Growth and Amino Acid Contents of *Spirulina platensis* with Different Nitrogen Sources

Aeran Choi, Song-Gun Kim, Byung-Dae Yoon, and Hee-Mock Oh*

Environmental Biotechnology Laboratory, Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-600, Korea

Abstract The growth and amino acid contents of the cyanobacterium, *Spirulina platensis* strain NIES 46, were investigated using ammonium, nitrate, nitrite, or urea as the sole nitrogen source in a batch culture. Chlorophyll *a* concentration was highest at 2,096 μg/L in the nitrate group after 10 days of cultivation, while the dry weight of *S. platensis* was highest at 4.5 g/L in the ammonium group after 30 days of cultivation. The total amino acid content was highest at 174 mg/g dry weight of *S. platensis* in the urea group at the end of the cultivation period, yet the amino acid patterns for *S. platensis* were similar for all the experimental groups. Therefore, it seemed that the growth and amino acid composition of *S. platensis* varied depending on the type of nitrogen sources, while the amino acid patterns were not changed. Also, the most efficient harvesting time for *S. platensis* seemed to be approximately 10 days after cultivation.

Keywords: amino acid, growth, harvesting time, nitrogen source, *Spirulina platensis*

INTRODUCTION

Spirulina platensis is a blue-green photoautotroph found in both aqueous and saline habitats. The alga has long been used by human being as a source of food in Mexico and Africa [1,2] and is one of the most potent sources of natural nutrition for animals and human being. Due to its high protein content of up to approximately 60-70% on a dry weight basis [3], amino acids present in this organism match the proportions recommended by the FAO [2,4].

The specific growth rate of microalgae is under the control of several biological factors (*e.g.*, biomass concentration and yield) and physicochemical variables (*e.g.*, pH, temperature, irradiance and deficiency of nutrients) [5,6].

Nitrogen is an essential element required for the synthesis of primary and secondary amino acids, proteins, chlorophyll and other accessory photosynthetic pigments [7,8]. Yet, nitrogen deficiency has been found to stimulate the synthesis of all carbohydrate fractions (intracellular, capsular, and soluble) of cyanobacteria [9]. Cyanobacteria, like most microorganisms, can use nitrate, nitrite, or ammonium and, to some extent, organic compounds as nitrogen sources [10]. Nitrogen comprises about 10% of the cell dry weight in cyanobacteria [11], and yet, there are specific distinctions in the uptake of different nitrogen sources by microalgae [12]. The nutritional quality of protein is determined by its amino acid profile, *i.e.* the content, proportion, and availability of the amino acids. In addition, amino acids can be a sensitive indicator of the state of nitrogen nutrition as well as the general synthetic activity of algae [13].

Accordingly, the aim of the current study was to determine the effects of several nitrogen sources on the growth and amino acid composition of *S. platensis*, which in turn would provide information on the proper harvesting time.

***Corresponding author**
Tel: +82-42-860-4321 Fax: +82-42-860-4594
e-mail: heemock@kribb.re.kr

MATERIALS AND METHODS

Microorganism and Culture Conditions

A culture of *Spirulina platensis* strain NIES 46 was obtained from the Microbial Culture Collection of the National Institute for Environmental Studies, Japan. The organism was maintained in an SOT medium [14], containing per litre 16.8 g of $NaHCO_3$, 0.5 g of K_2HPO_4, 2.5 g of $NaNO_3$, 1 g of K_2SO_4, 1 g of NaCl, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.04 g of $CaCl_2 \cdot 2H_2O$, 0.01 g of $FeSO_4 \cdot 7H_2O$, 0.08 g of Na_2EDTA, and 1 mL of trace solution A_5. One litre of the trace solution A_5 contained 2.86 g of H_3BO_3, 2.5 g of $MnSO_4 \cdot 7H_2O$, 0.222 g of $ZnSO_4 \cdot 7H_2O$, 0.079 g of $CuSO_4 \cdot 5H_2O$ and 0.021 g of $Na_2MoO_4 \cdot 2H_2O$.

To produce an inoculum, the organism was grown in 250-mL Erlenmeyer flasks containing 100 mL of the SOT medium, except in the case of nitrogen starvation, where the combined nitrogen ($NaNO_3$) was omitted. Thereafter, the inoculum was cultured in 250-mL Erlenmeyer flasks containing 100 mL of a modified SOT medium (150 μg N/L) with ammonium, nitrate, nitrite, or

Table 1. *In vivo* fluorescence-based maximum specific growth rate (μ_{max}), maximum chlorophyll *a* concentration (Chl_{max}), and maximum dry weight (DW_{max}) for four experimental groups using different nitrogen sources

Parameter	Nitrogen source				
	Ammonium	Nitrate	Nitrite	Urea	Days after cultivation
μ_{max} (day^{-1})	0.121	0.178	0.190	0.194	3
Chl_{max} (μg/L)	1,090	2,096	1,947	1,763	10
DW_{max} (mg/L)	4,533	4,050	3,000	2,825	30

urea as a nitrogen source. The cultivation was carried out under 14/10-h light/dark cycle, with illumination of 120 μmol photon m^{-2} s^{-1} provided by cool white fluorescent lamps at 25 ± 1°C, and 100 rpm. The initial pH was 9.3.

Algal Growth Estimation

The growth was monitored as an increase in *in vivo* fluorescence, chlorophyll *a* concentration, and dry weight. The *in vivo* fluorescence was measured using a fluorometer (Turner 450, Barnstead/Thermolyne). Up to 10 mL of the culture samples were filtered through Whatman GF/C filters. The filters containing biomass were then extracted in 90% acetone, and the chlorophyll *a* contents were determined spectrophotometrically (UV-160A, Shimadzu) [15]. The dry weight was determined using 5 mL samples. Following the procedure set up by Olguín *et al.*, the algal materials were filtered through a preweighted Whatman GF/C filter paper, washed twice with distilled water, and dried at 80°C for 4 h [16]. All measurements were performed in triplicate.

Nitrogen and Amino Acid Analysis

To determine the nitrogen content, the culture samples were collected on precombusted GF/C filters. Then, the filtered samples were placed in a screw cap test tube with 15 mL of a 1% potassium peroxodisulfate solution and was autoclaved at 120°C, 1.5 atm for 2 h. The nitrogen in the algae and water was measured according to the second-derivative method [17]. The cells were then harvested by centrifugation (MF550, Hanil, Korea) for 20 min at 2,500 rpm, and general amino acid composition analyses of the algal materials were conducted using a Waters Pico-Tag amino acid analysis system after hydrolysis in constant boiling HCl for 24 h at 110°C. The hydrolyzed samples were dried and redried using a redry solution (Ethanol/DW/Triethylamine, 2/2/1, v/v/v). The derivatization of the hydrolyzed samples and free amino acids samples was accomplished using a derivatizing solution (Ethanol/DW/Triethylamine/Phenylisolthiocyanate (PITC), 7/1/1/1, v/v/v/v) for 15 min. The PITC-derivatized free amino acids were then fractionated on a Pico-Tag column (300 mm × 39 mm), which was equilibrated with buffer A equipped with a Waters HPLC system (510 HPLC pump, 717 automatic sampler, 996 photodiode array detector, and Millennium 32 chromatography manager) and was eluted with a linear gradient composed of buffer B (0, 14, 20, 46, and 100%) at a flow rate of 1 mL/min at 46°C. The absorbance was measured at 254 nm. Buffer A consisted of 140 mM sodium acetate (6% acetonitrile), while buffer B consisted of 60% acetonitrile.

Data Analysis

Two-way analyses of variances (ANOVAs) were used to determine the amino acid composition of *S. platensis* at each stage of cultivation and with the different nitrogen sources after 30 days of cultivation.

RESULTS AND DISCUSSION

Most photosynthetic microorganisms depend on either ammonium or nitrate as their sole source of combined nitrogen. Generally, the preferential order of nitrogenous compounds for cyanobacteria and other algae is ammonium > nitrate or urea > other organic compounds [18].

The *in vivo* fluorescence-based specific growth rate (μ_{max}), after adaptation, was highest in all groups after 3 days of cultivation, with the maximum at 0.194 day^{-1} in the urea group (Table 1). Nitrate is normally used as the nitrogen source for *Spirulina* sp. cultivation. However, Stanca and Popovici [19] demonstrated that using urea as the nitrogen source for *S. platensis* cultivation caused an increase in both the biomass production and the chlorophyll content. It was reported that urea utilization as a nitrogen source provided an energetic gain due to its spontaneous hydrolysis to ammonium in the alkaline medium, which was then easily assimilated by *Spirulina* [2]. The dry weight of each nitrogen group continuously increased and exhibited a maximum value of 4.5 g/L in the ammonium group. In the current study, the nitrogen source exhibiting the highest growth and productivity changed depending on the growth parameters.

The chlorophyll *a* concentration was highest after 10 days of cultivation in all groups, with the maximum at 2,096 μg/L in the nitrate group (Fig. 1). The chlorophyll *a* concentration suddenly decreased after 10 days of cultivation that coincided with the decrease of total nitrogen content. Previous studies have already found that nitrogen-starvation induced a lower chlorophyll *a* content [20,21], and in the current study, the concentration of chlorophyll *a* abruptly decreased after 10 days of cultiva-

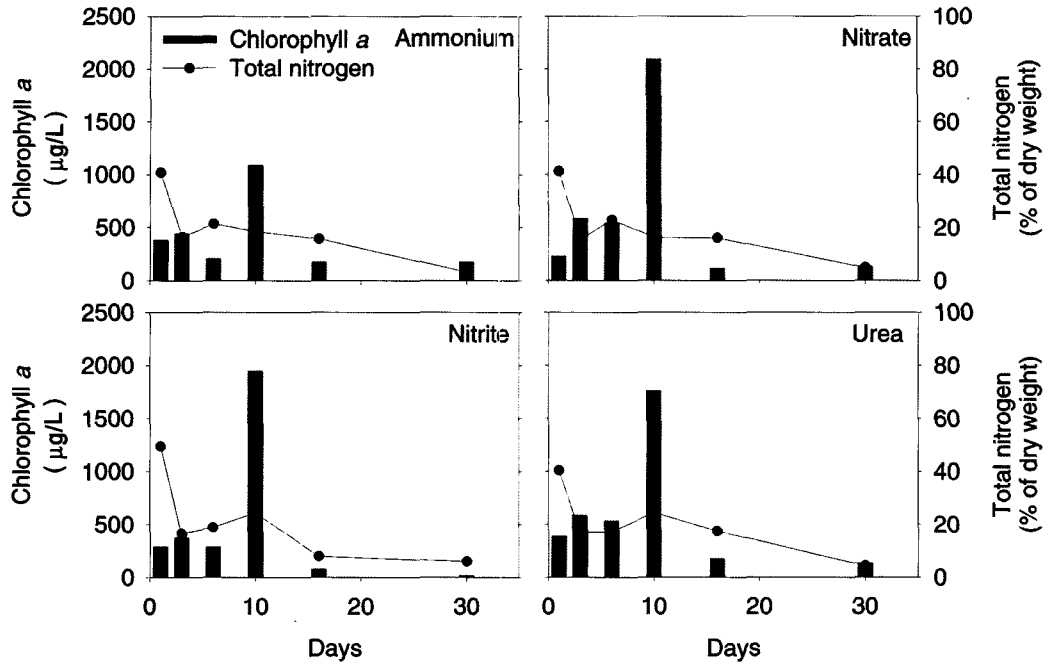

Fig. 1. Chlorophyll *a* concentration and total nitrogen percentage in *Spirulina platensis* with different nitrogen sources.

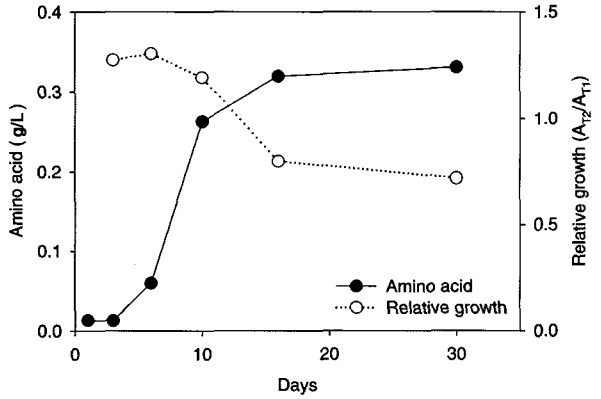

Fig. 2. Time course of amino acid concentration and relative growth of *Spirulina platensis* culture using ammonium as the nitrogen source.

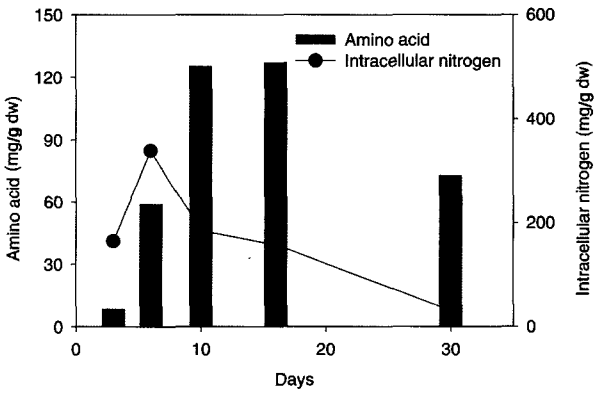

Fig. 3. Time course of amino acid content and intracellular nitrogen content in *Spirulina platensis* culture using ammonium as the nitrogen source.

tion in all groups, suggesting nitrogen limitation at this point (Fig. 1). As such, it seemed that the chlorophyll *a* concentration was affected by the nitrogen content in the cells.

The time course of the amino acid concentrations in *S. platensis* was examined for the ammonium group with the maximum dry weight (Fig. 2). The total amino acid concentration increased rapidly after 6 days of cultivation, which corresponded to the period of maximum relative growth (1.30), and was highest at 0.33 g/L after 16 days of cultivation. That is, the relative growth rate was dependent on the amino acid concentration.

The amino acid content was highest at 127.0 mg/g dry weight after 16 days of cultivation and exhibited a particular increase after 6 days, corresponding with a rapid decrease in the total intracellular nitrogen (Fig. 3). As such, a relationship was exhibited among the start of nutrient deficiency, the deceleration in the growth rate, and the accumulation of carbohydrates [22].

When culturing *Spirulina*, the demand for amino acids is reduced and the growth rate is decreased at low temperatures [1]. In addition, excess nitrogen is metabolized into cyanophycin, a copolymer of amino acids that forms granules occupying up to 18% of the cell volume. In the current study, the percentage and pattern of amino acids in the *S. platensis* culture, with ammonium as the nitrogen source, exhibited no change ($P < 0.01$) according to the sampling time. The amino acid content after 30 days

Table 2. Essential amino acid contents of *Spirulina platensis* under different nitrogen sources after 30 days of cultivation

Amino acid	Nitrogen source (mg/g dw)			
	Ammonium	Nitrate	Nitrite	Urea
Threonine	3.6 (5.0)	4.2 (4.4)	6.7 (4.8)	8.4 (4.9)
Valine	5.5 (7.5)	6.7 (7.1)	11.9 (8.5)	13.2 (7.6)
Methionine	1.1 (1.5)	1.6 (1.7)	2.3 (1.6)	2.5 (1.5)
Leucine	6.5 (8.9)	8.2 (8.7)	14.2 (10.1)	15.6 (8.9)
Phenylalanine	3.9 (5.4)	4.7 (5.0)	7.9 (5.6)	8.7 (5.0)
Tryptophan	0.5 (0.6)	0.7 (0.7)	0.8 (0.6)	1.1 (0.7)
Lysine	3.3 (4.5)	4.6 (4.8)	6.7 (4.8)	7.0 (4.0)
Total	73.0 (100)	94.5 (100)	140.4 (100)	173.9 (100)

Data in parenthesis indicate a percentage of each amino acid to total amount.

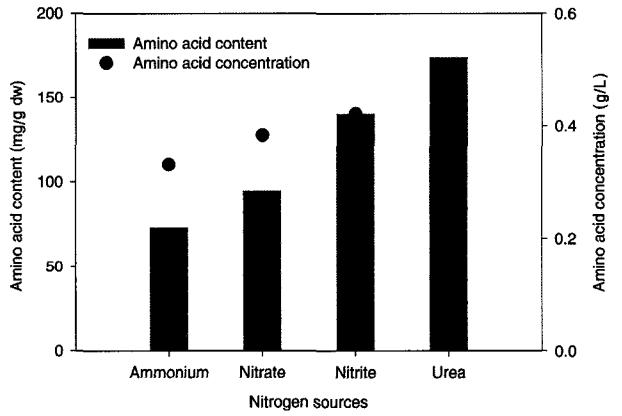

Fig. 4. Amino acid content and concentration in *Spirulina platensis* cultured with different nitrogen sources after 30 days of cultivation.

of cultivation was highest at 174 mg/g dry weight in the urea group, which also exhibited the highest concentration at 0.4913 g/L (Fig. 4).

As shown in Table 2, the contents of the essential amino acid for *S. platensis* were determined. After 30 days of cultivation, all groups of *S. platensis* contained 18 amino acids. The urea group particularly contained amino acids, including threonine (4.9%), valine (7.6%), methionine (1.5%), leucine (8.9%), phenylalanine (5.0%), tryptophan (0.7%), and lysine (4.0%). Meanwhile, *Spirulina maxima* contains 16 amino acids, eight of which are essential, including leucine (10.9% of total amino acids), valine (7.5%) and isoleucine (6.8%) [23]. A percentage of the total amount was analogous in the experiments conducted using different nitrogen sources ($P < 0.01$).

Accordingly, the current study confirmed that the use of different nitrogen sources during cultivation affected the growth and final products of *S. platensis* in various ways. Based on the growth rate, chlorophyll *a* concentration, dry weight, and amino acid content, the most efficient harvesting time for *Spirulina platensis* was found to be after approximately 10 days of cultivation.

Acknowledgement This research was supported by a grant (code DC1-104) from the Carbon Dioxide Reduction & Sequestration Research Center, a 21st Century Frontier Program funded by the Korean Ministry of Science and Technology.

REFERENCES

[1] Ciferri, O. (1983) *Spirulina*, the edible microorganism. *Microbiol. Rev.* 47: 551-578.
[2] Danesi, E. D. G., C. O. Rangel-Yagui, J. C. M. Carvalho, and S. Sato (2002) An investigation of effect of replacing nitrate by urea in the growth and production of chlorophyll by *Spirulina platensis*. *Biomass Bioenerg.* 23: 261-269.
[3] Koksharova, O. A. and C. P. Wolk (2002) Genetic tools for cyanobacteria. *Appl. Microbiol. Biotechnol.* 58: 123-137.
[4] Dillon, J. C., A. P. Phuc, and J. P. Dubacq (1995) Nutritional value of the alga *Spirulina*. *World Rev. Nutr. Diet.* 77: 32-46.
[5] Yang, C., Q. Hua, and K. Shimizu (2000) Energetics and carbon metabolism during growth of microalgal cells under photoautotrophic, mixotrophic and cyclic light-autotrophic/dark-heterotrophic conditions. *Biochem. Eng. J.* 6: 87-102.
[6] Olguín, E. J., S. Calicia, O. Angulo-Guerrero, and E. Hernández (2001) The effect of low light flux and nitrogen deficiency on the chemical composition of *Spirulina* sp. (*Arthrospira*) grown on digested pig. *Bioresource Technol.* 77: 19-24.
[7] Raven, P. H., R. F. Evert, and S. E. Eichhorn (1992) *Biology of Plants*. Worth Publishers, New York, USA.
[8] D'Souza, F. M. L. and G. J. Kelly (2000) Effects of a diet of a nitrogen-limited alga (*Tetraselmis suecica*) in growth, survival and biochemical composition of tiger prawn (*Penaeus semisulcatus*) larvae. *Aquaculture* 181: 311-329.

[9] Fresnedo, O. and J. L. Serra (1992) Effect of nitrogen starvation on the biochemistry of *Phormidium laminosum* (Cyanophyceae). *J. Phycol.* 28: 786-793.
[10] Gillor, O., A. Harush, O. Hadas, A. F. Post, and S. Belkin (2003) A *Synechococcus* P*glnA*::*luxAB* fusion for estimation of nitrogen bioavailability to freshwater cyanobacteria. *Appl. Environ. Microbiol.* 69: 1465-1474.
[11] Flores, E. and A. Herrero (1994) Assimilatory nitrogen metabolism and its regulation. pp. 487-517. In: D. A. Bryant (ed.). *The Molecular Biology of Cyanobacteria*. Kluwer Academic, Dordrecht, The Netherlands.
[12] Molloy, C. J. and P. T. Syrett (1988) Interrelationships between uptake of urea and uptake of ammonium by microalgae. *J. Exp. Mar. Biol. Ecol.* 118: 85-95.
[13] Admiraal, W., H. Peletier, and R. W. P. M. Laane (1986) Nitrogen metabolism of marine planktonic diatoms; excretion, assimilation and cellular pools of free amino acids in seven species with different cell size. *J. Exp. Mar. Biol. Ecol.* 98: 241-263.
[14] Ogawa, T. and G. Terui (1970) Studies on the growth of *Spirulina platensis*: I. On the pure culture of *Spirulina platensis*. *J. Ferment. Technol.* 48: 361-367.
[15] APHA, AWWA, WEF (1992) *Standard Methods for the Examination of Water and Wastewater*, 18th ed. American Public Health Association, Washington, DC, USA.
[16] Olguín, E.J., S. Galicia, R. Camacho, G. Mercado, and T. J. Pérez (1997) Production of *Spirulina* sp. in sea water supplemented with anaerobic effluents in outdoor raceways under temperate climatic conditions. *Appl. Microbiol. Biotechnol.* 48: 242-247.
[17] Crumpton, W. G., T. M. Isenhart, and P. D. Mitchell (1992) Nitrate and organic N analysis with second derivative spectroscopy. *Limnol. Oceanogr.* 37: 907-913.
[18] Shehawy, R. M. and D. Kleiner (2001) Nitrogen limitation. pp. 45-64. In: L. C. Rai and J. P. Gaur (eds.). *Algal Adaptation to Environmental Stresses: Physiological, Biochemical and Molecular Mechanisms*. Springer-Verlag, Berlin, Germany.
[19] Stanca, D. and E. Popovici (1996) Urea as nitrogen source in modified Zarrouk medium. *Rev. Roum. Biol. Ser. Biol. Veg.* 41: 25-31.
[20] Collier, J. L., S. K. Herbert, D. C. Fork, and A. R. Grossman (1994) Changes in the cyanobacterial photosynthetic apparatus during acclimation to macronutrient deprivation. *Photosynth. Res.* 42: 173-183.
[21] Miller, S. R., M. Martin, J. Touchton, and R. W. Castenholz (2002) Effects of nitrogen availability on pigmentation and carbon assimilation in the cyanobacterium *Synechococcus* sp. strain SH-94-5. *Arch. Microbiol.* 177: 392-400.
[22] Kaplan, D., A. E. Richmond, Z. Dubinsky, and S. Aaronson (1986) Algal nutrition. pp. 147-198. In: A. Richmond (ed.). *Handbook of Microalgal Mass Culture*. CRC Press, New York, USA.
[23] Cohen, Z (1997) The chemicals of *Spirulina*. pp. 175-204. In: A. Vonshak (ed.). *Spirulina platensis (Arthrospira) – Physiology, Cell-biology and Biotechnology*. Tayler & Ransis Ltd., London. UK.

[Received October 15, 2003; accepted December 17, 2003]