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The Effect of Extracted Salt from Urmia Lake on the Growth, βeta-Carotene and Chlorophyll a Content of Halophilic Alga *Chlorella* sp.

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Abstract

Chlorella is a microalga that has been widely used in medicine, agriculture and environment. At present, there has been a founded equipped cultural pond to mass-produce of this alga all over the world. In this study, the effect of extracted salt from Urmia Lake with concentrations of 10, 30 and 50 g. L^{-1} on *Chlorella* sp. Biological parameters were investigated. The results indicated that in all treatments, the highest number of cells was observed on the 20th day while there were no significant differences among various salt concentrations. The highest and the lowest chlorophyll a values were reported in 50 g.L^{-1} and 30 g.L^{-1} salinities respectively although, the highest chlorophyll a value was obtained from 50 g.L^{-1} salinity on the 5th day. This study indicated that 50 g. L^{-1} salinity produced the highest Beta-carotene on days 10 and 15.

Keywords: Urmia Lake, *Chlorella*, salinity, βeta-carotene, chlorophyll a, cell number.

Introduction

Algae are one of the most variable organisms which are classified in the plant kingdom (Britton *et al*., 1995; Takaichi, 2011). They are highly helpful natural resources that contain higher values of iodide, Iron, Niacin, Magnesium, proteins, Vitamins (B6, B2, B1, C, A), Unsaturated fatty acids, antioxidants, carotenoids and astaxanthin. In addition, algae are the main source of omega-3 in water ecosystems (Horincar *et al*., 2011). Green algae are the best known as primary producers in water environment (Chmielewská and Medved, 2001; Kar *et al*., 2008). This group is considered as live food for aquatic organisms due to higher organic material content (Grossa and Lockwood, 2004). *Chlorella* is an eukaryotic unicellular green microalga that belongs to Chlorophyta. It contains green and yellow pigments as chlorophyll a, b and beta-carotene (Bilgrami and Saha, 2002; Bagulia, 2008). The most prominent properties of the *Chlorella* are the production of hermetical drugs, hygienic and make-up products (Kar *et al*., 2008). Besides this Genus is used for remedying cancer, heart and vessels diseases (Chmielewská and Medved, 2001; Horincar *et al*., 2011). *Chlorella* is considered as a crucial food source for man, livestock, and poultry and also used as a live food for aquatic organisms (Niu *et al*., 2011). The production of unpolluted fuel (biodiesel) which may be substituted for fossil fuels is another important application for the alga *Chlorella* (Chisti, 2007; Lawal and Babakano, 2011). It is a source of vitamins A, B, C, Niacin, Iodide, Potassium, Magnesium, Calcium, Iron and contains high antioxidant property. The alga *Chlorella* has amino acids, nucleotide acids, fats, fibre and nutrients too (Bertram and Bortkiewicz, 1995; Horincar *et al*., 2011). Beta-carotene is a natural orange pigment belongs to carotenoids with chemical formula as C40H56, which is sensitive against temperature and light (Ax *et al*., 2001). Algae contain an appropriate concentration of beta-carotene, which is an antioxidant (Borowitzka and Borowitzka, 1990). Therefore, algae are considered as suitable alternatives for plants to extract beta-carotene (Kaur and Khattar, 2009). Chlorophyll is a photosynthetic pigment, which is located in plant cell chloroplasts, and chlorophyll a is a general photosynthetic pigment in plant cells (Rajesh *et al*., 2001) which has a crucial role in photosynthesis. Indeed, it is a special form of chlorophyll, which one used in photosynthesis. Chlorophyll a absorbs the natural light spectrum more efficiently in violet, blue and orange-red regions (Raven *et al*., 2005). This pigment is necessary for photosynthesis in eukaryotes, cyanobacterial and prochlorophytes (Papageorgiou and Govindjee, 2004).

In the present study, the effect of extracted, natural salt from Urmia Lake in salinities 10, 30 and 50 g.L^{-1} on the growth (cell number), beta-carotene and chlorophyll a concentration was investigated.

Materials and Methods

The Sample is Preparing

Liquid stock of halophilic alga (*Chlorella* sp.) purified at the *Artemia* and Aquatic Animals Institute phycolab- Urmia University was prepared.

Treatments

Purified *Chlorella* sp. The sample was cultured in water with 30 g. L^{-1} salinity. Then, the volume was increased to 3.3×10^{-6} cells/mL and cultured in three different salinities (10, 30 and 50 g. L^{-1}) in three replicates in 500 mL volumes respectively. At first, TMRL culture medium (1 ml per L of the culture medium) was used to improve the cultural conditions (Faramarzi *et al*., 2010).

Other conditions were fixed during the cultural period as follows:

pH = 7.5-8.0, Light intensity: 3000-4000 μ $mol^{-2}s^{-1}$, Temperature: 25±1°C.

To create an equal condition direct airing method was used.

Measurement of Beta-Carotene and Chlorophyll a

5 ml of each treatment was centrifuged at 4000 rpm for 10 minutes. The supernatant was discarded. Due to the presence of salts the sediment was centrifuged again. The resulted sediment was dissolute in 5 ml acetone 80%. The solution was centrifuged at 4000 rpm for 10 minutes. Then it was placed in spectrophotometer to measure the light absorbance at 412, 431, 460 and 480 nm. Beta-carotene and chlorophyll a was calculated according to (Eijckelhoff and Dekker, 1997).

Results

The results of study have been summarized in Table 1.

Table 1. Mean (S.D) of biological parameters of *Chlorella* sp. In different salinity. (Same letters in each column show non-significant difference, ANOVA, Tukey, P>0.05)

Salinity	Day 1	Day 5	Day 10	Day 15	Day 20	Day 25	Day 30
Cell (no.ml^{-1})							
10 g. L^{-1}	3.3×10^6 (2.3×10^5)a	13.9×10^6 (10×10^5)a	17.7×10^6 (1.7×10^6)a	17.8×10^6 (3×10^6)a	29.1×10^6 (3.9×10^6)a	25.2×10^6 (3.4×10^6)a	13×10^6 (4.5×10^6)a
30 g. L^{-1}	3.3×10^6 (2.3×10^5)a	10.5×10^6 (1.3×10^5)b	14.7×10^6 (5×10^6)a	19.8×10^6 (3.9×10^6)a	22.7×10^6 (1.3×10^6)a	16.2×10^6 (4×10^4)a	17×10^6 (7×10^4)a
50 g. L^{-1}	3.3×10^6 (2.3×10^5)a	19.5×10^6 (8×10^4)c	24.8×10^6 (3×10^5)a	20.1×10^6 (5.3×10^6)a	30.2×10^6 (6×10^5)a	20.5×10^6 (1.9×10^6)a	16×10^6 (3.1×10^6)a
βeta-carotene (μg.ml^{-1})							
10 g. L^{-1}	0.227 (0.016)a	0.549 (0.002)a	0.349 (0.086)a	0.524 (0.032)ab	0.636 (0.012)a	0.36 (0.039)a	0.297 (0.002)a
30 g. L^{-1}	0.227 (0.016)a	0.388 (0.011)a	0.355 (0.064)a	0.297 (0.052)a	0.319 (0.063)a	0.289 (0.014)a	0.385 (0.037)a
50 g. L^{-1}	0.227 (0.016)a	0.662 (0.176)a	0.762 (0.002)b	0.859 (0.204)b	0.738 (0.163)a	0.489 (0.156)a	0.428 (0.072)a
Chlorophyll a (μg.ml^{-1})							
10 g. L^{-1}	0.106 (0.012)a	0.776 (0.049)a	0.277 (0.016)a	0.225 (0.014)a	0.211 (0.000)a	0.128 (0.028)a	0.079 (0.012)a
30 g. L^{-1}	0.106 (0.012)a	0.618 (0.000)b	0.396 (0.034)a	0.183 (0.003)a	0.121 (0.009)b	0.123 (0.041)a	0.108 (0.005)a
50 g. L^{-1}	0.106 (0.012)a	0.910 (0.038)a	0.507 (0.121)a	0.343 (0.036)b	0.317 (0.005)c	0.129 (0.015)a	0.154 (0.071)a
βeta-carotene (pg.$cell^{-1}$)							
10 g. L^{-1}	0.068 (0.000)a	0.039 (0.002)a	0.019 (0.003)a	0.029 (0.003)a	0.022 (0.002)a	0.014 (0.000)a	0.022 (0.007)a
30 g. L^{-1}	0.068 (0.000)a	0.036 (0.000)a	0.025 (0.004)a	0.015 (0.000)b	0.014 (0.001)a	0.017 (0.000)a	0.022 (0.002)a
50 g. L^{-1}	0.068 (0.000)a	0.034 (0.008)a	0.030 (0.000)a	0.043 (0.001)c	0.024 (0.004)a	0.023 (0.004)a	0.025 (0.000)a
Chlorophyll a (pg.$cell^{-1}$)							
10 g. L^{-1}	0.031 (0.001)a	0.055 (0.000)a	0.015 (0.000)a	0.013 (0.001)a	0.007 (0.000)a	0.005 (0.000)a	0.006 (0.001)a
30 g. L^{-1}	0.031 (0.001)a	0.058 (0.000)a	0.028 (0.007)a	0.009 (0.002)a	0.005 (0000)b	0.007 (0.002)a	0.006 (0.000)a
50 g. L^{-1}	0.031 (0.001)a	0.046 (0.002)b	0.020 (0.004)a	0.017 (0.002)a	0.010 (0.000)c	0.006 (0.000)a	0.009 (0.002)a

Cell (no. ml^{-1})

As indicated in Table 1 and Figure 1, the highest cell number among treatments was observed on the 20th day. There was no significant difference between salinities on day 20.

Beta-Carotene (µg. ml^{-1})

The highest values of beta-carotene were observed on day 20, day 5 and day 30 of salinities 10 g. L^{-1}, 30 g. L^{-1} and 50 g. L^{-1} respectively (Tab. 1 and see also Figure 2).

Chlorophyll a (µg. ml^{-1})

Table 1 indicates that the highest value of chlorophyll a in all treatments was observed on day 5, among which the 30 g. L^{-1} salinity showed the lowest value and significantly different to other two treatments (Figure 3).

Beta-Carotene (pg. cell^{-1})

According to Table 1 and Figure 4, the highest beta-carotene value in all salinities was observed on the first day and did not indicate any statically significant differences between treatments.

Chlorophyll a (pg. cell^{-1})

With regard to Table 1 the highest value of chlorophyll a per cell was observed on the 5th day. On the other hand, the lowest value of chlorophyll a was reported from 50 g. L^{-1} salinity on the 5th day which showed a significant difference compared to 10 and 30 g. L^{-1} salinities (Figure 5).

Discussion

Vonshak *et al*. (1996) studied the direct role of photosynthesis on the Cyanobacter *Arthrospira platensis* (Nordstedt) Gomont under high salinity stress. Their results indicated that in salinities of 0.1

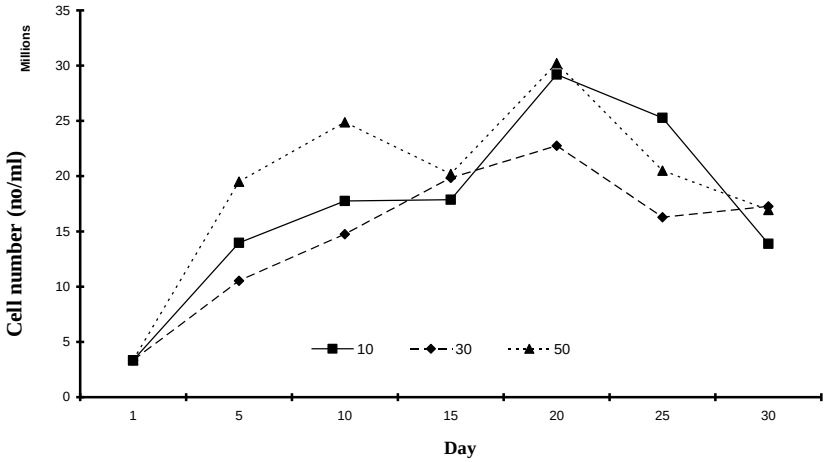

Figure 1. Mean of Cell number (no. ml^{-1}) in different salinities.

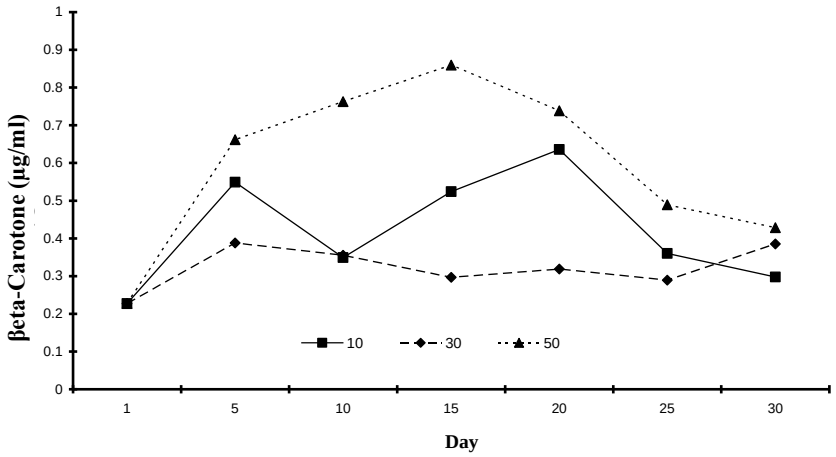

Figure 2. Mean of βeta-carotene (µg.ml^{-1}) in different salinities.

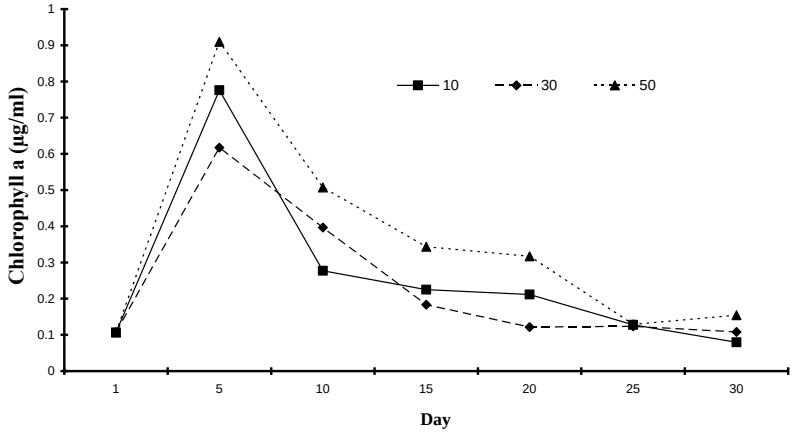

Figure 3. Mean of Chlorophyll a (µg.ml^{-1}) in different salinities.

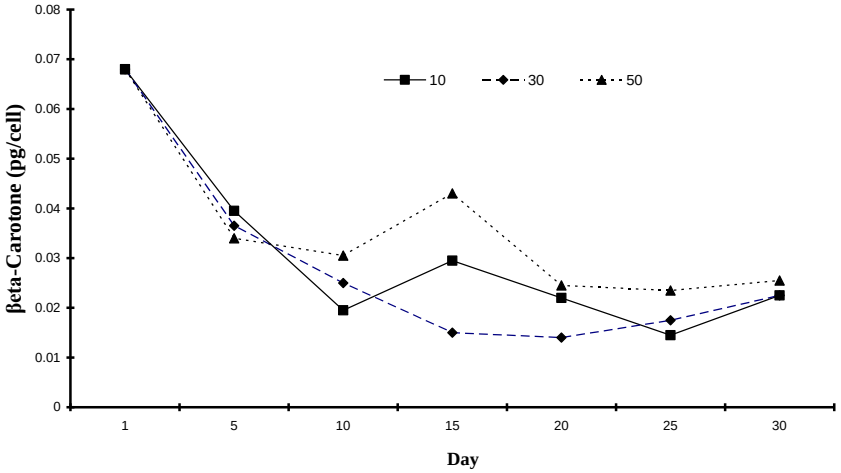

Figure 4. Mean of βeta-carotene (pg. cell^{-1}) in different salinities.

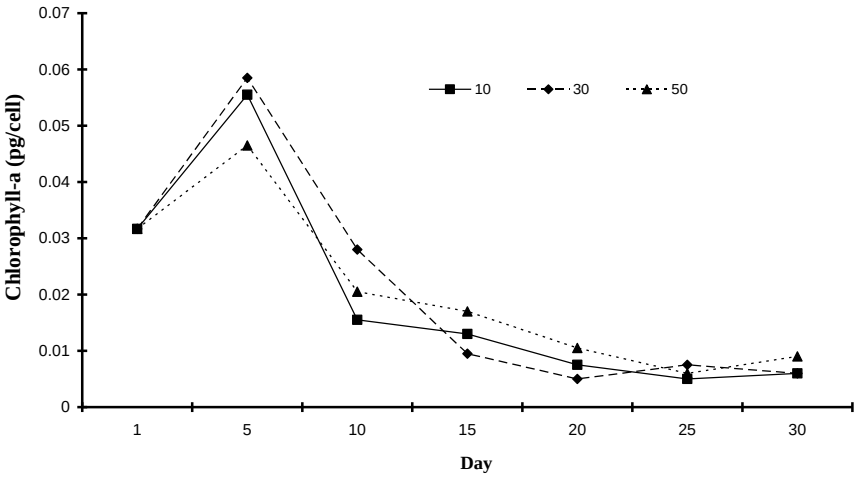

Figure 5. Mean of Chlorophyll a (pg. cell^{-1}) in different salinities.

and 0.2M the chlorophyll a content increased, while in salinities of 0.3 and 0.4M the chlorophyll a content decreased. Reddy *et al*. (2003) investigated the high Na stress on the isolates of cyanbacteria. The results indicated that beta-carotene content increased in 0.1, 0.2 and 0.3 m and decreased in 0.4 m NaCl. Abdel-Rahman *et al*. (2005) studied the effects of decreased sodium chloride concentration on *Chlorella vulgaris* Beyerinck [Beijerinck] and *Chlorococcum infusionum* (Schrank) Meneghini by riboflavin. Both of them

algae stimulated in 150-250 mM of NaCl concentrations, while the growth rate of *C. infusionum* decreased by 50 -100 mM of NaCl concentrations. Analyzed the salinity effects on beta-carotene production in *Dunaliella tertiolecta* Butcher DCCBC26 isolated from Urmia Lake. They suggested that the highest beta-carotene (11.73 mg L^{-1}) was produced in 0.5 m concentration of NaCl in this alga (Fazeli *et al*., 2005). Ranga *et al*. (2007) investigated the effect of high salinities (34 and 85 mM NaCl) on the growth of the green alga *Botryococcus braunii* Kützing. The results showed that in 85 mM concentration of NaCl palmitic and oleic acids increased by two folds. They also suggested that salinity increased the biomass of this alga. Qingtian and Guikun (2009) studied the effects of salinity on the microalga *Isochrysis* and suggested that high salinities had noticeable effects on the growth of the alga. By the way, the results showed that this alga had the highest growth rate in 27 $g.L^{-1}$ salinity. Lei and Yong (2009) analyzed the effect of salinity on the lipid contents and fatty acid composition in four species of marine green algae. They suggested that salinity could affect the total lipids in these species. Cho *et al*. (2011) investigated the effects of temperature and salinity on the *Thalassiosira pseudonana* Hasle and Heimdal (Bacillariophyceae)isolated from ballast water of ships. *T. pseudonana* was not able to survive in fresh-water and temperatures 5-10 °C, but it had suitable growth in brackish water and 5°C. Gu *et al*. (2012) had shown the effects of salinity changes on the biomass and biochemical composition in *Nannochloropsis oculata* (Droop) D.J.Hibberd. The fluctuations of salinity are a crucial factor in microalgae. In this study the effects of salinities: (Tr-1:35-15 g. L^{-1}, Tr-2:35-25 g. L^{-1}, Tr-3:35-35 g. L^{-1}, Tr-4:35-45 g. L^{-1} and Tr-5:35-55 g. L^{-1}) on the growth and biochemical composition in *N. oculata* was investigated. The results indicated that in this alga the absorbance and dry weight biomass have been decreased. Increased salinity reduced either the Special Growth Rate (SGR) or chlorophyll and beta-carotene contents. Jiménez and Niell (1991) analyzed the effects of salinity, temperature and nitrogen concentration on the green alga *D. viridis* Teodoresco. They suggested that this alga had an appropriate growth in 1m of NaCl and 30° C, while increasing the salinity up to 4M NaCl acted as a depressor on the growth and cell division. Increased salinity enhanced the pigments efficiency but increased temperature reduced their efficiency. Pick (1992) proved the effect of Cadmium on the growth indexes of *D. salina* (Dunal) Teodoresco. At the presence of various NaCl concentrations. In this study the effect of Cadmium on the cell, number and total chlorophyll in Iranian line of *D. salina* in the presence of 1M (control), 1.5 and 2M NaCl was investigated during 36 days. The results indicated that increased salinity could reduce the destructive effects of Cadmium on the cell number and total chlorophyll content in *D. salina*. It appeared that reduced effects of Cadmium on the growth rate and total chlorophyll content with increased NaCl concentration occurred due to $CdCl_2$ formation and reduced Cd absorbance by cells. Dong and Gui (1999) confirmed the effect of salinity on the chloroplast ultra-structure in *D. salina*. The results indicated that salinity fluctuations were effective on the outer structure of *Dunaliella*. They indicated that the 4.3 m NaCl was more effective than 2.7 m NaCl on the chloroplast structure. Raja *et al*. (2007) studied on the beta-carotene production from alga *Dunaliella* and indicated that temperature, salinity and feeding stress were all effective in the beta-carotene production in these microalgae. Gomez *et al*. (2003) showed the effects of salinity on the quality and quantity of carotenoids on the algae *Dunaliella salina* and *D. bardawil*. Ben-Amotz and Avron These two algae were cultured on (ART and PES) culture mediums at 3M and 2.1M NaCl concentrations respectively. *D. bardawil* produced higher amounts of beta-carotene in 3M NaCl and ART culture medium, while *D. salina* showed higher efficiency in 2M NaCl and ART culture medium. These results indicated that both culture mediums had positive effects on carotenoid production. Although *D. bardawil* is synonym with *D. salina* but the different results in recent study can be attributed to different strains of one species. Chun and Shun-shan (2006) analyzed the *Dunaliella* response against salinity fluctuations. In this study chlorophyll a, beta-carotene, proteins and carbohydrates changes were studied. First, 15, 20, 25, 30 ppt salinities were tested. The results indicated that *Dunaliella* had a good growth in 20 ppt. In the second phase 50, 70, 90 and 110 ppt salinities were tested. In these salinities, beta-carotene and chlorophyll a concentrations were higher than that of 20 ppt. Besides, proteins and carbohydrates of *Dunaliella* were highest in 15 ppt among other treatments, while these elements reduced in other salinities. Fazeli *et al*. (2005) suggested that the salinity was so effective in cellular efficiency for beta-carotene production. Several studies confirm the effect of environmental factor on beta-carotene production in *Dunaliella* sp. (Fazeli *et al.,* 2005; Celekli and Donmez, 2006; Borowitzka and Siva, 2007). Alyabyev *et al*. (2007) analyzed the effect of salinity fluctuations on the energetic processes in *C. vulgaris* and *D. maritima* Massyuk. Their results indicated that higher salinities had negative effects on energy production but in 50mM NaCl better results was obtained, while *D. maritima* showed higher adaptation for 59 mM NaCl. Wang and Yuan (2009) studied the effects of salinity changes on the growth and pigment accumulation in *D. salina*. The results have been showed that the growth and cell division had higher performance in lower salinities than higher salinities. Therefore, the highest beta-carotene and chlorophyll values were obtained in 60 mg L^{-1} and 30.17 mg L^{-1} respectively. Pasqualetti *et al*. (2010) analyzed the effect of salinity and nitrate concentration on the growth and carotenoids

accumulation in a line of salt Lake *Dunaliella* in laboratory. The effects of 14.9% and 22% NaCl (w/v) and 212, 435 and 882 µm nitrates were analyzed on the growth and carotenoids production. The results indicated that the highest growth rate and cell density was observed in 22% (w/v) NaCl and 882-µm nitrate. On the other hand, the highest carotenoids concentration was reported in 22% (w/v) NaCl and 212-µm nitrate. Rad *et al*. (2011) studied the effect of salinity on the growth and beta-carotene production in *Dunaliella* sp. isolated from Urmia Lake in Northwest Iran. In this study, *Dunaliella* sp. was cultured in 3 and 2.1M NaCl by Johnson culture medium. The results have been shown that the highest carotenoids values and cell growth rates were obtained in 3 and 1M NaCl. Narváez-Zapata *et al.* (2011) studied the various physiological interactions of *Dunaliella* genetically. In this study one sample of DUNS-1 and two samples of coastal wetlanda *Dunaliella* (DUNS-2 and DUNS-3) were analyzed in 14% and 30% (w/v) salinities respectively. Mingjiang *et al*. (1983) studied the effects of temperature and salinity on the growth of *C. vulgaris*. The results indicated that salinity was more effective than temperature on the growth of this alga. They also suggested that the appropriate growth condition for the alga were at temperature 28 °C and 30 ppt salinity. Moronta *et al.* (2006) analyzed the response of microalga *C. sorokiniana* Shihira and R.W.Krauss against pH, temperature and salinity changes. Roohnavaz (2008) studied the effects of salinity on the *C.vulgaris* and observed that with increasing the salinity the cell number was properly increased. On the other hand, the increased salinity may decrease the chlorophyll a content. Cho *et al*. (2007) analyzed the effects of salinity and temperature on the growth of *Chloroidium ellipsoideum* (Gerneck) Darienko, Gustavs, Mudimu, Menendez, Schumann, Karsten, Friedl and Proschold and *Nannochloropsis oculata* (Droop) D.J.Hibberd. The results indicated that the most appropriate temperature and salinity for *C. ellipsoide* and *N. oculata* were 15°C and 10 ppt and 25°C and 10, 30 ppt respectively. Hiremath and Mathad (2010) studied the effect of salinity on the physiological and biochemical characteristics of *C. vulgaris*. The results demonstrated that chlorophyll was stimulated in 0.1 and 0.2 M NaCl but decreased in 0.3 and 0.4 m NaCl, while beta-carotene and carbohydrates increased in 0.3MNaCl. Totally, this alga showed different responses against various stresses.

Totally, the results indicated that 50 g L^{-1} salinity was more appropriate than 10 and 30 g L^{-1} salinities for cellular growth in *Chlorella* sp. However, there were no significant differences between treatments. Between all treatments, the highest number of cells was observed in day 20^{th} of 50 g. L^{-1} salinity.

Beta-carotene concentration fluctuation did not obey a regular pattern. The highest value of beta-carotene belonged to day 15^{th} of 50 g L^{-1} salinity. Statistical comparison indicated that beta-carotene value in 50 g L^{-1} salinity had no significant differences between days 15^{th} and 20^{th}.Therefore; we may conclude that the day 20^{th} of 50 g L^{-1} salinity is the most appropriate state for higher values of beta-carotene production in *Chlorella* sp.

The highest value of beta-carotene per cell was observed on the first day in all treatments. This indicated that the beta-carotene content of cells may decrease with time but due to cell division and growth, its value in volume unit of medium culture (ml) increased.

The highest value of chlorophyll a per ml of culture medium per cell was observed on day 5^{th} in all treatments. The chlorophyll a value either in volume unit or in each cell decreased with time.

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