the γ-linolenic acid content can be carried out in a smaller space, such as indoor tanks, compared to primary incubation for cell proliferation.

ACKNOWLEDGMENTS

We would like to thank S. Miyachi, M. Tsuzuki and M. Ohmori of University of Tokyo, and J. G. Burgess of Tokyo University of Agriculture and Technology for suggestions.

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