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Effect of saltiness on the growth and production of β-carotene in isolated *Dunaliella* sp. microalga from qom salt lake of Iran

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Abstract

The aim of this study was to achieve the highest β-carotene production by *Dunaliella* sp. isolates from under salinity stress factor. In this study, water samples from hyper-saline Qom Salt Lake were cultured in modified Johnson media and were treated at different salinities (1, 2 and 3 M of NaCl). 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 0.46 and 2.12×10^6 cell.ml^{-1} and 0.16 to 9.88 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 2 and 3 M NaCl concentrations, as 1.78×10^6 cell.ml^{-1} and 7.40 pg.cell^{-1}, respectively.

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Introduction

Microalgae, are a group of algae that produce biochemical products, include production of a wide array of carbohydrates, lipids, and proteins that are commercially valuable. *Dunaliella* sp. is a type of halophile green-orange microalgae and widely distributed and has been recorded from an extremely wide range of habitats. Some species such as *D. lateralis* live in fresh water, while in hypersaline environments species like *D. salina* predominates (Borowitzka and Borowitzka, 1998). When some species of *Dunaliella* are subjected to environmental stress conditions such as high salt concentrations, high light intensities or nutrient deprivation, they overproduce and accumulate very large amounts of β-carotene (Ben-Amotz and Avron, 1983, Ben-Amotz *et al.*, 1989, Lers *et al.*, 1990, Ben-Amotz, 1995, Nikookar *et al.*, 2004).

Studies using the halophilic *D. bardawil* have indicated a direct relationship between β-carotene content and salinity (Ben-Amotz and Avron, 1990). β-Carotene is a lipid-soluble orange pigment and antioxidant that is mainly used in cosmetics and as a colorant for feed and food. A rich source of natural β-Carotene is the unicellular green microalga *D. salina;* including a strain previously known as *D. bardawil* (Borowitzka and Siva, 2007), which can accumulate β-Carotene to as much as 10% of the cellular dry weight under certain extreme environmental conditions, such as high light intensity, nutrient deprivation, high salinity, and extreme temperatures (Ben-Amotz and Avron, 1983; Ben-Amotz, 1996; Ben-Amotz *et al.*, 1982; Borowitzka *et al.*, 1990; Kleinegris, *et al.*, 2010; Krol *et al.*, 1997; Shaish *et al.*, 1993). β-Carotene is the predominant carotenoid in the marketplace, with other carotenoids such as lycopene, astaxanthin, canthaxanthin, and lutein having a much smaller market share.

β-carotene is still the most prominent carotenoid. While consumption developed only very moderately, products across all types of formulation and origin are no longer available at the price range seen in 2007. A general rise in prices resulted in an increase of the overall market value. The current market value of commercially used carotenoids is estimated at nearly $1.2 billion in 2010, with a chance to grow to $1.4 billion in 2018 with a compound annual growth rate of 2.3%. The market value of β-carotene, estimated at around $250 million in 2007, increased to just $261 million in 2010. This market is expected to grow to $334 million by 2018 at a compound annual growth rate of 3.1%. The price of natural *β*-carotene ranges from about US$300 to 3000 kg^{-1}, depending on the product type and the market demand. In 2010, the total market value of *β*-carotene, both synthetic and natural, was about US$260 million, and this is expected to increase to over US$300 million by 2018. Most carotenoids are still produced by chemical synthesis and will remain so over the study period (http://www.bccresearch.com/marketresearch/food-and-beverage/carotenoids-market-fod025c.html).

Qom Salt Lake is a large salt lake located north-east of Kashan and south-east of Tehran, with a surface of about 2400 km^2 and it is situated 800 m above sea level. Water salinity is above 200 $g.l^{-1}$. It is fed by four permanent and/or seasonal rivers (Agh, 2007).

The purpose of the present work was to study the role of salinity and light intensity parameters in β-carotene production content by *Dunaliella* sp. isolated from a hyper-saline Qom Lake in Iran.

Material and methods

Culture of alga

Wild types of *Dunaliella* sp. were collected via sterile plastic bottles from hyper-saline Qom Lake and transferred to laboratory, collected samples were brought to the laboratory and microscopic analysis was carried out to find out the presence of *Dunaliella* sp. cells. *Dunaliella* sp. cells were identified under microscope based on the morphological description given by Borowitzka and Siva (2007). Collected *Dunaliella* sp. samples were treated with 5 ml (per lit.) 1M KNO_3 and 0.1M KH_2PO_4 and were placed for 7-10 days in a static phytotron (25±2°C, 1.5 M NaCl). Modified Johnson's medium was prepared for culturing microalgae. Different concentrations of NaCl (1-3 M) were added to the medium and the pH of the growth medium was adjusted to 7.5 by dilute

(0.01M) and concentrated (1M) sulfuric acid or sodium hydroxide solutions. 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. Enriched indigenous isolates were cultivated after 10 days in 250 ml Erlenmeyer flasks with 100 ml of modified Johnson's medium with 1.5 M NaCl concentration under a continuous photon flux density of 100 µmol photons.$m^{-2}.s^{-1}$ and 25±2°C. Flasks each containing 100 ml of modified Johnson's medium (ASW) were inoculated with 50 ml of enriched samples (microalgae). For elimination of culture media contamination, samples were sub-cultured several times after microalgae growth and Selection of the clone was done based on the method described by Shaish *et al*. (1991). 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 sub-cultured every fortnight to have a log phase growth as described in Ben-Amotz (1980), Borowitzka and Siva (2007). The taxonomy of the genus *Dunaliella* Chlorophyta, Dunaliellales, with emphasis on the marine and halophilic species (Fazeli *et al*, 2006). Carotenoids accumulation by *Dunaliella tertiolecta* (Lake Urmia isolate) and *Dunaliella salina* (CCAP 19/18 & WT) under stress conditions (Fazeli *et al*, 2005; Olmos *et al*, 2000; Gomez *et al*, 2003; Hejazi and Wijffels, 2003).

Non-stress and stress phases

Isolates were placed in culture media with 1.5 M (8.75% w/v) NaCl concentration for thirty days, and then the most efficient isolate was placed again for ten days in culture medium with same conditions and then exposed to three salinities 1 (5.85% w/v), 2 (11.7% w/v) and 3 M NaCl (17.53% w/v) stresses for another twenty days. under a photon flux density of 100 µmol $m^{-2}s^{-1}$ in the photoperiods of 16:8 h under Light/Dark cycle, 25±2 °C and pH value of the culture media was set at 7.5 in static phytotron. Finally, cell counting (haemocytometer; NEUBAUER) and carotenoid measuring (spectrophotometry) were done. Samples was shaked to homogenize, then 950 µl of sample was transferred to screw-cap glass bottle and 50 µl of Lugol's Iodine solution was added for fixing. Number of cells was calculated as follows: Algae number (per ml) = n. 1000 x 0.1. 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), each experiment was carried out in triplicate (Fazeli *et al*, 2005 and 2006; Olmos *et al*, 2000; Gomez *et al*, 2003; Hejazi and Wijffels, 2003).

Pigment extraction and analysis

Carotenoid production ability of the desired samples according to the protocol provided by method Çelekli and Dönmez (2006) was took place, In the end of first phase (tenth day) and throughout of second phase the amount of carotenoid content (β-carotene content was measured at 453^{nm} by spectrophotometry, every two days (Fazeli *et al*, 2005 and 2006; Olmos *et al*, 2000; Gomez *et al*, 2003; Hejazi and Wijffels, 2003).

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 IBM SPSS-22 (©2013 IBM SPSS Inc., USA) and Microsoft Excel (©2013 Microsoft corporation, USA) softwares, respectively; and the statistical significance was determined at 99% confidence limit.

Results and discussion

Macroscopic and microscopic study of algae

The isolated and purified ovoid algal strain, which has one cup-shaped chloroplast and two equal flagella without cell wall, was identified as *Dunaliella* sp. by morphological examination under light microscope as

(Borowitzka and Siva, 2007). 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 15-30 days after inoculation of algae on the solidified agar medium. Algae were seen elliptical (pear shape) to round.

Number of cells and ß-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 ($\times 10^6$ 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 (Table 1).

Table 1. 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±2 °C and 100 µmol photons.m^{-2}.s^{-1} illumination, 1.5 M NaCl), Data are expressed as means of three replicates.

Stations No.	1.5 M NaCl and 100 µmol photons.m^{-2}.s^{-1}		
	Number of cells (cell.ml^{-1})	Total carotenoid (mg.l^{-1})	ß- carotene(pg[a].cell^{-1})
1 N:34.50729′ E: 51.72439′	1.38×10^6	5.41	3.91
2 N: 34.50106′ E: 51.76385′	1.49×10^6	5.22	3.5
3 N: 34.64378′ E: 51.83838′	1.01×10^6	4.72	4.67

a. pictogram.

According to preliminary results, among the isolates of Qom Lake, satiation no. 3 samples were considered for more productive, because they have the highest amount of ß-carotene production per cell. Therefore, in subsequent experiments only the effect of stress on these isolates was evaluated.

Table 2. Analysis of variance for number of cells and β-carotene content per cell of *Dunaliella* sp. in three different concentrations of salt.

Source	df	Mean Squares	
		Number of cells	β-carotene content
Molarity(M)	2	5.20**	132.53**
Day(D)	10	0.431**	32.54**
M×D	20	0.370**	1.52**
Error	66	0.00089	0.001

**, means significant at 1% probability levels.

Number of cells and carotenes content under salt stress conditions

The results of 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 is depicted in Fig. 1,2 and 3. The number of cells of *Dunaliella* sp. ranged

from 0.46 to 2.12×10⁶ cell.ml⁻¹ and total carotenoids content ranged from 0.072 to 11.02 mg.ml⁻¹ for the culture broth and 0.15 to 9.88 pg.cell⁻¹ for per cell basis during 30 days at all tested NaCl concentrations.

Analysis of variance of the traits are expressed as means of Correlation test showed the cell density of *Dunaliella* sp. was significantly correlated with the 1,2 and 3 M NaCl salinities (P<0.01) (Table 2).

Table 3. Comparison of the mean number of cells and β-carotene content per cell in three different salt concentrations.

Molarity (NaCl)	Number of cells (x10⁶.ml⁻¹)	β-carotene content (pg.cell⁻¹)
1	1.46[b]	3.69[c]
2	1.78[a]	4.28[b]
3	0.986[c]	7.42[a]

Each value represents the average of three replicates, values followed by the same letter(s) are not significantly different at 1% level by Duncan's multiple range test.

Dunaliella sp. showed its maximum cell density mean at 2 M NaCl concentration (1.78×10⁶ 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 1-3 M NaCl concentration during 30 days. As well as *Dunaliella* sp. showed its maximum total carotenoid content mean at 2 M NaCl concentration (7.76 mg.ml⁻¹). There were significant differences (P<0.01) at total carotenoids content mean of *Dunaliella* sp. grown in defined inorganic medium with 1-3 M NaCl concentration (salinity and it's relation with carotenoid production). In other words different molarities of NaCl concentration influenced on cell density and ß-carotene content per cell. Carotenoid to cell density ratio (ß-carotene content per cell) increased with salinity in *Dunaliella* sp. and high ß-carotene content mean observed at 3 M NaCl concentration that was 7.41 pg.cell⁻¹ after 30 days of incubation period (Table 3).

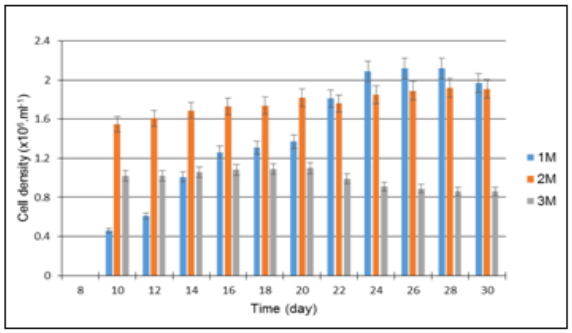

Fig. 1. The effect of the different salinity on number of cells of *Dunaliella* sp. during the incubation period (T: 25±2 °C; 100 µmol photons.m⁻².s⁻¹ illumination).

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 were 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.

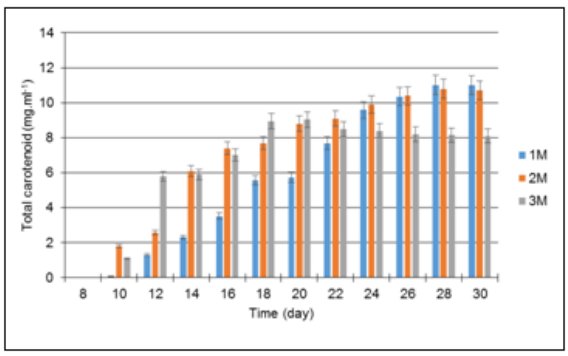

Fig. 2. The effect of different salinity on total carotenoids accumulation in *Dunaliella* sp. during the incubation period (T: 25±2 °C; 100 µmol photons.m⁻².s⁻¹ illumination).

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 0.46x10⁶ to 2.12x10⁶ at

defined conditions. Besides, β-carotene content (per cell) of *Dunaliella* sp. increased with increment of culture medium salinity from 0.15 to 9.88 pg.cell^{-1} per cell basis during 30 days at all tested NaCl concentrations.

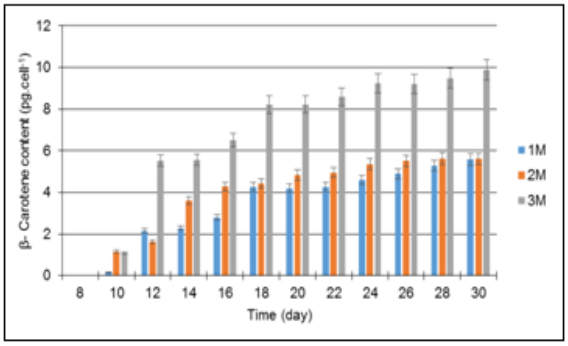

Fig. 3. The accumulated β-carotene concentration on a per cell basis by *Dunaliella* sp. at different salinity during the incubation period (*T*: 25±2 °C; 100 µmol photons.m^{-2}.s^{-1} illumination).

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.

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 (Tab. 3).

These results are in accordance with those reported by Ben-Amots and averon, (1982), Gomez *et al.* (2003) and Fazeli *et al.* (2005), despite lower cell density at 3 M NaCl concentration, this isolate, were produced higher β-carotene, that it is noteworthy in the viewpoint of biotech industries.

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