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Research Paper

Variation of *Spirulina maxima* biomass production in different depths of urea-used culture medium

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Abstract

Fewer studies have assessed the outdoor cultivation of *Spirulina maxima* compared with *S. platensis*, although the protein content of *S. maxima* is higher than *S. platensis*. *Spirulina* growth medium requires an increased amount of $NaHCO_3$, Na_2CO_3, and $NaNO_3$, which increases the production cost. Therefore, the current study used a low-cost but high-efficiency biomass production medium (Medium M-19) after testing 33 different media. The medium depth of 25 cm (group A) was sub-divided into A1 (50% cover with a black curtain (PolyMax, 12 oz ultra-blackout), A2 (25% cover), and A3 (no cover). Similarly the medium depths of 30 and 35 cm were categorized as groups B (B1, B2, and B3) and C (C1, C2, and C3), respectively, and the effects of depth and surface light availability on growth and biomass production were assessed. The highest biomass production was 2.05 g L^{-1} in group A2, which was significantly higher ($p < 0.05$) than that in all other groups and sub-groups. *Spirulina maxima* died in B1 and C1 on the fifth day of culture. The biochemical composition of the biomass obtained from A2 cultures, including protein, carbohydrate, lipid, moisture, and ash, was 56.59%, 14.42%, 0.94%, 5.03%, and 23.02%, respectively. Therefore, *S. maxima* could be grown outdoors with the highest efficiency in urea-enriched medium at a 25-cm medium depth with 25% surface cover or uncovered.

Key words: *Spirulina maxima*, fertilizer-grade urea, medium depth, surface cover, outdoor mass culture.

Introduction

Spirulina (Arthrospira) includes various species of primitive unicellular blue-green algae, most commonly *S. platensis* and *S. maxima*. *Spirulina* grows in shallow and highly alkaline water in tropical areas (Johnston, 1970). The Aztecs have consumed *Spirulina* in Mexico since the 16th century, the first tribe of hunter-collectors used it for food when they found it on margins of lakes, and even today, indigenous people consume *Spirulina* growing in African lakes. For the last 2-3 decades, millions of people around the world have enjoyed *Spirulina* as a safe food supplement. *Spirulina* is being commercially cultivated as a human food supplement, as an animal feed ingredient, and for pharmaceutical uses because of its ability to produce compounds such as carotene and omega 3 and 6 polyunsaturated fatty acids (Alonso and Maroto, 2000). *Spirulina* is also a good source of vitamin B12 with immune-promoting effects and antioxidant activity (Estrada *et al.*, 2001; Xue *et al.*, 2002). There are many advantages of cultivating *Spirulina* over traditional agriculture, such as high protein

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biomass, absence of processing by-product discard, suitability for arid or semi-arid areas of the world, and the fact that *Spirulina* can be cultured in saline water.

However, there are some disadvantages, mainly due to the mineral costs required to make suitable media for high biomass production. *Spirulina* is being cultured mostly in Zarrouk's or modified Zarrouk's medium or Society of Toxicology (SOT) medium, which are expensive because they require increased amounts of $NaHCO_3$, Na_2CO_3, $NaNO_3$, and trace metals. Some studies have been conducted using seawater enriched with different minerals (Lamela and Rocha, 2000; Tredici *et al.*, 1986). *Spirulina* sp. have also been cultured directly in human urine in China and in effluents from pig wastewater treatment plants in Korea (Lun and Cheng, 2006; Hong and Lee, 1993). The *Spirulina* grown in saline water is known as *S. maxima* and has higher protein content than does *S. platensis* (Oliveira *et al.*, 1999). There are fewer studies on *S. maxima* than on *S. platensis*, especially studies examining outdoor cultivation. In Hawaii, *S. pacifica*® has been developed from an *S. platensis* strain (Cyanotech, Kailua-Kona, HI).

In the present study, an *S. maxima* scale-up culture was conducted in a medium that was selected after 33 different culture media were tested using distilled water, underground water (UGW), natural seawater (NSW), and treated natural seawater (TNSW). Thereafter, the highest efficiency biomass production with the lowest cost medium was selected for scale-up mass culture. The best medium among the 33 media was made of urea, soil extract, and other nutrients from SOT medium. Urea appears to be an alternative nitrogen source that is less expensive than the conventional nitrogen source nitrate used to grow *Spirulina* sp. The nitrogen of urea is metabolized by cyanobacteria through enzymes such as urease and urea starch lyase (Meeks *et al.*, 1983). The two atoms of nitrogen yielded from urea are efficiently assimilated by microalgae (Faintuch and Sato, 1992). Urea levels higher than 300-500 mg L^{-1} strongly inhibited the growth of *Spirulina* in batch culture (Torre *et al.*, 2003). The area with dissolved urea particles becomes a zone of high pH with a higher ammonia concentration, which becomes quite toxic for several hours. At the end of the first step of the study, broken *S. maxima* trichomes with fewer coils (3-5 coils) and an ammonia odor were found in some culture jars containing urea-enriched medium. Costa *et al.* (2001) reported that ammonia or an ammonium complex compound might reduce the number of trichrome coils.

In addition, the accumulation of ammonia within the cell can occur due to low glutamine synthetase activity at low light intensities because of culture self-shadowing, and the accumulation of ammonia in cells represses urease activity (Costa *et al.*, 2001). In addition, it is often necessary to shadow the basins to prevent the temperature or light intensity becoming too high for the application of outdoor cultures of *Spirulina* (Bast, 1986). Therefore, the *S. maxima* scale-up culture was performed at different depths and with different surface light availability using the best growth medium to identify the optimum outdoor culture conditions. Finally, the effects of depth and light availability on specific growth rate, biomass production, biochemical composition, amino acid composition, and mineral content were investigated in cultured *S. maxima*.

Materials and Methods

Study site and estimate of environmental factors

This study was conducted at Chuuk State, which belongs to the Federated States of Micronesia (7°29' N, 151°50' E, Figure 1). The Chuuk climate is warm and tropical, with air temperatures ranging from 26 to 32 °C and water temperatures of approximately 28 to 30 °C throughout the year. In the first step of the study, 28 of 33 media were made using a soil extract instead of a trace metals solution. A local soil (100 g) was diluted with 1 L of UGW and

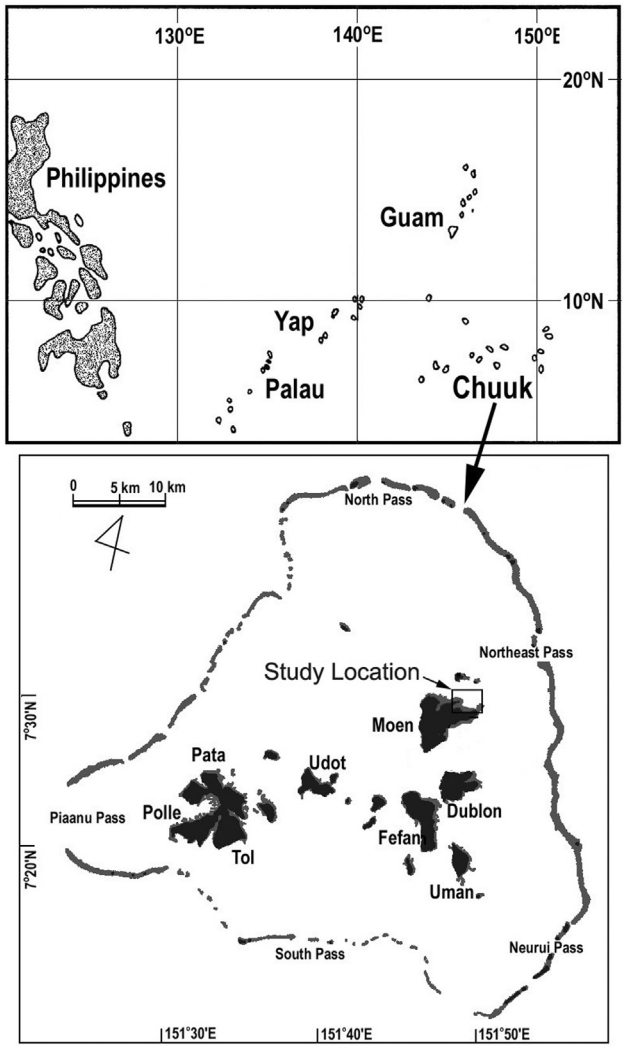

Figure 1 - Location of the study area.

heated at 90 °C for 2 h. The soil-diluted turbid water was kept overnight at room temperature, and a 2% supernatant was added with water to create a media with the nutrients described in Table 1. The micronutrient and vitamin solution (Microvitsol) was produced by dilution of a solution with the following chemical composition: 10.00 mg L^{-1} H_3BO_3, 2.00 mg L^{-1} $MnSO_4.5H_2O$, 1.00, mg L^{-1} $ZnSO_4.7H_2O$, 1.00 mg L^{-1}, $Co(NO_3)_2.6H_2O$, 1.00, mg L^{-1} $Na_2MoO_4.2H_2O$, 0.01 mg L^{-1} $CuSO_4.5H_2O$, and 0.0001 mg L^{-1} cyanocobalamin. Then, 1 mL of Microvitsol was added to SOT, M-2, M-4, M-6, and M-20 media, respectively. In addition, media from M-21 to M-32 were made using TNSW, which was prepared after pretreating NSW with 8.00 g L^{-1} of $NaHCO_3$. All media were prepared according to the recipes described in Table 1. All of the major nutrients ($NaHCO_3$, $NaNO_3$, Na_2CO_3, and K_2HPO_4) were food grade, and all micronutrients were laboratory grade. Cooking salt (KOOKA iodized cooking salt, Cheetham Salt Ltd, Australia) was used instead of NaCl in M-8 to M-19 media (Table 1).

Spirulina maxima was grown outdoors for 2 weeks in a 10-L transparent plastic jar with two replications (R1 and R2). The biomass, pH, and salinity were measured during inoculation and at the end of the study. Therefore, the second step of the study was conducted for scale-up based on the highest biomass production with the cheapest medium, which was the M-19 medium. Fiberglass-reinforced plastic tanks (95 x 46 cm) were used with different depths and surface covers. The tanks were filled with culture medium at depths of 25, 30, and 35 cm and a black curtain (PolyMax, 12 oz ultra-blackout) was used to cover the surface of the tanks at the rate of 50%, 25%, and 0% or no cover. The tanks with culture medium at a depth of 25 cm (A group) were categorized as A1 (25 cm depth with 50% cover), A2 (25 cm depth with 25% cover), and A3 (25 cm depth without cover). Culture tanks with depths of 30 and 35 cm were categorized as groups B (B1, B2, and B3) and C (C1, C2, and C3), respectively. Continuous aeration was supplied to the culture with an aeration pump at a maximum airflow of 3.32 cfm. The light intensity was measured at 0900, 1300, and 1700 with a light meter (Lux/Fc light meter 205, Tenmars Electronics, Taipei, Taiwan). The lux data were converted to μmol photons m^{-2} s^{-1} according to the formula described by Clayton (Pierre *et al.*, 2008). Temperature, pH, and salinity were measured with a YSI probe (MPS556, YSI, Inc., Yellow Springs, OH, USA) every other day when samples were collected for estimating biomass.

Determination of specific growth and biomass production

The culture medium pH was maintained for 12 h after adjusting the initial pH to 7.5 by adding NaOH or HCl. Approximately 0.015 g L^{-1} (dry weight) *S. maxima* was inoculated and grown for 16 days. A 20-mL sample was collected from each culture tank every other day to estimate the biomass. The sample was filtered through preweighed GF/F Whatman filter paper. A preweighed filter paper that was soaked in distilled water and dried at the same time was used as a blank. The biomass filter paper was kept at 55 °C in an oven, dried and weighed, and the dry weight biomass was calculated as g L^{-1}, which was plotted as a growth curve. The specific growth rate (μ) was defined as the increase in biomass per unit time and calculated using the following formula (Pirt, 1975).

$$\mu\ (\text{day}^{-1}) = \frac{\ln\left(\frac{X_1}{X_0}\right)}{t_1 - t_0}$$

where X_0 and X_1 are the biomass at the beginning (t_0) and the end (t_1) of a selected time interval between inoculation and maximum biomass production.

Microscopic observation

Contamination with other plankton (phytoplankton or zooplankton) and the number of coils in an *S. maxima* trichome were observed under an inverted light microscope (Olympus, CKX41, Tokyo, Japan). A 10-mL sample was collected at the end of the study; 4 to 6 drops of live sample were gently put on a glass slide and mixed properly, and 2 to 3 drops were covered with a cover slip. The presence of contaminants was then assessed at 400x magnification. Broken and lysed trichomes with fewer coils were found in some cultures in urea-enriched media in the first step of the study. Therefore, the number of coils in trichomes was counted in outdoor cultures to assess the variation among the culture conditions. For counting the number of coils, 1 mL of sample was placed on a Sedgwick-Rafter counter chamber, and the number of coils was counted for 50 randomly selected trichomes in each culture. Finally, the entire biomass was collected after filtering through a 20-μm mesh plankton net and was freeze dried for further study.

Biochemical composition

The biomass from the best growth condition was selected for biochemical composition analysis, and carbohydrate, protein, lipid, moisture, and ash contents were determined following the methods of the Association of Official Analytical Chemists AOAC (1995). Crude lipid was determined by Soxhlet extraction; crude protein, by the Kjeldahl method; ash, by calcinations in a furnace at 550 °C; and moisture by heating to 105 °C for 20 h.

Amino acid composition was determined following the AOAC method. Briefly, a 10-g sample was dehydrated with acetone and dried on filter paper at 60 °C in a drying oven. The dried materials were hydrolyzed in 6 N HCl for 24 h at 110 °C. After cooling, the samples were washed with 0.01 N HCl, and 2 N NaOH was added for neutralization. Supernatants were collected by centrifugation at 5,000 rpm for 30 min. The supernatants were evaporated

Table 1 - *Spirulina maxima* culture media, medium component chemicals (g L^{-1}), soil extract (SE; 2mL L^{-1}), Micronutrients and vitamins solution (Microvitsol. 1 mL L^{-1}) and biomass production per liter (g L^{-1}) at the 1st step of experiment.

No.	$NaHCO_3$	Na_2CO_3	K_2HPO_4	K_2SO_4	$MgSO_4.7H_2O$	$FeSO_4.7H_2O$	Na_2-EDTA	$NaNO_3$	Urea	NaCl	Cooking salt	$CaCl_2.2H_2O$	Microvit sol	SE	Water	Biomass
SOT	13.61	4.03	0.50	1.00	0.20	0.01	0.08	2.50	NA	1.00	NA	0.04	*	NA	DW	2.35 ± 0.04
M-1	13.61	4.03	0.50	1.00	0.20	0.01	0.08	2.50	NA	1.00	NA	NA	NA	*	DW	2.32 ± 0.06
M-2	13.61	4.03	0.50	1.00	0.20	0.01	0.08	2.50	NA	1.00	NA	0.04	*	NA	UG	2.29 ± 0.02
M-3	13.61	4.03	0.50	1.00	0.20	0.01	0.08	2.50	NA	1.00	NA	NA	NA	*	UG	2.31 ± 0.02
M-4	13.61	4.03	0.50	1.00	0.20	0.01	0.08	NA	2.50	1.00	NA	0.04	*	NA	DW	2.250.13
M-5	13.61	4.03	0.50	1.00	0.20	0.01	0.08	NA	2.50	1.00	NA	NA	NA	*	DW	2.27 ± 0.04
M-6	13.61	4.03	0.50	1.00	0.20	0.01	0.08	NA	2.50	1.00	NA	0.04	*	NA	UG	2.28 ± 0.02
M-7	13.61	4.03	0.50	1.00	0.20	0.01	0.08	NA	2.50	1.00	NA	NA	NA	*	UG	2.24 ± 0.03
M-8	5.00	3.00	0.50	1.00	0.20	0.01	0.08	2.50	NA	NA	1.50	NA	NA	*	UG	1.81 ± 0.09
M-9	5.00	2.00	0.50	1.00	0.20	0.01	0.08	2.50	NA	NA	1.50	NA	NA	*	UG	1.79 ± 0.03
M-10	5.00	1.00	0.50	1.00	0.20	0.01	0.08	2.50	NA	NA	1.50	NA	NA	*	UG	1.76 ± 0.02
M-11	5.00	3.00	0.50	1.00	0.20	0.01	0.08	NA	2.50	NA	1.50	NA	NA	*	UG	1.95 ± 0.04
M-12	5.00	2.00	0.50	1.00	0.20	0.01	0.08	NA	2.50	NA	1.50	NA	NA	*	UG	1.68 ± 0.07
M-13	5.00	1.00	0.50	1.00	0.20	0.01	0.08	NA	2.50	NA	1.50	NA	NA	*	UG	1.74 ± 0.08
M-14	6.00	3.00	0.50	1.00	0.20	0.01	0.08	2.50	NA	NA	1.50	NA	NA	*	UG	1.89 ± 0.03
M-15	6.00	2.00	0.50	1.00	0.20	0.01	0.08	2.50	NA	NA	1.50	Na	NA	*	UG	1.79 ± 0.11
M-16	6.00	1.00	0.50	1.00	0.20	0.01	0.08	2.50	NA	NA	1.50	NA	NA	*	UG	1.74 ± 0.08
M-17	6.00	3.00	0.50	1.00	0.20	0.01	0.08	NA	2.50	NA	1.50	NA	NA	*	UG	1.98 ± 0.01
M-18	6.00	2.00	0.50	1.00	0.20	0.01	0.08	NA	2.50	NA	1.50	NA	NA	*	UG	2.01 ± 0.03
M-19	6.00	1.00	0.50	1.00	0.20	0.01	0.08	NA	2.50	NA	1.50	NA	NA	*	UG	2.03 ± 0.02
M-20	13.61	4.03	0.50	1.00	0.20	0.01	0.08	2.50	NA	NA	NA	NA	*	NA	TNS	X
M-21	5.00	3.00	0.50	1.00	0.20	0.01	0.08	2.50	NA	NA	NA	NA	NA	*	UG:TNS (1:1)	0.95 ± 0.03
M-22	5.00	2.00	0.50	1.00	0.20	0.01	0.08	2.50	NA	NA	NA	NA	NA	*	UG:TNS (1:1)	1.02 ± 0.07
M-23	5.00	1.00	0.50	1.00	0.20	0.01	0.08	2.50	NA	NA	NA	NA	NA	*	UG:TNS (1:1)	0.98 ± 0.19
M-24	5.00	3.00	0.50	1.00	0.20	0.01	0.08	NA	2.50	NA	NA	NA	NA	*	UG:TNS (1:1)	0.86 ± 0.03
M-25	5.00	2.00	0.50	1.00	0.20	0.01	0.08	NA	2.50	NA	NA	NA	NA	*	UG:TNS (1:1)	0.90 ± 0.08
M-26	5.00	1.00	0.50	1.00	0.20	0.01	0.08	NA	2.50	NA	NA	NA	NA	*	UG:TNS (1:1)	0.89 ± 0.02
M-27	6.00	3.00	0.50	1.00	0.20	0.01	0.08	2.50	NA	NA	NA	NA	NA	*	UG:TNS (1:1)	0.82 ± 0.03
M-28	6.00	2.00	0.50	1.00	0.20	0.01	0.08	2.50	NA	NA	NA	NA	NA	*	UG:TNS (1:1)	0.89 ± 0.02
M-29	6.00	1.00	0.50	1.00	0.20	0.01	0.08	2.50	NA	NA	NA	NA	NA	*	UG:TNS (1:1)	0.85 ± 0.01
M-30	6.00	3.00	0.50	1.00	0.20	0.01	0.08	NA	2.50	NA	NA	NA	NA	*	UG:TNS (1:1)	0.68 ± 0.02
M-31	6.00	2.00	0.50	1.00	0.20	0.01	0.08	NA	2.50	NA	NA	NA	NA	*	UG:TNS (1:1)	0.75 ± 0.05
M-32	6.00	1.00	0.50	1.00	0.20	0.01	0.08	NA	2.50	NA	NA	NA	NA	*	UG:TNS (1:1)	0.72 ± 0.09

*Indicates adding component, NA, Not adding; DW, Distilled water; UG, Underground water; TNS, Treated natural seawater and X, die off *S. maxima*.

with nitrogen gas at 60 °C and dissolved with 0.02 N HCl. The samples were filtered with a 0.45-µm filter and analyzed on a Beckman 6300 automated amino acid analyzer (Beckman Coulter, Fullerton, CA, USA). Areas of amino acid standards were used to calculate the quantity of each amino acid in the samples. Mineral content was also determined by AOAC methods using an inductively coupled spectrometer (Perkin Elmer Instruments, Shelton, CT, USA). *Spirulina maxima* was ashed at 550 °C for 6 h. The ash was dissolved in HNO_3, filtered, and diluted with distilled water. Thereafter, the absorbance of the sample was read directly on the spectrometer.

Statistical analysis

The mean values of each treatment for temperature, pH, salinity, light intensity, specific growth, and biomass production were compared using one-way analysis of variance (ANOVA) followed by Tukey's test. A P-value of < 0.05 was considered to be significant. All statistical analyses were performed using SPSS Statistical Software, version 12.0, 12.0.1 (Edinburgh, Scotland).

Results

Dynamics of environmental factors

During the study, the sunlight intensity varied from 479 to 2,492, 429 to 3,300, and 182 to 660 µmol photon $m^{-2}s^{-1}$ at 0900, 1,300, and 1,700, respectively, with the peak at 1,300 on the sixth and eighth days of the study (Figure 2). The temperature ranged from 28.20 to 34.00 °C, with an average of 30.62 °C. The highest temperature was in tank A3, and the lowest was in C1 tank (Figures 3A). The temperature was highest in the uncovered tanks, followed by the 25% and 50% covered tanks. Salinity fluctuated from 10.72 to 13.56 psu, and the highest salinity was in tank A3 (Figure 3B). The pH varied from 7.50 to 8.49 in the A group tanks, with the highest pH in A2, followed by A1 and A3; in the B and C group tanks, the pH values were 8.76 and 8.74 in B2 and C2, respectively, although B1 and C1 had the highest pHs of 8.98 and 8.83, respectively, during culture die-off (Figures 3C).

Specific growth rate and biomass production

The maximum specific growth rate (μ_{max} d^{-1}) was estimated as an informative way to ascertain microbial activity, which can increase at exponential rates. Significant biological information on *Spirulina* mass culture can be obtained by determining growth characteristics under controlled conditions, and this information can then be applied to create a high-density mass-culture system. The μ_{max} d^{-1} of *S. maxima* varied from 0.34 to 0.35, 0.33 to 0.36, and 0.31 to 0.35 d^{-1} among the A, B, and C culture groups, respectively; the highest μ_{max} d^{-1} was in B2 (Figure 3D). *Spirulina maxima* grew well in the entire A group tanks; biomass production varied from 1.80 to 2.05 g L^{-1} and was highest in A2, followed by A3 and A1 (Figure 3D). In the B group tanks, the biomass production ranged from 0.55 to 1.55 g L^{-1}, with the highest value in B3, followed by B2 (Figure 3D). In the C group tanks, the highest biomass was 1.15 g L^{-1} in C3 (Figure 3D). Among all nine culture conditions, A2 had the highest *S. maxima* biomass production.

Microscopic observation

Microscopic observations were performed to confirm the presence of other plankton in the culture and to count the numbers of trichomes coils of *S. maxima*. Microscopic observation of an *S. maxima* live sample revealed that the culture was a monostrain at the end of the study. The coil numbers in trichomes varied from 5 to 14 in the A culture

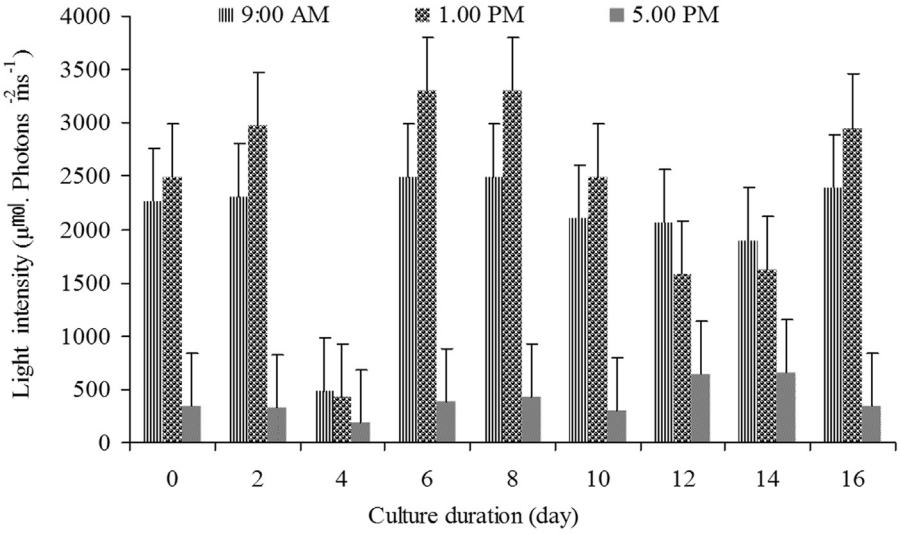

Figure 2 - Variation in daily sunlight intensity during the culture of *Spirulina maxim* in the FRP tanks. Values are mean ± SE.

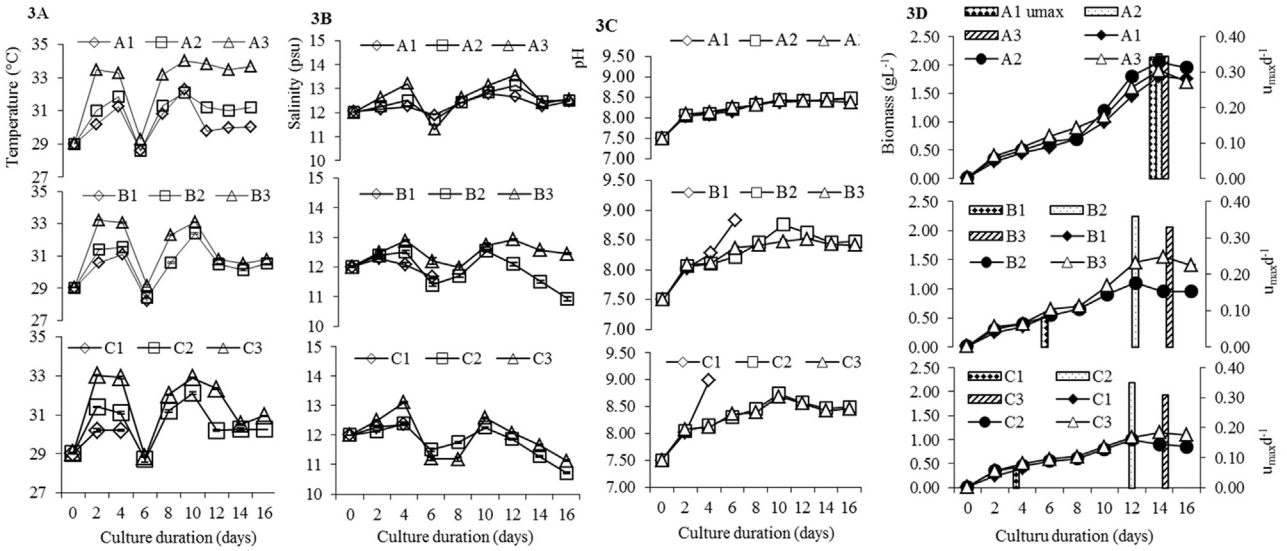

Figure 3 - (3A) Variation of temperature (°C) at depths of 25, 30, and 35 cm with 50, 25, and 0% surface shadow in the *S. maxima* culture FRP tanks. Culture tank conditions: A1 (25-cm depth with 50% surface shadow), A2 (25-cm depth with 25% surface shadow), and A3 (25-cm depth with 0% surface shadow). The covers of B (30-cm depth) and C (35-cm depth) were identical to those of A. (3B) Variation in salinity, (3C) variation in pH during different *S. maxima* culture conditions, and (3D) variation in maximum specific growth rate (μ_{max}, indicated in bars) and growth curve with biomass production (g L^{-1}, indicated in lines) of *S. maxima* at different depths and surface shadow cover. Values are mean ± SE.

group, with the maximum number in A2, and average coil numbers of 8.15 ± 1.60, 8.70 ± 2.30, and 7.75 ± 1.52 in A1, A2, and A3, respectively. Four to six coils were observed in the B group tanks, with the maximum number found in B3 (6.42 ± 0.15), followed by average values of 5.18 ± 1.1 in B2. In the C group tanks, there were 4 to 5 coils with averages of 4.29 ± 0.93 and 4.48 ± 1.53 in C2 and C3, respectively (Figure 4).

Biochemical and amino acid composition and mineral content

In the present study, the *S. maxima* biochemical composition was as follows: protein 56.59%, carbohydrate 14.42%, lipid 0.94%, moisture 5.03%, and ash 23.02% on a dry weight basis in the biomass of the A2 culture tank. Ten essential amino acids (threonine, valine, methionine, isoleucine, tyrosine, phenylalanine, lysine, histidine, tryptophan, and arginine) made up 5.26, 6.63, 1.37, 5.94, 4.57,

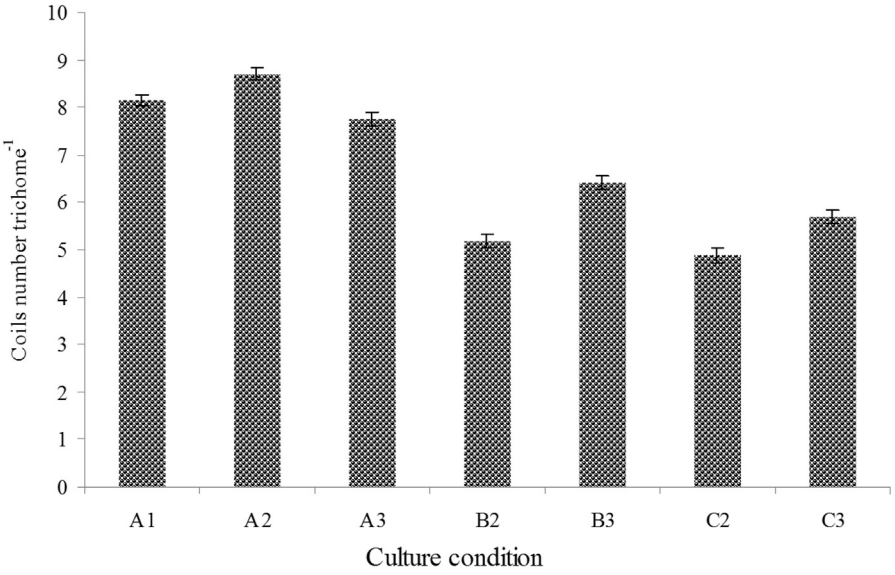

Figure 4 - Number of coils in the trichomes of *Spirulina maxima* grown at different depths and surface shadow covers (see Fig. 3 for culture conditions). Values are mean ± SE.

5.03, 5.26, 1.6, 0.07, and 6.86% of the *S. maxima* biomass, respectively, while eight nonessential amino acids (aspartic acid, serine, glutamic acid, proline, glycine, alanine, cystine, and leucine) made up 9.83, 5.03, 15.08, 3.43, 5.48, 8.23, 0.27, and 9.83%, respectively (Figure 5). Analysis of mineral content revealed that the Na, K, Fe, Mg, Ca, Mn, Zn, and P contents were 65.80, 24.26, 5.34, 3.52 2.38, 0.03, 0.01, and 13.31 mg 100 g^{-1}, respectively (Figure 6).

Discussion

Effects of environmental factors

Spirulina maxima grew well in all tanks of the A group with different biomasses. Meteorological factors had a large influence on the growth of *S. maxima*, especially cloudy sky and rainfall in the outdoor mass culture in urea nitrogen medium. On the fourth and fifth days of the study, the sky was cloudy and there was heavy rain. During rainy days, the sunlight intensity decreased to the same intensity as that at 1,700 on a sunny day. The water depth in all of the tanks increased 3-5 cm due to rain, and the salinity and temperature of each tank decreased approximately 1.00 psu and 3 °C, respectively. The 35-cm deep tanks increased to 38 to 41 cm. On rainy days, a strong ammonia odor came from the 30- to 35-cm-deep tanks with 50% surface covers, and higher pH values (8.98 and 8.83 in B1 and C1) were observed in those tanks. A weak ammonia odor came from the tanks with depths greater than 30 cm with a 25% surface cover or uncovered. The *S. maxima* culture died in the tanks in which the ammonia odor was very strong. Urea was partly hydrolyzed to ammonia under alkaline conditions and lost by off gassing (Costa *et al.*, 2001). Total ammonia in aqueous solution consists of two principal forms, the ammonium ion (NH_4^+) and un-ionized ammonia (NH_3). The form depends on the pH, with ammonium (NH_4^+) predominating when the pH is below 8.75 and ammonia (NH_3) predominating above pH 9.75 (Hydrolab, a Hatch Company). In addition, optimum activity of urease probably occurs in the pH range of 7 to 9, with little activity above a pH of 9.5 (Bast, 1986). Accumulation of ammonia within the cell can occur due to low glutamine synthetase activity at low light intensity because of self-shadowing of cultures, and accumulation of ammonia in cells represses urease activity (Costa *et al.*, 2001). NH_3 is quite toxic to seeds or plantlets because it is uncharged and lipid-soluble (AOAC, 1995), and thus traverses biological membranes more readily than the charged and hydrated NH_4^+ ions (Swigert, 1984; Wuhrmann and Woker, 1948). Therefore, it was assumed that the *S. maxima* died as a result of the ammonia derived from urea because of less light availability due to a cloudy sky, less surface area exposed to light due to the covers, or less light penetration due to greater depths. Additionally, the ammonia concentration increased due to weak mixing at increased depths during rainy days.

Specific growth rate and biomass production

Except for the *Spirulina* die-off tanks, the specific growth rate of *S. maxima* showed no significant ($p < 0.05$) differences among tanks of the A group. Biomass production was significantly higher ($p < 0.05$) in the A2 among group as well as in all culture group tanks. The $\mu_{max}d^{-1}$ and biomass production of *S. maxima* in this study were low in comparison with that in the SOT medium in which μ_{max} was 0.40 d^{-1} and 2.70 g L^{-1} maximum biomass production of *S. maxima* (Tredici *et al.*, 1986; Oliveira *et al.*, 1999). Oliveira *et al.* (1999) reported that *S. maxima* growth rate and biomass production were 0.45 d^{-1} and 2.40 g L^{-1} at 35 °C, respectively, under a continuous light condition in a Paoletti

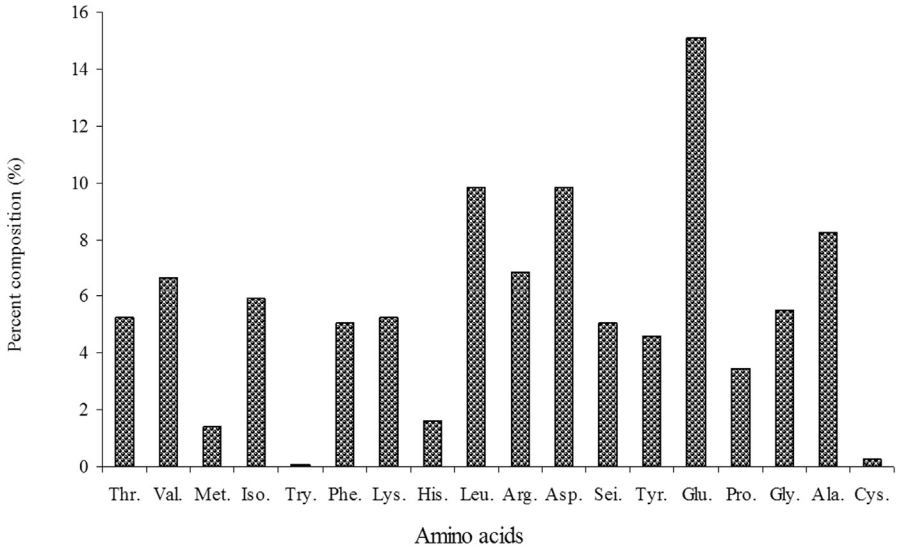

Figure 5 - Percent composition of mineral content of *Spirulina maxima* grown in A2 culture conditions. Values are mean ± SE.

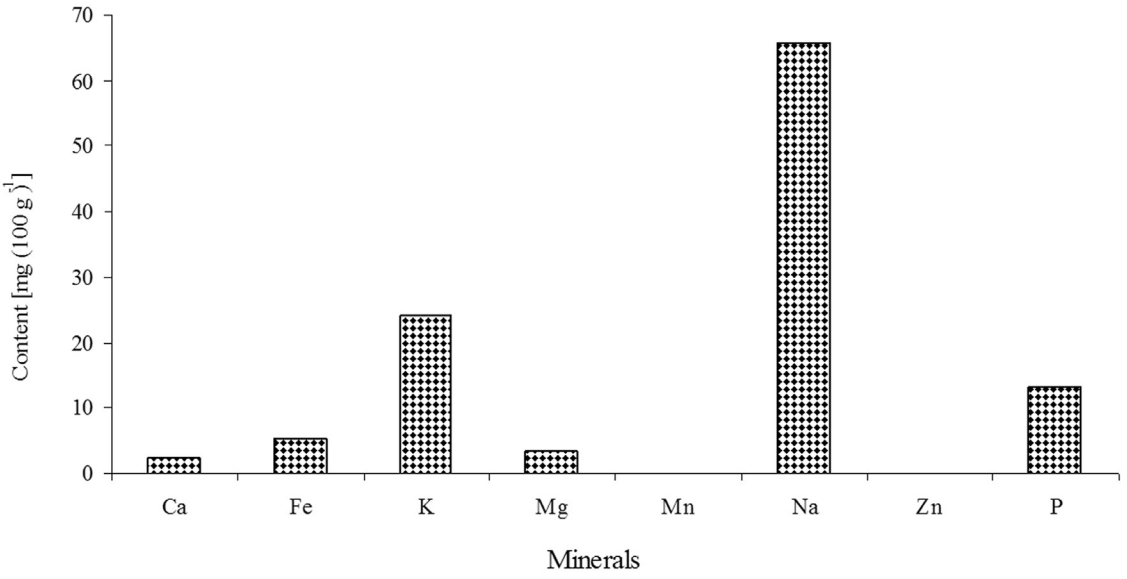

Figure 6 - Essential and nonessential amino acid composition of *Spirulina maxima* grown in A2 culture conditions. Values are mean ± SE.

medium (Downing and Merkens, 1955). The biomass production was significantly ($p > 0.05$) higher in B3 than C3 in tanks from groups B and C, respectively. However, the *S. maxima* production differed with variation in the surface cover in M-19 culture medium at the same depth. Biomass production from the A2 tanks was higher than the biomass production of *S. platensis* in urea using Zarrouk's medium, in which the biomass was 0.91 g L^{-1} in a microenvironment culture condition (Costa *et al.*, 2001) or urea-fed batch cultured biomass (Costa *et al.*, 2001). In most published studies, urea was used in culture media having a pH of 9.0 or above which is the alkaline condition triggering NH_3 formation rather than NH_4^+ and decreasing or stopping urease activity, as discussed above (Costa *et al.*, 2001; Sánchez-Luna *et al.*, 2006; Sánchez-Luna *et al.*, 2004; Abeliovich and Azov, 1976). In the A culture group, pH did not exceed 8.5, which might be the optimum condition in which to use urea for *S. maxima* growth.

Biomass production of *S. maxima* in M-19 medium is economically more viable, as the price of the chemicals is lower than that of Zarrouk's, SOT, or Paolett medium. Moreover, the culture system will also be effective to grow *S. maxima* under natural light conditions, as urea uptake by microalgae is greater in the light than in the dark (Stanca and Popovici, 1996). Therefore, a 25-cm culture medium depth with a surface cover of 50%, 25%, or 0% in regular sunlight will be a suitable condition for *S. maxima* growth in M-19 culture medium.

Microscopic observation

The presence of other plankton in the culture can create competition for nutrients or can be toxic if the contaminant species is toxic, like toxic cyanobacteria. In this study, the culture was contaminant-free. A culture medium prepared with 1.5% table salt using UGW protects the *Spirulina* from contamination with other plankton (Hodson and Thompson, 1968). Microscopic observation of a *S. maxima* live sample revealed that the culture was a monostrain at the end of the experiment. The number of coils in the trichomes varied among the A, B and C culture groups. There were more coils in the A group tanks than in the B and C group tanks, and the number of coils in the C tanks was almost half that in the A tanks. Short and broken *S. maxima* trichomes were found in tanks with a higher pH and an ammonia odor. The occurrence of ammonia in association with higher pH might have an adverse effect on the coils formation in trichomes. Similarly, Costa *et al.* (2001) found broken trichomes and lysed *S. platensis* cells in a culture medium composed of 0.01 M ammonium acid phosphate.

Biochemical and amino acid composition and mineral content

Information on the biochemical composition of *Spirulina* sp. grown under specific culture conditions is important for profitable commercial production, as the chemical composition may differ with variations in the physico-chemical conditions of the culture system. For example, *S. maxima* protein content was 70.24, 68.01, 68.67, 64.58, and 62.81% at temperatures of 20, 25, 30, 35, and 40 °C, respectively (Oliveira *et al.*, 1999). Low protein synthesis and high carbohydrate content were found in *S. maxima* grown in seawater due to physiological stress (Lamela and Rocha, 2000). In our study, the protein and carbohydrate content of *S. maxima* also showed similar results, which might have been related to the use of cooking salt containing 50 mg g^{-1} potassium iodate as well as other unspecified contents rather than NaCl alone.

Protein content and amino acid composition also varied in *S. platensis* (Kim *et al.*, *2007*). The amino acid analysis revealed that leucine was proportionally the highest (9.83%), followed by arginine, valine, and isoleucine. Leucine is very important for health, as it is used for growth and repair of muscle tissue. It also helps to prevent muscle protein breakdown. Additionally, leucine is directly linked to the maintenance of glucose homeostasis by enhancing glucose recycling via the glucose-alanine cycle and is directly linked to the translational regulation of muscle protein synthesis through the insulin signaling cascade (Volkmann *et al.*, 2008). Similarly, the non-amino acid *S. maxima* analysis showed that glutamic acid was 15.08%, followed by aspartic acid and alanine. Glutamic acid is a precursor of gamma-aminobutyric acid, which functions as an inhibitory neurotransmitter. It is one of the few nutrients that crosses the blood-brain barrier, and it is the only means by which ammonia in the brain can be detoxified. It is considered nature's "brain food" due to its association with improved mental capacity. Therefore, more leucine and glutamic acid can be obtained from *S. maxima* using this culture methodology. Among the *S. maxima* minerals, the amount of potassium was second highest in ranking. High potassium diets are beneficial for protecting against cardiovascular disease partly because of their blood-pressure-lowering effect. Low blood pressure, decreased renal tubulointerstitial injury, and suppressed renal inflammation were observed in male Sprague-Dawley rats after 8 weeks of feeding a potassium-supplemented diet (2.1% potassium) (Layman, 2003). Thus, abundant potassium can be obtained from *S. maxima* as a dietary source.

Conclusions

Spirulina maxima grew well at a depth of 25 cm in M-19 medium consisting of urea, soil extracts, and other chemicals. The highest biomass production was 2.05 g L^{-1} at a depth of 25 cm in a tank with 25% surface cover. In terms of quality, the protein content and amino acid composition were remarkable as regards to reported study elsewhere. Moreover, cost-wise calculations suggest that the preparation of 1,000 L of SOT medium would cost KRW 164205 ($147 USD), compared with KRW 61093 ($55 USD) for M-19 medium. In addition to this low cost, M-19 medium also yields high *S. maxima* production. In contrast, less light availability due to medium depth (> 30 cm) or meteorological conditions might have stimulated the formation of unionized ammonia (NH_3), which might have been responsible for breaking, whitening trichomes and the death of *S. maxima*. Therefore, outdoor mass culture of *S. maxima* at a 25-cm depth in M-19 medium is suitable and profitable for home consumption and/or industrial-scale production, as most of the major ingredients were low-cost and locally available.

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