

Full Length Research Paper

Effect of salinity on cell growth and β -carotene production in *Dunaliella* sp. isolates from Urmia Lake in northwest of Iran

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Urmia Lake, the second largest hyper-saline lake by area in the world, has fluctuated in salinity over time, but recently, it has reached a maximum of 360 g/l. *Dunaliella* is a type of halophile green-orange microalgae especially found in lake and salty fields and is known for its antioxidant activity; because of its ability to create large amount of carotenoids. In the present study, *Dunaliella* sp. isolates from hyper-saline Urmia Lake water were cultured in modified Johnson media and were treated at different salinities (1, 2 and 3 M NaCl), then their cell proliferation rate and β -carotene production were studied. In order to determine the optimal salinity required for the highest β -carotene accumulation, cell count of *Dunaliella* sp. isolates; total carotenoids and concentration of the β -carotene were determined by direct microscopic counting and spectrophotometry. In the samples with different salinities, the cell count and the β -carotene content of *Dunaliella* sp. ranged between 0.53 and 2.21×10^6 cell.ml⁻¹ and 0.2 to 11.4 pg.cell⁻¹, respectively. At the end of the experiments, the maximum cell content mean and the highest β -carotene content mean were obtained at 1 and 3 M NaCl concentrations, as 1.68×10^6 cell.ml⁻¹ and 8.94 pg.cell⁻¹, respectively.

Keywords: *Dunaliella*, microalgae, Urmia Lake, chlorophyceae, β -carotene.

INTRODUCTION

Algal biotechnology has made major advances in the past three decades and several microalgae like *Botryococcus*, *Chlorella*, *Dunaliella*, *Haematococcus* and *Spirulina* are cultivated to produce proteins, astaxanthin, β -carotene, glycerol, liquid fuels and pharmaceutical formulations and also fine chemicals (Raja et al., 2007).

Dunaliella sp. green-orange microalgae (Chlorophyceae) are motile, unicellular, rod to ovoid shaped (9 to 11 μ m), occurs in a wide range of marine habitats such as oceans, brine lakes, salt marshes, salt lagoons and salt water ditches near the sea, predominantly in water bodies containing more than 2 M salt and high-levels of magnesium (Ben-Amotz, 2004). Few organisms can

survive in high saline conditions such as salt evaporation ponds. To survive, these organisms synthesize high concentrations of β -carotene and glycerol to protect against the intense light and osmotic pressure, respectively. *Dunaliella* sp. is also known for its antioxidant activity because of its ability to create large amounts of carotenoids.

Genus *Dunaliella* has 22 species and a number of varieties and forms of the marine and halophilic species (Borowitzka and Siva, 2007), the organisms are relatively simple to cultivate and do not clump or form chains. The best-known species is the halophile *Dunaliella salina* (Borowitzka and Siva, 2007).

Dunaliella is the main natural source which accumulates a massive amount of carotenoids. For example, *D. salina* is a microalgae naturally occurring in some locations worldwide. In the marine environment, *D. salina* appears green, however, in conditions of high salinity and

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Figure 1. View of the shore of Urmia Lake, increasing salinity and the red color of lake. (Shahid Kalantari causeway, Urmia Lake, Iran, photos by: F. Arash Rad, 2010).

light intensity, the microalgae turns red due to the production of protective carotenoids in the cells. Carotenoids are chemicals with significant commercial interests, which are used as coloring agents in nutraceuticals, pharmaceuticals, cosmetics and foods (Fazeli et al., 2006).

These compounds have antioxidant properties and have attracted attention as potential agents in chemoprevention of cancers (Nishino et al., 2002). Of about 1000 carotenoids found in nature, only a few of them occur in abundance in fruits and vegetables. These include β -carotene (carrots), lycopene (tomatoes) and lutein (spinach) (Prasad et al., 1999). Although, some carotenoids for example β -carotene and zeaxanthin are available in synthetic forms, there is growing interest on natural microalgal as well as bacterial and yeast sources of carotenoids derived by the world public opinion on additives (Bhosale et al., 2004).

This offers an opportunity for commercial biological production of these substances. Nowadays, microalga *Dunaliella* is under increasing attention for its β -carotene production, its applications in nutritional, pharmaceutical and cosmetics industries and in research such as genetic engineering; hence, identification of high productive isolate and optimization of its culture media for natural β -carotene production is of great importance.

The β -carotene is a terpenoid pigment that is highly valuable for the sake of its nutritional benefits as a precursor of vitamin A and for its antioxidant properties (Gomez et al., 2003). One molecule of β -carotene is found to neutralize up to 1000 molecules of free radical oxygen (Foote et al., 1970).

Urmia (or Orumiyeh) salt Lake, is one of the largest permanent hypersaline lakes in the world and resembles the Great Salt Lake in the western USA in many respects of morphology, chemistry and sediments (Kelts and Shahrabi, 1986) and lies in northwest of Iran. The lake is between the provinces of west Azerbaijan and east

Azerbaijan, west of the southern portion of the similarly shaped Caspian Sea. It is the largest lake inside Iran and the second salt water lake on earth, with a surface area of approximately 4750 to 6100 km² (1834 to 2355 mile²) depending on evaporation and water influx (Azari, 1987).

Due to drought and increased demands for agricultural water in the lake's basin, the salinity of the lake has raised up to 360 g.l⁻¹ during recent months and large areas (\approx 3000 km²) of the lake bed have dried. The lake is divided into north and south parts separated by a causeway in which a 1500 m gap provides little exchange of water between the two parts.

The purpose of the present work was to study the role of salinity parameter in β -carotene production content by *Dunaliella* sp. isolated from a hyper-saline Urmia Lake in Iran. Recently, due to rising temperature, plenty of water evaporation and thus, increasing salinity; *Artemia* population (a crustacean) declined and therefore, the abundance of *Dunaliella* density in the Urmia Lake in response to salt and high temperature, the red color of the lake is given (Figure 1).

MATERIALS AND METHODS

Sampling and microalgae isolation

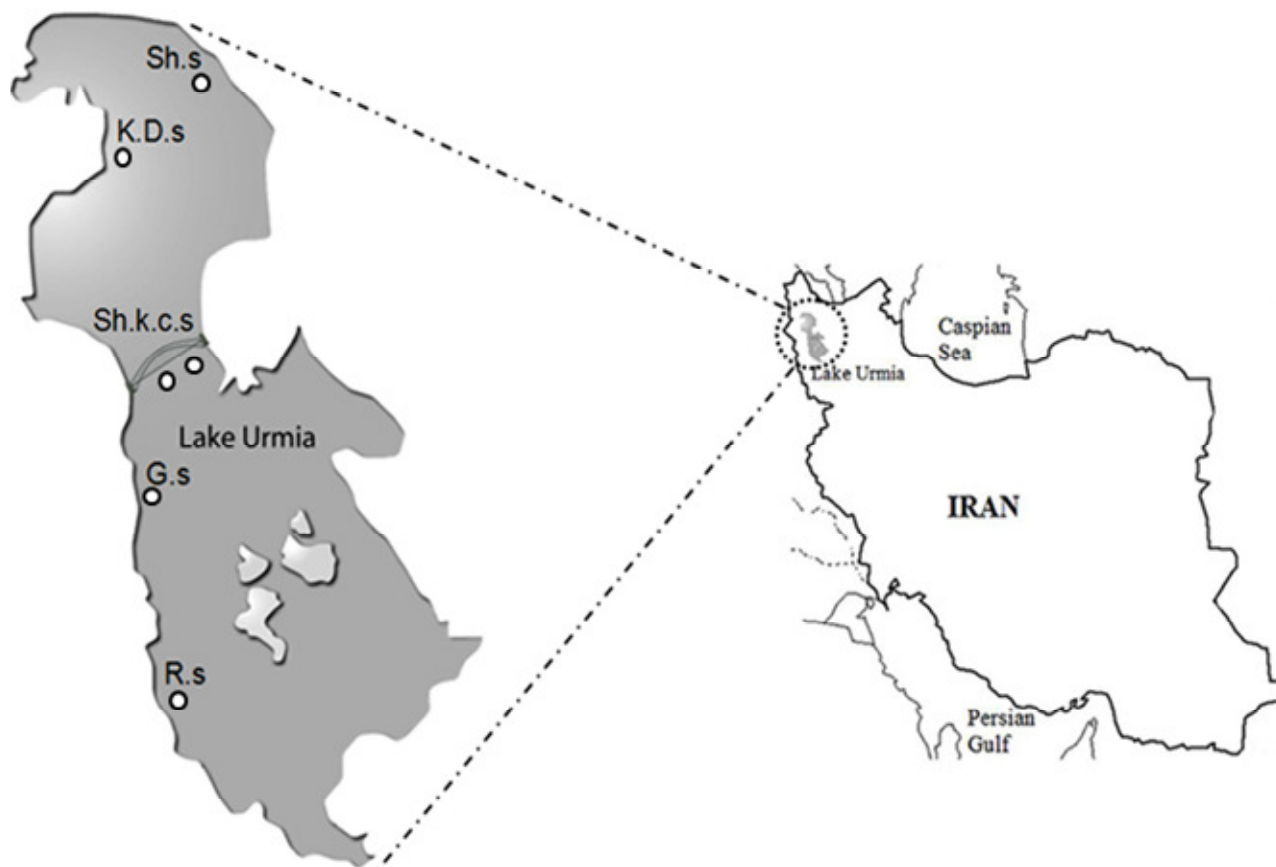
Five stations or locations (Table 1) were selected from the middle, northern and southern parts of Urmia Lake (Figure 2), then, wild types of *Dunaliella* sp. were collected and transferred to laboratory. Samples were collected in sterile plastic bottles from the Urmia Lake during midsummer (drought months) and second month of autumn (rainfall month) and were transferred to the laboratory within 24 h.

Viability and morphological study

The samples were brought to the laboratory and microscopic analysis was carried out to find out the presence of *Dunaliella* sp.

Table 1. Locations and sampling stations and their geographic coordinates.

Station or location	Map legend	Geographic coordinates of sampling station
The Rashakan station	R.s	37°29' N 45°30' E
The Sharafkhane station	Sh.s	38°15' N 45°48' E
The Golmankhane station	G.s	37°59' N 45°28' E
The Kazem-Dashi location	K.D.s	37°06' N 45°20' E
The Shahid Kalantari causeway location	Sh.K.c	37°78' N 45°35' E and 37°79' N 45°38' E

**Figure 2.** Sampling stations or locations of Urmia Lake.

cells. *Dunaliella* sp. cells were identified under microscope based on the morphological description given by Borowitzka and Siva (2007). Viability of the cells was determined by light microscope. Then, 950 μ l of collected green-orange cells were mixed with 50 μ l Lugol's iodine solution and were seen by 100x objective lens. Microalgae had ovoid and spherical shapes and some had two flagella under microscopic observation (Figure 3).

Culture condition

Enrichment and preparation of samples

Collected *Dunaliella* sp. samples were transferred to the laboratory for enrichment and were treated with 5 ml (per lit.) 1M KNO_3 and 0.1M KH_2PO_4 and were placed for 7 to 10 days in a static phytotron ($25 \pm 2^\circ\text{C}$, 1.5 M NaCl).

Preparation of artificial sea water (ASW) medium for microalgal culturing

Modified Johnson's medium (Olmos et al., 2000) was prepared for culturing microalgae with the following components per liter of artificial sea water; macronutrients: $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 9.8 $\text{g} \cdot \text{l}^{-1}$ or 0.05 M; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.53 $\text{g} \cdot \text{l}^{-1}$ or 4 mM; Na_2SO_4 , 3.2 $\text{g} \cdot \text{l}^{-1}$ or 17 mM; K_2SO_4 , 0.85 $\text{g} \cdot \text{l}^{-1}$ or 5 mM; Tris-Base, 12.11 $\text{g} \cdot \text{l}^{-1}$ or 0.1 M; KNO_3 solution (1 M), 5^{cc}; KH_2PO_4 solution (0.1 M), 5^{cc} (after autoclaving); micro-nutrients and trace elements: $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 4 $\text{mg} \cdot \text{l}^{-1}$ or 17 μM ; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.72 $\text{Mg} \cdot \text{l}^{-1}$ or 3.64 mM; $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$, 0.25 $\text{mg} \cdot \text{l}^{-1}$ or 1.5 μM ; $\text{CuSO}_4 \cdot 7\text{H}_2\text{O}$, 10.4 $\text{mg} \cdot \text{l}^{-1}$ or 37 μl ; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 17 $\text{mg} \cdot \text{l}^{-1}$ or 0.06 mM; Na_2EDTA , 4.578 $\text{g} \cdot \text{l}^{-1}$ or 13.54 mM; $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 1.259 $\text{g} \cdot \text{l}^{-1}$ or 4.658 mM (5 ml of trace elements solution were prepared and were added to the medium).

Different concentrations of NaCl (1 to 3 M) were added to the medium and the pH of the growth medium was adjusted to 7.5 by

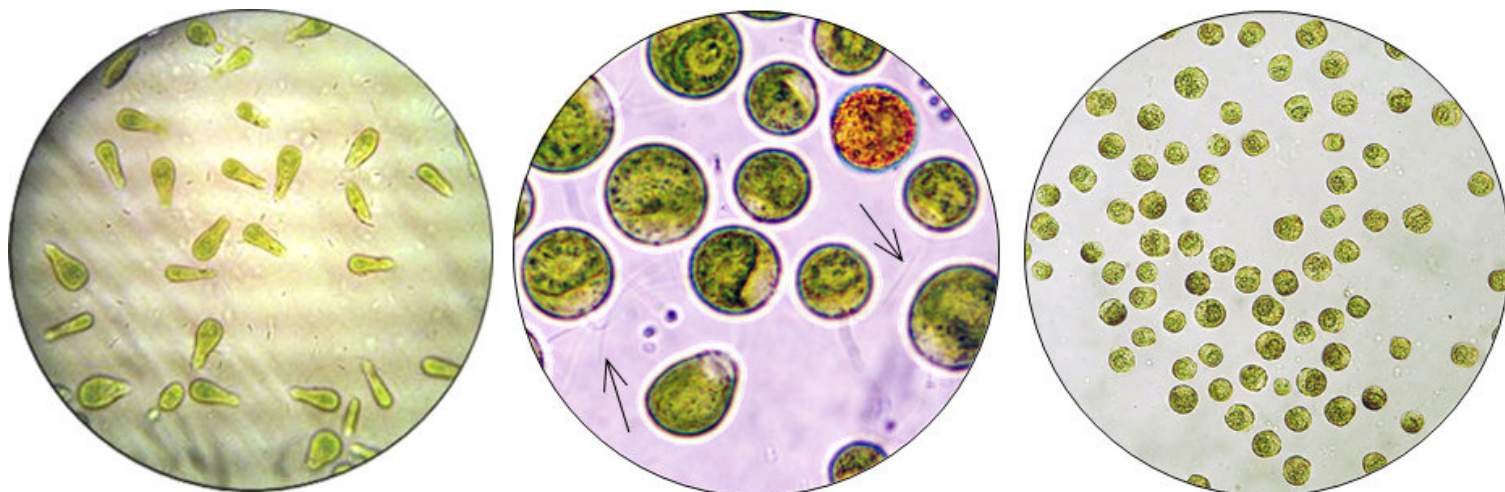


Figure 3. Various forms of isolated *Dunaliella* sp. (100x objective lens).

dilute (0.01 M) and concentrated (1 M) sulfuric acid or sodium hydroxide solutions (Celekli and Donmez, 2006). In order to avoid the precipitation of certain compounds, all stock solutions were sterilized separately and pooled aseptically. Sterilization was accomplished by autoclaving at 121°C. Sodium bicarbonate stock was heat-sterilized at 130°C (Hejazi and Wijffels, 2003).

Culture of enriched *Dunaliella* sp. in broth media

Enriched indigenous isolates were cultivated after 10 days in 250 ml Erlenmeyer flasks with 100 ml of modified Johnson's medium (Olmos et al., 2000) with 1.5 M NaCl concentration under a continuous photon flux density of $100 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and $25 \pm 2^\circ\text{C}$. Flasks each containing 100 ml of modified Johnson's medium (ASW) were inoculated with 50 ml of enriched samples (micro-algae).

Sub-culture and pure-culture

Samples were sub-cultured several times after microalgae growth, in this stage salinity and pH of ASW gradually were increased from 1.5 M to 3 M NaCl (1.5, 2 and 3 M NaCl) and pH = 7.5 to pH = 10 (7.5, 8.5 and 10), then, salinity was decreased to 1.5 M NaCl ($25 \pm 2^\circ\text{C}$, static phytotron, $100 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and 24 h daylight). Each treatment was continued for a fortnight at five times. The aim of treatments was elimination of culture media contamination. Because of organic compound absence in ASW, secondary contamination was eliminated after many sub-culturing of samples. After two-week of last treatment, the culture was again observed under microscope.

Selection of the clone was done based on the method described by Shaish et al. (1991). One ml of medium with a week old culture was mixed with 3 ml of melted 0.4% agrose at 37°C and spread on a layer of 15 ml pre-gelled ASW medium containing 1.5% Bacto-agar in Petri dishes. The plates were incubated under illumination in culture room where colonies could be observed in 15 days. A well-developed colony was picked up and used as a single clone for further studies. Pure culture of this clone was obtained by sub-culturing on nutrient agar surface. Finally, two-week old colonies were carefully picked and inoculated into 100 ml fresh ASW medium in 250 ml flasks. This pure culture was maintained and subcultured every fortnight to have a log phase growth (Phadwal

and Singh, 2003).

Non-stress and stress phases

Isolates were placed in culture media with 1.5 m NaCl concentration for thirty days, then, the most efficient isolate was placed again for ten days in culture medium with same conditions and then exposed to different salinities stresses for another twenty days. Finally, cell counting and carotenoid measuring were done.

Cell counts

To compare cell growth in different salinities, cell counting was done using a light microscope and haemocytometer (NEUBAUER). Each concentration at the desired light intensity was replicated in triple. Sample was shaken to homogenize, then, 950 μl of sample were transferred to screw-cap glass bottle. 50 μl of Lugol's iodine solution was added for fixing. Number of cells was calculated as follows:

$$\text{Algae number (per ml)} = n \cdot 1000 \times 0.1 \cdot X$$

In this formula n was the number of *Dunaliella* sp. cells counted in the large square (total volume of 0.1 mm^3) and X was dilution factor applied in combination with Lugol's iodine solution which was 0.95.

Number of cells was evaluated in two phases. First phase was at the end of tenth day and the second phase was a period of twenty days (once every two days). Cells counting were conducted in three salinities: 8.76% (w/v) NaCl or 1.5 M NaCl (at the end of thirty days), 5.8% (w/v) NaCl or 1M NaCl (twenty-day period), 11.68% NaCl (w/v) or 2 M NaCl (twenty-day phase), 17.53% (w/v) NaCl or 3 M NaCl (twenty-day phase) under a photon flux density of $100 \mu\text{mol m}^{-2}\cdot\text{s}^{-1}$ 100 in the photoperiod of 16:8 h under light/dark cycle, $25 \pm 2^\circ\text{C}$ and pH value of the culture media were set at 7.5. in static phytotron. Each experiment was carried out in triplicate.

Pigment extraction and analysis

Carotenoid production ability of the desired samples according to the protocol provided by method Celekli and Donmez (2006) took place, Unlike the first step (ten-day phase), at the stage of stress,

Table 2. The results of initial pH value and NaCl concentration on number of cells and β -carotene content of *Dunaliella* sp. after 30 days (pH = 7.5; $25 \pm 2^\circ\text{C}$ and $100 \mu\text{mol photons.m}^{-2}.\text{s}^{-1}$ illumination, Sh.s: The Sharafkhane station, K.D.s: The Kazem-Dashi station, Sh.K.c: The Shahid Kalantari causeway, G.s: The Golmankhane station and R.s: The Rashakan station). Data are expressed as means of three replicates.

Station	1.5 M NaCl and $100 \mu\text{mol photons.m}^{-2}.\text{s}^{-1}$		
	Number of cell (cell.ml^{-1})	Total carotenoid (mg.l^{-1})	β - carotene (pg.cell^{-1})
Sh.s	1.97×10^6	6.63	3.36
K.D.s	1.19×10^6	5.18	4.35
Sh.K.c.s	2.01×10^6	9.52	4.74
G.s	1.71×10^6	5.94	3.47
R.s	1.88×10^6	6.11	3.28

algal samples were treated with different salinities (1-3 M NaCl) for twenty days (after the tenth day). In the second phase, the amount of carotenoid content was measured, every two days.

Statistical analyses

Each result shown was the mean of three replicated studies. Statistical analysis and drawing graphs of the data were performed by using the SPSS-18 (© 2010 SPSS Inc., USA) and Microsoft excel (© 2010 Microsoft corporation, USA) programs, respectively and the statistical significance was determined at 95 or 99% confidence limit.

RESULTS

Macroscopic and microscopic study of algae

Water samples from different stations were evaluated under a microscope. The isolated and purified ovoid algal strain, which has one cup-shaped chloroplast and two equal flagella without cell wall, was identified as *Dunaliella* species by morphological examination under light microscope (Borowitzka and Siva, 2007). Population density of motile algae was low at this moment. Other small creatures, along with the algae, could be seen under a microscope (*Artemia* sp.; a genus of phyllopod Crustacean found in salt lakes and brines). During subsequent culturing, various forms of algae were observed that showed the possibility of multiple species. In the enrichment step, after adding food sources of phosphate and nitrate, growth and reproduction of algae were found. Gradual color change medium could be seen because of algae growth after about 10 days, from colorless to a mixture of green and orange. Algae grew well after culturing in liquid modified Johnson's medium. Spherical shaped colonies appeared 15 to 30 days after inoculation of algae on the solidified agar medium. Algae were seen elliptical (pear shape) to round (Figure 2). These cells had two flagella (Figure 3, arrows), which were shown rapid and sometimes slow motility.

Purification of *Dunaliella* sp.

Bacterial contamination was eliminated from the environment by frequently doing sub-culturing and gradually increasing NaCl concentration and pH. The sub-culturing was considered every 14 days, because if this time gets longer, organic medium matter will be provided in the result death of algae for the growth of microbes; therefore, no bacterial colonies were observed in agar media.

Number of cells and β -carotene accumulation properties

Samples were investigated in a batch system at different salinity concentrations. The results were given as the number of cells per ml ($\times 10^6 \text{ cell.ml}^{-1}$) and accumulated β -carotene concentration per cell basis (pg.cell^{-1}) or per ml of culture broth (mg.ml^{-1}). The average β -carotene production and mean number of cells for the most efficient isolated from the relevant station (after 30 days of treatment) is tabulated in Table 2.

According to preliminary results, among the isolates of Urmia Lake, Shahid Kalantari station samples were considered for more productive, because they have the highest amount of β -carotene production per cell. Therefore, in subsequent experiments, only the effect of stress on these isolates was evaluated.

Number of cells and carotenes content under salt stress conditions

The effect of salt concentration on number of cells, total carotenoids accumulation and accumulated β -carotene concentration per cell basis by *Dunaliella* sp. during incubation period was performed at different salinities. The results are shown in Figures 3, 4 and 5. The number of cells of *Dunaliella* sp. ranged from 0.51 to $2.21 \times 10^6 \text{ cell.ml}^{-1}$ and total carotenoids content ranged from 0.1 to 13.41 mg.ml^{-1} for the culture broth and 0.19 to 11.9

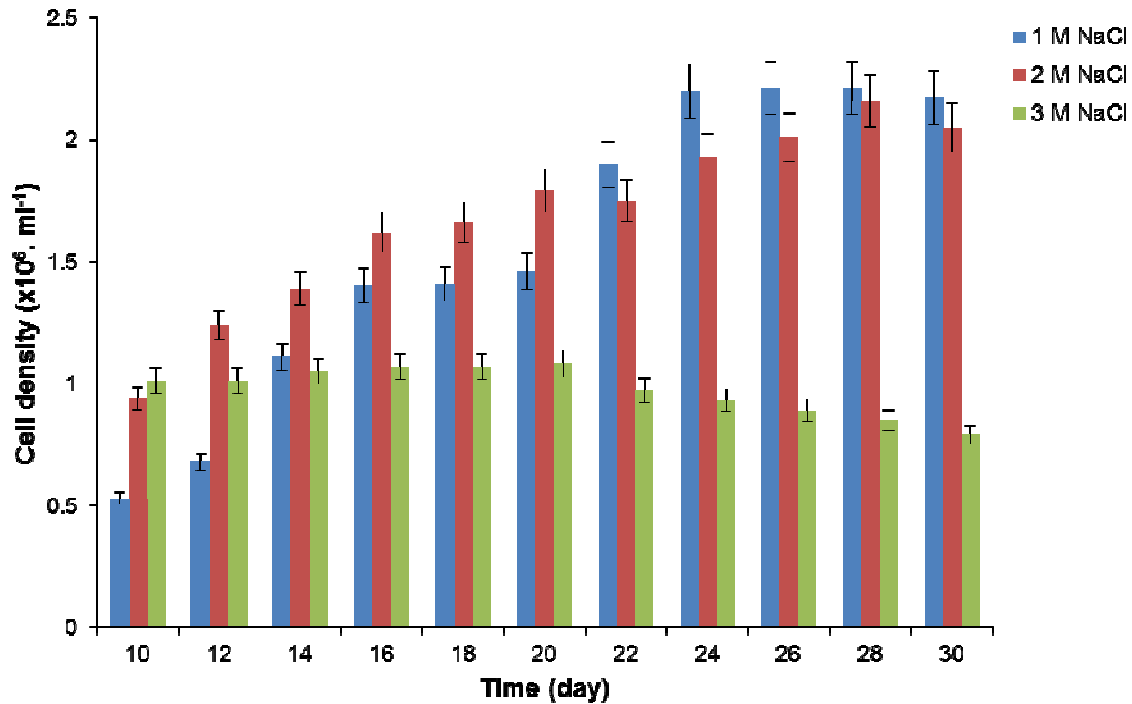


Figure 4. The effect of the salinity on number of cells of *Dunaliella* sp. (T: $25 \pm 2^\circ\text{C}$; $100 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ illumination).

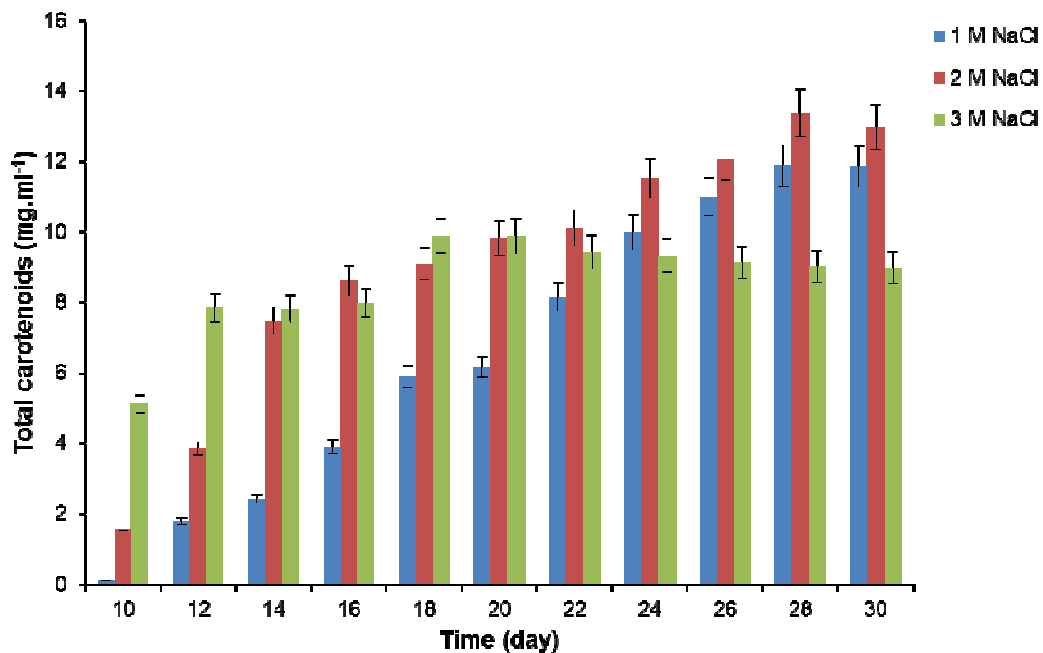


Figure 5. The effect of different salinity on total carotenoids accumulation in *Dunaliella* sp. during the incubation period (T: $25 \pm 2^\circ\text{C}$; $100 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ illumination).

$\text{pg}\cdot\text{cell}^{-1}$ for per cell basis during 30 days at all tested NaCl concentrations.

Correlation test showed the cell density of *Dunaliella*

sp. was significantly correlated with the 3 M NaCl salinity ($P < 0.01$, Table 3). One-way analysis of variance (ANOVA) test showed the growths of the three groups

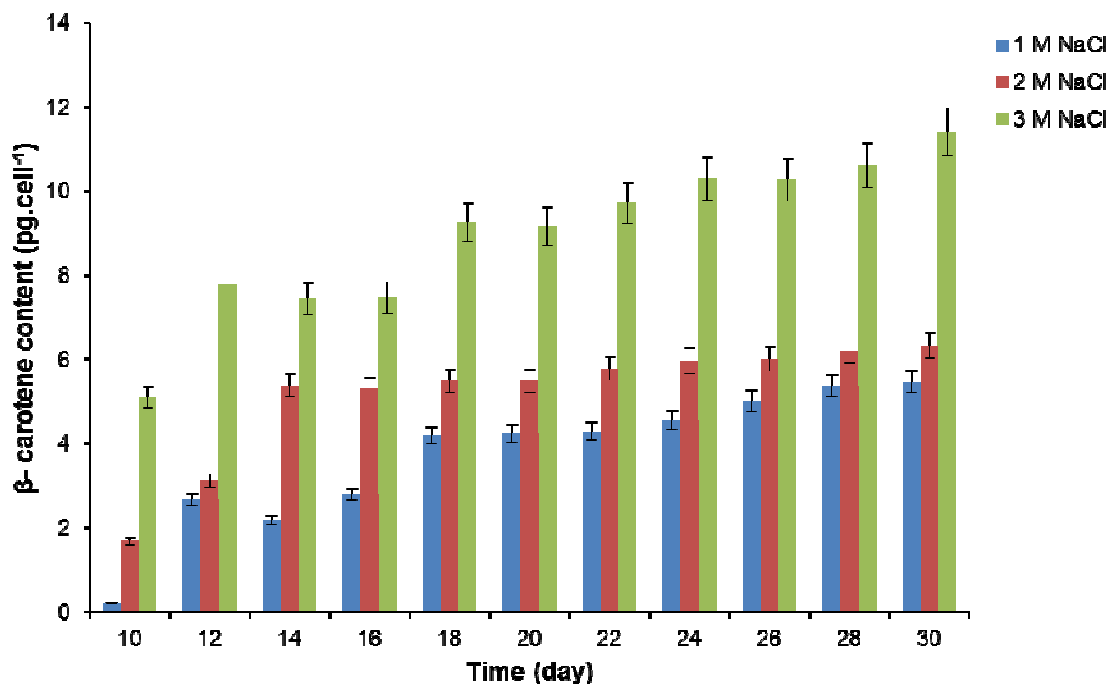


Figure 6. The accumulated β -carotene concentration on a per cell basis by *Dunaliella* sp. at different salinity during the incubation period ($T: 25 \pm 2^\circ\text{C}$; $100 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ illumination).

had significant difference at the 0.05 level ($P < 0.05$).

Dunaliella sp. showed its maximum cell density mean at 2 M NaCl concentration (1.68×10^6 cells. ml^{-1}). There were significant differences ($P < 0.01$) in the daily measurements of carotenoid contents of *Dunaliella* sp. grown in defined inorganic medium with 1 to 3 M NaCl concentration during 30 days. Also, *Dunaliella* sp. showed its maximum total carotenoid content mean at 2 M NaCl concentration ($9.13 \text{ mg}\cdot\text{ml}^{-1}$). There were significant differences ($P < 0.05$) at total carotenoids content mean of *Dunaliella* sp. grown in defined inorganic medium with 1 to 3 M NaCl concentration (salinity and its relation with carotenoid production, Table 4). In other words, different molarities of NaCl concentration influenced cell density and total carotenoid contents. Carotenoid to cell density ratio (β -carotene content per cell) increased with salinity in *Dunaliella* sp. (Figure 5) and high β -carotene content mean observed at 3 M NaCl concentration that was $8.94 \text{ pg}\cdot\text{cell}^{-1}$ after 30 days of incubation period.

DISCUSSION

As previously described by Mironyuk and Einior (1968), Semenenko and Abdullayev (1980), Ben-Amotz and Avron (1982), Borowitzka et al. (1984), Ramazanov et al. (1988), Celekli and Donmez (2006), Fazeli et al. (2006) and Borowitzka and Siva (2007), environmental factors played most effective rules on the level of carotenoid

production by *Dunaliella* sp. The highest production of β -carotene is observed in high salinity, high temperature and high light intensity.

Production of carotenoids is influenced by the growth rate usually, as the growth rate decreases, the carotenoid production rate increases (Ben-Amotz and Avron, 1982; Borowitzka et al., 1984). During the present study, the number of cells of *Dunaliella* sp. increased from 0.51×10^6 to 2.21×10^6 at defined conditions. Besides, β -carotene content (per cell) of *Dunaliella* sp. increased with increment of culture medium salinity from 0.2 to $11.4 \text{ pg}\cdot\text{cell}^{-1}$ per cell basis during 30 days at all tested NaCl concentrations.

However, productivity on a cellular basis was significantly higher at extreme salt concentration (3 M NaCl). These results are in accordance with those reported by Cifuentes et al. (2001) and Fazeli et al. (2005), who found that total carotenoid production as well as cell productivity was affected by salinity. The highest carotenoid contents per cell were obtained at 2 M NaCl. These results are in accordance with those reported by Gomez et al. (2003).

To study the effects of different salt concentrations, maximum biomass production and carotenoid concentrations were compared in three molarities by Duncan's test that showed, cell growth rate in the 3 molar salinity significantly less than 1 and 2 concentrations, while the production rate per cell at this concentration (3 M NaCl) is significantly higher (Figures 4, 5 and 6). These results are in accordance with those reported by Ben-Amotz and

Table 3. Analysis of variance for number of cells and β -carotene content (pg.) per cell of *Dunaliella* sp. in three different concentrations of salt.

Source	Mean squares	df
Molarity	4.80**	2
	240**	2

** Significant at 1% level.

Table 4. Comparison of the mean number of cells and β -carotene content per cell in three different salt concentrations.

Molarity	Number of cell ($\times 10^6 \cdot \text{ml}^{-1}$)	β -Carotene content ($\text{pg} \cdot \text{cell}^{-1}$)
1	1.57 ^a	3.72 ^c
2	1.68 ^a	5.16 ^b
3	0.97 ^b	8.94 ^a

*Each value represents the average of three replicates. Values followed by the same Letter(s) are not significantly different at 5% level by Duncan's multiple range test.

averon, (1982), Gomez et al. (2003) and Fazeli et al. (2005), despite lower cell density at 3 M NaCl concentration, this isolate produced higher β -carotene, that it is noteworthy in the viewpoint of biotech industries.

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