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The Effect of the Environmental Factors on the Vitamin C (Ascorbic Acid), E (Alpha-tocopherol), β-carotene Contents and the Fatty Acid Composition of *Spirulina platensis*

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Özet: ***Çevre faktörlerinin* Spirulina platensis'*in C (askorbik asit) ve E (Alfa-tokoferol) vitaminleri, β-karoten içeriği ve yağ asidi kompozisyonuna etkisi.*** Bu çalışma, subtropik bir bölgede mevsime bağlı iklim değişikliğinin havuzlarda kültüre alınan *Spirulina platensis*'in C (askorbik asit) ve E (alfa-tokoferol) vitaminleri, β- karoten içeriği ve yağ asiti kompozisyonuna etkilerini incelemek amacıyla yürütülmüştür. Işık yoğunluğu, pH ve tuzluluk günlük olarak ölçülürken, sıcaklık ve çözünmüş Oksijen ölçümleri gece ve gündüz yapılmıştır. Yaz mevsiminde ortalama gündüz sıcaklığı 33.9±0.4 ºC olarak belirlenirken kış mevsimi için 18.6±0.5 ºC değeri belirlenmiştir. Yaz ve kış için gece ortalama sıcaklık değerleri ise sırasıyla 29.9±0.2 ºC ve 14.4±0.2 ºC olarak saptanmıştır. Ortalama ışık yoğunluğu yaz mevsiminde 848.3 µmol/m^2/s iken kışın 506.26±48 µmol/m^2/s olmuştur. Kış büyüme döneminde *S. platensis*'e ait C vitamini içeriği 39.31±3.63 mg/100 g ile yaz dönemine gore daha yüksek bulunmuştur. Alfa-tokoferol içeriği, 6.57±1.18 mg/100 g ile yazın daha yüksek saptanmıştır. B-karoten miktarı ise yaz ve kış mevsimleri için benzer bulunmuştur. Yağ asitleri kompozisyonu çevre koşullarından önemli ölçüde etkilenmiştir. En yüksek γ-linolenik asit miktarı (22.221±0.388 %) yaz koşullarında saptanmıştır.

Anahtar Kelimeler: *Spirulina platensis*, alfa-tokoferol, β-karoten, yağ asitleri, C vitamini.

Abstract: The purpose of this study was to clarify the seasonal variation of vitamin C (ascorbic acid), E (alpha-tocopherol), β-carotene contents and fatty acid composition of *Spirulina platensis* grown in the ponds in the subtropic area. While the light intensity, pH and salinity were measured daily, the temperature and dissolved oxygen were measured in day and night. The mean day temperatures were 33.9±0.4 ºC in summer and 18.6±0.5 ºC in winter. The mean night temperatures were measured 29.9±0.2 ºC and 14.4±0.2 ºC in summer and winter, respectively. The mean light intensity of 848.3 µmol/m^2/s was determined in summer. It was 506.26±48 µmol/m^2/s in winter. The vitamin C content (39.31±3.63 mg/100 g) of *S. platensis* grown in winter was found to be higher than in summer. The alpha-tocopherol content (6.57±1.18 mg/100 g) was higher in summer. However, -carotene contents were found to be similar both in summer and winter. Fatty acid composition was affected from the ambient factors significantly. The higher γ-linolenic acid content (22.221±0.388 %) was found in summer.

Key Words: *Spirulina platensis*, alpha-tocopherol, β-carotene, fatty acids, vitamin C.

Introduction

Spirulina platensis, a filamentous cyanobacterium, is widely used in many countries as health food due to its protein content and biochemical substances for immune system. Temperature is one of the major factors controlling the multiplication of *Spirulina* species. The optimum temperature for *Spirulina* growth lies in the range of 30 to 35 ºC. In winter, *Spirulina* does not grow significantly in open ponds (except in tropics), resulting in lower yields (Richmond, 1992). In order to enhance culture conditions and to lower the costs, algae manufacturers frequently cover the ponds with transparent polythylene to keep the medium warmer and to reduce the risk of contamination (Vonshak, 1992). Low temperatures cause a decrease in *Spirulina* growth. Therefore, the regions where winter temperatures are below 15 ºC are not suitable to grow *Spirulina* (Richmond et all, 1990). It is known that the environmental conditions, especially culture temperature, greatly influence the composition and physiological state of phytoplankton (Reynolds, 1984), in particular, changing fatty acids (FA) metabolism, vitamins and carotenoids contents in the cells (*5-8*). *Spirulina* contains high level of γ-linolenic acid (C18:3) that is associated with pharmaceuticals and nutraceuticals (Cohen et all, *1987*). The other important factor pH also determines the carbon dioxide solubility and minerals in the medium, directly or indirectly influencing the metabolism of algae (Becker, 1993). Light is the other environmental factor which affects to the cell composition. The chlorophyll *a* and other light-harvesting pigments response to the low light intensity with the increase. On the other hand, in response to high light intensity secondary carotenoids increase (Ben-Amotz et all, 1982). Numerous studies with microalgae of various groups suggest that the cellular content of lipids and total polyunsaturated fatty acids is inversely related to growth light intensity (Cohen et all, *1987*).

The aim of this work was to compare the influence of

physico-chemical variations on the vitamin C (ascorbic acid), E (alpha-tocopherol), β-carotene contents and the FA composition of the cyanobacterium *Spirulina platensis*, grown in the subtropic region.

Materials and Methods

Spirulina platensis was used in this study. The starter culture was obtained from Ben Gurion University of the Negev, The Jacob Blaustein Institue for Desert Research, Israel. *Spirulina* stock cultures were maintained at 30±2 °C on continuous illumination with flourescent (Phlips) lights in erlenmayers (250 mL, 500 mL, 2 L) and carboys (5 L and 10 L) in laboratory conditions. The irradiance, as measured by a Radiation Sensor LI-COR (LI-250), was 80 µmol/m^2/s. The stock cultures in 10 L volume were transferred to outdoors earlier 24 hours from inoculation.

The cultures were grown in *Spirulina* medium. The content of the medium consists of the following composition (g/L): 18.6 $NaHCO_3$, 8.06 Na_2CO_3, 1.00 K_2HPO_4 5.00 $NaNO_3$, 2.00 K_2SO_4, 2.00 NaCl, 0.40 $MgSO4.7H_2O$, 0.02 $CaCl_2.2H_2O$, 0.02 $FeSO_4.7H_2O$, 0.16 $EDTANa_2$ and micronutrient elements (0.001 $ZnSO_4.7H_2O$, 0.002 $MnSO_4.7H_2O$, 0.01 H_3BO_3, 0.001 $Na_2MoO_4.2H_2O$, 0.001 $Co(NO_3)_2.6H_2O$, 0.00005 $CuSO_4.5H_2O$, 0.7 $FeSO_4.7H_2O$, 0.8 $EDTANa_2$) were added 10 mL to 1 L.

The experiments were carried out with four fiberglass ponds, 1m^3 capacity for each, in the greenhouse in summer (July) and winter (January). The cultures were circulated by the paddle wheels at a flow rate about 20 cm/s continuously. Culture depth was maintained at 10 cm in the ponds. The experiment lasted 38 days in January. Fresh culture medium was added to the ponds to compensate the lost water via evaporation on the 23th day of the experiment. In summer (July), the cultivation was completed in nine days. The temperature, pH, light intensity, salinity and chlorophyll *a* (chl *a*) were determined daily. Five mL of the culture samples were filtered through GF6 glass fiber filter papers (Schleicher & Schuel) for the chl *a* measurements. Concentrations were measured by a UV-VIS SHIMADZU-1240 Spectrophotometer after extraction in 90 % acetone and held at 4 ºC 24h in the darkness. The chl *a* was calculated from the following equation,

Ca = 11.6. D_{665}-1.31 D_{645}-0.14 D_{630}, Chl *a* (µg/L) = Chl *a*.v / V.l

V=Volume of water filtered for extraction

v=Volume of acetone used

l= pathlenght (in cm) of cuvette (*12*).

The amounts of cellular chl *a* were calculated using the highest cell density and chl *a* values. The chl *a* quantity (µg/L) was divided by cell number (cell/L).

The temperature and dissolved oxygen (DO) were measured twice a day; midday (12:00) and midnight (24:00). pH of the growth medium was measured using WTW-330 pH meter daily. The salinity was recorded by using YSI 30 salinometer. Wet and dry weight of the biomass and the ash amounts were determined. Water content was decided by the weight loss of 1 g of wet material, maintained at 105 ºC for 4 hour while ash content was obtained by the weight loss of 1 g wet material, maintained at 550 ºC for 4 h (*13*). The daily sampling was made using 5 mL culture, and samples were fixed in 4 % formaldehid solution. The filaments were counted in triplicate by sedgwick-rafter counting chamber. The growth rates (µ) were determined as in the following formula;

(div./day) = $\log(N_1/N_0)$ x (3.322/t), N_1 and N_0 are the cell concentrations at the end and beginning of a period of time (t), days (*14*).

The vitamins and FA were analyzed at The Scientific and Technical Research Council of Turkey, TUBITAK-Marmara Research Center-Food Institute. Vitamin C was analyzed by AOAC (*15*), alfa-tocoferol (vitamin E) by HPLC (*16*), β- carotene by spectrophotometric method, AOAC (*15*) and FA were analysed by IUPAC 2D-19 method (*17*) with Thermoquest Trace GC. FID detector (250 ºC) and SP-2330 Fused Silica Capillary Column 30m.-0.25 mm ID-0.20 µm (film thickness) were also used. Air was adjusted 350 ml/min. 35 ml/min H_2 and 30 ml/min He were used. The range, carrier ratio, split flow and split ratio were 1, 0.5 ml/ min, 75 ml/min and 1/150, respectively. Oven temperature was 120 ºC (up to 220 ºC with the addings of 5 ºC). The sample injection was 0.5 µL. The FA was identified by comparing them in their retention time with standards obtained from Sigma (no: 189-19).

When the cultures reached to the stationary phase which was determined with the growth parameters of cell density and the chl *a* contents, the samples were harvested with the 40 µm mesh size cloth. The wet algal slurry was kept at -20 ºC for vitamins and fatty acids analyses. The analyses were made with two replicates for each ponds to determine the fatty acids composition in summer and winter experiment period.

Statistical evaluations were carried out with SPSS Windows version 10.0. A one-way ANOVA test was used to compare means at 0.01 and 0.05 probability level (*18)*.

Results and Discussion

The measurements of the environmental factors, temperature, light intensity, DO, pH, salinity, obtained from four ponds in the greenhouse in two different seasons are shown in Table 1. The mean temperature, light intensity, DO and pH values were found different between summer and winter and day and night ($p<0.01$) except salinity. The same culture medium was used in both experimental periods.

At the end of the growth periods in summer and winter, the filament densities and dry weights were found to be different ($p<0.05$). The filament densities were 123458±11011 and 94708±3534 in summer and winter respectively. The dry weight obtained in winter was found to be almost one quarter of in summer. Although the environmental factors were far from optimum for *S. platensis*, the yield could be obtained in winter. It was also observed that the growth rate was higher in summer than winter.

The chl-*a* quotas of *Spirulina* cultures grown in winter were found to be higher than in summer. The cellular

response to decreasing light intensity is to increase chlorophyll *a* and other light-harvesting pigments such as chlorophyll *b*, chlorophyll *c* and primary carotenoids. On the other hand, in response to high light intensity, chlorophyll *a* and other pigments directly involved in photosynthesis decrease, while the β-carotene, which serve as photo-protective agents, increase (Ben-Amotz, 1992*)*.

The vitamin C, alpha-tocopherol and β-carotene contents of *S. platensis* (mg/100 g wet samples) grown in summer and winter are shown in Table 3.

Table 1. The growth conditions, temperature, light, DO, pH, salinity, of *S. platensis* cultures in summer and winter.

	Summer (July-9 days)		Winter (January-38 days)	
	Day	Night	Day	Night
Temp.(ºC)(mean)	33.9±0.4**	29.9±0.2	18.6±0.5**	14.4±0.2
(max.)	36.5±0.2	30.9±0.1	24.2±0.1	17.1±0.1
(min.)	31.7±0.4	28.5±0.2	11.6±0.04	10.4±0.02
Light (µmol/m²/s) (mean)	848.36±52**	-	506.26±48**	-
(max.)	1085.25±28	-	1084.72	-
(min.)	481.95±44	-	23.6±0.4	-
DO (mg/L) (mean)	24±3.5**	3.8±0.2	18.7±1.7**	5.9±0.1
(max.)	35.77±0.6	4.9±0.1	41.6±0.9	7.4±0.04
(min.)	6.65±0.06	2.53±0.2	6.6±0.04	4.8±0.1
pH(mean)	9.25±0.006**	-	9.52±0.004**	-
(max.)	9.77±0.01	-	9.84±0.001	-
(min.)	9.22±0.007	-	9.52±0.004	-
Salinity(max.)	29.85±0.04	-	28.4±0.2	-
(min.)	19.5±0.6		22.8±0.2	-

** $p<0.01$

Table 2. The growth parameters, filament density, chl *a* quota, growth rate and the biomass data, dry weight, ash weight of *S. platensis*.

Growth parameters / Biomass data	Summer (July-9 days)	Winter(January-38 days)
Filament density (max.)	123458±11011*	94708±3534
(filament/mL) (the beginnig)	1625±79	1782±44
Chl *a* quota (max.) (pg/filament)	7.451*	13.09
Growth rate (mean)	0.78*	0.19
(max.)	1.57	0.47
Dry weight (mg/L)(max.)	1352±0.02*	297±0,01
Ash weight (%)	16,7±0,01*	23.1±0,01

* $p<0.05$

Table 3. Vitamin C, alpha tocopherol and β-carotene levels of *S. platensis* (mg/100 g wet samples) grown in summer and winter.

Vitamins (mg/100 g wet samples)	Summer	Winter
C	14.58±1.09*	39.31±3.63
E (alpha-tocopherol)	6.57±1.18*	0.88± 0.05
β-carotene	21.44±0.42	17.50±1.56

* $p<0.05$

In the present study, β-carotene contents of *S. platensis* grown in summer and winter were found to be similar, statistically ($p<0.05$). It appeared that light intensity didn't affect the amount of the carotenoid, β-carotene and the increasing light intensity didn't create a stress on the cells to the increment of β-carotene in summer. The differences in the contents of ascorbic acid and alpha-tocopherol of *S. platensis* observed in our experiments were correlated with the variations of the environmental factors. The vitamin C contents were found to be 14.58±1.09 mg/100 g in summer and 39.31±3.63 mg/100 g in winter. The low temperature caused the increase of the vitamin C content. While the alpha-tocopherol level of 6.57±1.18 was higher in summer, it was found to be 0.88±0.05 mg/100 g in winter. Borowitzka (*1988*) reported that the alpha-tocopherol content of *S. platensis* were 50-70 µg/g dry weight. In the other study, the soluble vitamins (mg/100 g) were identified in the *Spirulina* biomass and the content of ascorbic acid was found 42.8 mg in winter, 42.0 mg in spring, 106.2 mg in summer, 195.3 mg in spring (Babadzhanov et all, 2004).

FA composition

The fat contents of *S. platensis* biomass grown in summer and winter were found to be 0.69±0.03 and 0.80±0.07, respectively ($p>0.05$). The FA composition was influenced by ambient factors varied according to the seasons.

Table 4. Variation of total fatty acid composition (% total fatty acids) from *S. platensis* grown in summer and winter.

Fatty acids (% of total fatty acids)	Summer	Winter
C6:0**	0.006±0.000	0.195±0.014
C8:0**	0.048±0.008	0.016±0.001
C10:0**	8.910±0.532	0.318±0.006
C11:0	0.015±0.001	0.020±0.002
C12:0*	0.094±0.020	0.043±0.010
C13:0**	0.351±0.071	0.033±0.004
C14:0**	0.244±0.015	0.106±0.009
C14:1	0.026±0.003	0.024±0.001
C15:0**	0.028±0.003	0.014±0.001
C16:0**	36.891±0.827	27.945±0.379
C16:1**	2.394±0.025	8.188±0.064
C17:0	0.163±0.003	0.159±0.004
C17:1cis10**	0.321±0.047	0.634±0.010
C18:0*	1.289±0.032	1.659±0.119
C18:1n9t**	0.248±0.013	0.038±0.037
C18:1n9c**	8.015±0.229	2.635±0.134
C18:2n6t	0.044±0.007	0.051±0.005
C18:2n6c**	23.019±0.220	20.182±0.131
C18:3n6g**	11.392±0.357	22.221±0.388
C20:0**	0.218±0.007	0.123±0.056
C18:3n3a**	0.066±0.004	0.086±0.001
C20:1n9	0.032±0.002	0.023±0.003
C21:0**	0.013±0.000	0.059±0.006
C20:2cis11,14**	0.151±0.005	0.263±0.004
C20:3n3cis 11.14.17**	0.099±0.001	0.275±0.004
C22:0	0.018±0.000	0.017±0.003
C20:4n6	0.013±0.002	-
C22:2cis 13,16	0.009±0.001	-
C20:5n3 cis 5,8,11,14	0.008±0.006	-
C23:0	0.005± -	-
C24:0	0.009±0.000	-
C22:6n3	0.008±	-
Unknown	5.853	14.673
Total	100.0	100.0

** $p<0.01$, * $p<0.05$

Qualitative and quantitative differences between the FA content of *Spirulina platensis* cultivated in open ponds in summer and winter were observed. The fatty acids of which

levels were found different statistically in summer and winter were as in the following: C6:0, C8:0, C:10, C12:0, C13:0, C14:0, C15:0, C16:0, C16:1, C17:1cis10, C18:0, C18:1n9t,C18:1n9c, C18:2n6c, C18:3n6g, C20:0, C18:3n3a, C21:0, C20:2cis11,14, C20:3n3cis 11,14,17 ($p<0.05$).

Tomaselli and *et al*. (1988) were studied on the influence of temperature on *Spirulina platensis* M2 and determined the fatty acids contents of *S. platensis* at the different temperatures, e.g., 20, 25, 30, 35 and 40ºC. They observed that the lessening temperature led to the decrease of the C16:0 content of *S. platensis*. The similar reduction of C16:0 was observed in lower temperatures by Romano *et al*. (2000). Mühling *et al*. (2005) were also observed that the lower value of C16:0 when the temperature decreased to 20ºC from 30ºC. Quoc and Dubacq (1997) observed the variations in the fatty acids composition of *S. platensis* at 24, 30 and 35 ºC in the laboratory conditions. Accordingly, C16:0 decreased and C16:1 increased by the decrease in ambient temperature. Similarly, Tomaselli *et al*. (1988) reported the increasing C16:1 level by the low temperatures. Romano *et al*. (2000) observed that by the decrease in temperature to 26 ºC, a doubling of C16:1 (25.7 %) was found with respect to 30 ºC (11,2 %) at the expense of C18:2. The percentage of C18:2 (10.4 %) at the temperature of 30 ºC decreased to 1.4 % at the 26 ºC. In agreement with the studies, in the present work the level of C16:0 was found to be 36.891±0.827 % in summer and 27.945±0.379 % in winter. In our study, it was also determined that C16:1 content increased to 8.188±0.064 from 2.394±0.025 with lowering the temperature at the expense of C18:2. On the contrary, Quoc and Dubacq (1997) reported the increase in the level of C18:2 with the lowering of the temperature. It was shown that cyanobacteria responded to a decrease in ambient growth temperature by desaturating the fatty acids of membrane lipids to compensate for the decrease in membrane fluidity at low temperatures (*24, 25*). In addition, the proportion of desaturated fatty acids increases by the decrease in temperature (*22, 26, 27, 28*). Oliveira *et al*. (1999) reported the increase of the C18:3 with lowering temperature. In this study, the contents of C18:3n6g, C18:3n3a, C21:0, C20:2cis11, 14, C20:3n3 cis-11.14.17 were higher in winter. The result is similar with the relative content of unsaturated fatty acids increased with the lowering of the temperature (*22, 23*, 30). It was known that *S. platensis* is a very rich source in γ-linolenic acid. The amount of γ-linolenic acid was higher (22.221 %) in winter than in summer (11.392 %). While the C22:0 level was found to be similar in summer and winter, γ-linolenic acid content doubled in winter. The FA of C20:4n6, C22:2cis 13,16, C20:5n3 cis 5,8,11,14, C23:0, C24:0, C22:6n3 were detected only in summer.

Conclusions

Photosynthetic, filamentous, multicellular blue-green microalga *Spirulina platensis* is cultivated in tropic and subtropic areas generally. The present study indicated that the biochemical composition of the cells was influenced by the environmental factors. With the lower temperatures and light intensities, chlorophyll *a* quota, vitamin C and gamma-linolenic acid contents increased. The other polyunsaturated fatty acids were detected only in winter conditions. However, the vitamin E (alpha-tocopherol) content decreased with the lessening temperature.

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