

## Effect of Different Salinity Levels on $\beta$ -Carotene Production by *Dunaliella* sp. Isolates from the Maharlu Lake, Iran

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### ABSTRACT

**Background and Objective:** Microalgae are a group of algae that produce biochemical products consisting of a wide range of carbohydrates, lipids and proteins that are commercially valuable. Interest in microalgal cultivation is currently blossoming globally. Species of *Dunaliella* are found in freshwater, euryhaline habitats of all continents, oceans including the Dead Sea and even the salt lakes of the Antarctic. This study investigates the effect of different salinity levels on  $\beta$ -carotene production by *Dunaliella* sp.

**Methods:** Water samples from a hyper-saline lake (the Maharlu Lake in Shiraz) were cultured in modified Johnson media. The  $\beta$ -carotene content was measured after the samples were treated with different salinities (1, 2 and 3M NaCl).

**Results:** The cell count and  $\beta$ -carotene content of *Dunaliella* sp. samples ranged between  $0.46 \times 10^6$  to  $2.12 \times 10^6$  cell.mL<sup>-1</sup> and 0.15 to 9.98 pg.cell<sup>-1</sup>, respectively. At the end of the experiments, the mean maximum cell content ( $1.78 \times 10^6$  cell. mL<sup>-1</sup>) and the highest mean  $\beta$ -carotene content (7.41 pg. cell<sup>-1</sup>) were obtained at 2 and 3M NaCl concentrations, respectively.

**Conclusion:** Salinity of the medium might affect the quantity and composition of carotenoids in *Dunaliella* sp. isolates. Alteration of the culture medium's salinity to 3M NaCl significantly increases the accumulation of  $\beta$ -carotene and total carotenoids in *Dunaliella* sp. isolates.

**Keywords:** *Dunaliella*, Microalgae, Maharlu Lake,  $\beta$ -carotene.

## INTRODUCTION

Microalgae are unicellular species, commonly found in marine, freshwater and salt lakes with the size ranging from a few micrometers to a few hundreds of micrometers. This group of algae produces biochemical products consisting of a wide range of carbohydrates, lipids and proteins that are commercially valuable. *Dunaliella* sp. is a widely distributed halophile green-orange microalgae with an extremely wide range of habitats. Some species such as *Dunaliella lateralis* live in fresh water, while *Dunaliella salina* predominates in hypersaline environments (1). When some species of *Dunaliella* are subjected to environmental stress conditions such as high salinity, high light intensity or nutrient deprivation, they overproduce and accumulate large amounts of  $\beta$ -carotene (2-6). Studies on the halophilic *Dunaliella bardawil* have shown a direct relationship between  $\beta$ -carotene content and salinity [7]. Carotenogenesis is also enhanced under nitrate-limiting conditions (3). In *D. salina*, low nitrate concentrations negatively affect growth, but enhance carotenoid accumulation (8). The Maharlu Lake is located 27 kilometers southeast of Shiraz, Iran, at the latitude and longitude coordinates of 29.4491 ° N and 52.8176 ° E and altitude of about 1460m. This fishless lake has an estimated average area of 230-280 km<sup>2</sup> (depending on evaporation and water influx), maximum depth of 0.5-3 m, and salinity of 119.5-280 g l<sup>-1</sup> (2- ~4.8 M) (9).  $\beta$ -carotene is a lipid-soluble orange pigment antioxidant, mainly used in cosmetics and food coloring. *D. salina* is a unicellular green microalga and a rich source of natural  $\beta$ -carotene (10), which can accumulate  $\beta$ -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 (3, 11-16).  $\beta$ -carotene is the predominant carotenoid in the marketplace, while other carotenoids such as lycopene, astaxanthin, canthaxanthin and lutein have a much smaller market share. While its consumption developed only very moderately, a general rise in prices resulted in an increased overall market value. In 2010, the Baden Aniline and Soda Factory took over Cognis, the only sizable company that produces  $\beta$ -carotene from algae. In 2011,

Royal DSM N.V. company acquired Vitatene, an important producer of  $\beta$ -carotene by fermentation. The manufacturing industry structures have been consolidated, which led to disappearance of several companies that were in the market during the mid-2000s. Currently, a limited number of companies dominate this global business. 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  $\beta$ -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  $\beta$ -carotene ranges from about US\$300 to 3000 kg<sup>-1</sup>, depending on the product type and the market demand. In 2010, the total market value of  $\beta$ -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 some studies are being conducted to produce them naturally. (17).

Ginzburg et al. [18] first reported the presence of *Dunaliella* sp. in the Maharlu Lake. The purpose of the present work was to study the effect of salinity and light intensity on  $\beta$ -carotene content produced by *Dunaliella* sp. isolated from the hyper-saline Maharlu Lake in Iran. Recent temperature rise, increase in water evaporation and decrease in water influx have led to increased salinity. As a result, the population of *Artemia* sp. (a crustacean) declined, and the abundance of *Dunaliella* sp. density in the Maharlu Lake in response to high salinity and high temperature has given the lake a red color.

## MATERIAL AND METHODS

### Sampling and microalgae isolation

Three stations/locations were selected from the middle and southern parts of the Maharlu Lake. Wild types of *Dunaliella* sp. were collected during mid-spring (drought months) and mid-summer in sterile plastic bottles and then transferred to a laboratory. Microscopic examination was carried out to identify *Dunaliella* sp. Cells based on

The collected samples were treated with 5 ml (per lit.) of 1M KNO<sub>3</sub> and 0.1M KH<sub>2</sub>PO<sub>4</sub>, and placed for 7-10 days in a static phytotron (at 25±2 °C, with 1.5M NaCl). The microalgae was cultivated in modified Johnson's medium with different concentrations of NaCl (1-3 M) and pH of 7.5, adjusted by dilute (0.01M) and concentrated (1M) sulfuric acid or sodium hydroxide solutions. In order to avoid precipitation of certain compounds, all stock solutions were sterilized separately (by autoclaving at 121 °C) and pooled aseptically. Sodium bicarbonate stock was heat-sterilized at 130 °C. After 10 days, enriched indigenous isolates were cultivated at 25±2 °C in 250 ml Erlenmeyer flasks with 100 ml of modified Johnson's medium (ASW) containing 1.5 M NaCl under a continuous photon flux density of 100 µmol m<sup>-2</sup> s<sup>-1</sup>. Then, 100 ml of modified Johnson's medium in each flask were inoculated with 50 ml of enriched samples (microalgae). The samples were sub-cultured several times after microalgae growth to avoid contamination. Colony selection was done according to the following method. First, a well-developed colony was picked up and used as a single clone for further studies. Then, pure culture of the clone was obtained by sub-culturing on nutrient agar. Finally, two-week old colonies were carefully picked and inoculated into 100 ml of fresh Artificial Sea Water (ASW) medium in 250 ml flasks. This pure culture was maintained and sub-cultured every fortnight to reach a log phase as described in the previous studies [10, 19-25].

#### **Non-stress and stress phases**

Isolates were placed in culture media containing 1.5M NaCl concentration for 30 days. The most efficient isolate was placed again in the culture medium for 10 days under the same conditions. It was then exposed to different salinities (1, 2 and 3 M NaCl) for another 20 days. Finally, cell counting and carotenoid measurement (using spectrophotometry) were performed. 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 three times. The samples were shaken to homogenize, and 950 µl of the sample were transferred to carotenoids accumulation and accumulated β-carotene concentration per cell basis by

screw-cap glass bottle. Then, 50 µl of Lugol's Iodine solution were added for fixation. The number of cells was calculated using the following formula: Algae number (per ml) =  $n \cdot 1000 \times 0.1 \cdot X$ .

In this formula, n is the number of *Dunaliella* sp. cells counted in the large square (total volume of 0.1 mm<sup>3</sup>), while X was dilution factor applied in combination with the Lugol's Iodine solution (0.95). Cell counting was conducted at the end of 10th day and in a period of 20 days (once every two days). Number of cells was evaluated in 1M NaCl [5.8% (w/v)], 2M NaCl (11.68%) and 3M NaCl (17.53%). The samples were cultivated under a photon flux density of 100 µmol m<sup>-2</sup> s<sup>-1</sup> and a 16:8 h light-dark cycle, at 25±2 °C. The pH value of the culture media was set at 7.5 in static phytotron. Each experiment was repeated three times [20, 25].

#### **Pigment extraction and analysis**

Carotenoid production capability of the samples was evaluated according to the protocol provided by Celekli and Donmez [26]. Unlike the first step (ten-day phase), at the stage of stress, the samples were treated with different salinities (1-3 M NaCl) for twenty days (after the tenth day). In the second phase, the β-carotene content was measured at 453nm by spectrophotometry every two days [20, 25].

#### **Statistical analysis**

All data were expressed as means of the three replicated studies. One-way analysis of variance (ANOVA) was used in XLSTAT (ver. 2014.5.03) software, followed by Duncan's test (statistical significance at 99% confidence interval). Graphs were plotted using the Microsoft Excel (Microsoft Corporation, USA) software.

## **RESULTS**

Based on morphological examination under light microscope, cell wall-less isolated and purified ovoid algal strains with one cup-shaped chloroplast and two equal flagella were identified as *Dunaliella* sp. In the enrichment step, after adding phosphate and nitrate, growth and reproduction of algae were achieved. Gradual color change of the medium from colorless to a green-orange mixture was noted because of algae growth morphological characteristics. Viability of the cells was determined by a light microscope.

after about 10 days. Algae grew well after culture in liquid modified Johnson's medium. Spherical shaped colonies appeared 15-30 days after inoculation of algae onto the solidified agar medium. The algae had elliptical (pear-shaped) to rounded shapes. The samples were investigated in a batch system under different salinity levels. The results were presented as the number of cells per mL ( $\times 10^6$  cell.mL<sup>-1</sup>) and accumulated  $\beta$ -carotene concentration per cell basis (pg.cell<sup>-1</sup>) or per mL of culture broth (mg.mL<sup>-1</sup>). Table 1 shows the average  $\beta$ -carotene content and mean number of cells in most efficient isolates from the relevant stations (after 30 days of treatment). According to the preliminary findings, the isolates from station no.3 were considered more productive, because they had the highest amount of  $\beta$ -carotene per cell. Therefore, the effect of stress conditions in subsequent experiments was evaluated only on these isolates. The effect of different salinity levels on total *Dunaliella* sp. during the incubation period is

shown in Figures 1-2. The number of *Dunaliella* sp. cells ranged from 0.46 to  $2.12 \times 10^6$  cells. mL<sup>-1</sup>. The total carotenoids content ranged from 0.07 to 11.03 mg. mL<sup>-1</sup> in the broth culture, and from 0.15 to 9.98 pg. cell<sup>-1</sup> on per cell basis after 30 days at all tested NaCl concentrations. Results of ANOVA showed a significant correlation between *Dunaliella* sp. cell density and salinity level ( $P < 0.01$ ) (Table 2). There were significant differences in the daily measurements of carotenoid contents of *Dunaliella* sp. grown in defined inorganic medium with 1-3 M NaCl concentration after days. The highest mean of maximum total carotenoid content in *Dunaliella* sp. was recorded at 2M NaCl concentration (7.76 mg.mL<sup>-1</sup>). Carotenoid to cell density ratio ( $\beta$ -carotene content per cell) of *Dunaliella* sp. increased with rising the salinity level. The highest mean of  $\beta$ -carotene content (7.41 pg.cell<sup>-1</sup>) was observed at 3M NaCl after 30 days of incubation.

Table 1- Effect of initial pH value and NaCl concentration on number of cells and  $\beta$ -carotene content of *Dunaliella* sp. after 30 days (pH=7.5;  $25 \pm 2$  °C and  $100 \mu\text{mol m}^{-2} \text{s}^{-1}$  illumination, 1.5 M NaCl).

Stations No.	1.5 M NaCl and $100 \mu\text{mol m}^{-2} \text{s}^{-1}$		
	Number of cells (cell.mL <sup>-1</sup> )	Total carotenoid (mg.L <sup>-1</sup> )	$\beta$ -carotene (pg <sup>a</sup> .cell <sup>-1</sup> )
1	$1.46 \times 10^6$	6.20	3.69
2	$1.78 \times 10^6$	7.76	4.27
3	$0.98 \times 10^6$	7.20	7.41

a. pictogram

Data are expressed as means of three replicates.

Table 2- Results of ANOVA for number of cells and  $\beta$ -carotene content per cell of *Dunaliella* sp. at different salinity levels (1-3 M NaCl).

Source	Df <sup>1)</sup>	Mean Squares	
		Number of cells	$\beta$ -carotene content
Salinity	2	3.13**	205.59**
Day(D)	10	0.39**	56.82**
Salinity×D	20	0.45**	7.47**
Error	66	0.0002	0.0061

<sup>1)</sup> Degrees of Freedom

\*\* significant at 1% probability level

Figure 1- The effect of different salinity levels on total carotenoids accumulation in *Dunaliella* sp. during the incubation period (T: 25±2 °C; 100 μmol m<sup>-2</sup> s<sup>-1</sup> illumination, 1-3 M NaCl).

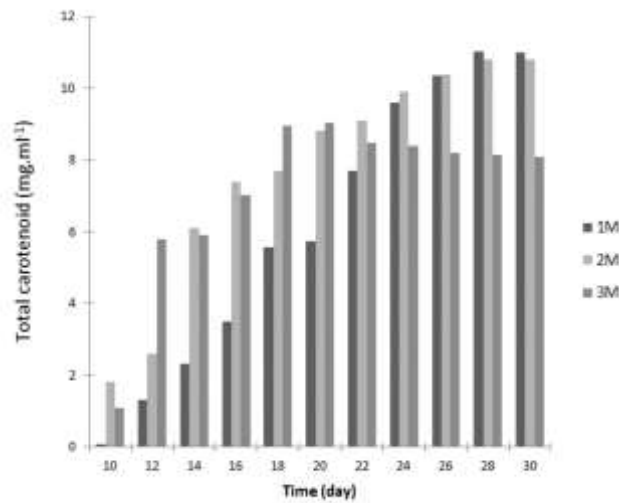
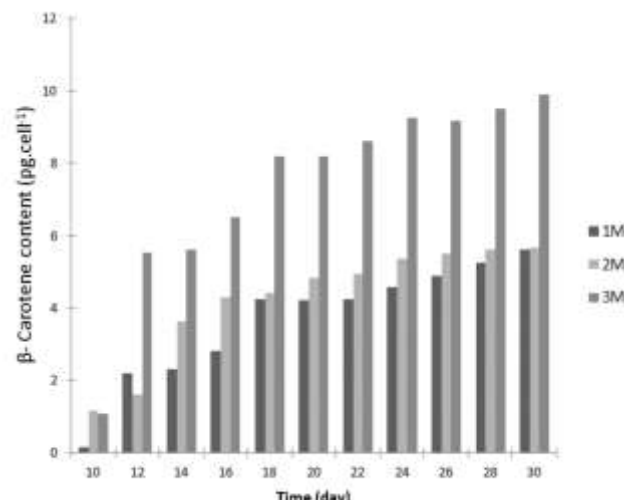


Figure 2- Accumulated β-carotene concentration on a per cell basis by *Dunaliella* sp. under different salinity levels during the incubation period (T: 25±2°C; 100 μmol m<sup>-2</sup> s<sup>-1</sup> illumination, 1-3 M NaCl).



## DISCUSSION

There were significant differences in the mean total carotenoids content of *Dunaliella* sp. grown in defined inorganic medium with 1-3 M NaCl concentrations. In other words, salinity level affected the cell density and total carotenoid contents.

According to the previous studies, the environmental factors have the biggest impact on carotenoid production by *Dunaliella* sp. (10, 12, 21, 26-28). The highest β-carotene content is observed at high salinity, high temperature and high light intensity. Production of carotenoids is usually influenced by the growth rate, as decrease in the growth rate increases the carotenoid production rate (12, 28). In the present study, the number of *Dunaliella* sp. cells increased from 0.46 to 2.12 × 10<sup>6</sup>.ml<sup>-1</sup> under the tested

conditions. Moreover, the increase in the culture medium salinity level increased the β-carotene content (per cell) of *Dunaliella* sp. from 0.15 to 9.98 pg.cell<sup>-1</sup> per cell basis after 30 days. However, the productivity on a cellular basis was significantly higher at high salinity concentrations (3M NaCl). Consistent with these findings, the studies of Gomez et al. [24] and Fazeli et al. [22] reported that total carotenoid production and cell productivity were affected by salinity level. Maximum biomass production and carotenoid concentrations were compared at different salinity levels. The cell growth rate at 3M NaCl was significantly less than that at 1 and 2M NaCl concentrations, while the production rate per cell at 3M NaCl was significantly higher. These results are in

accordance with Ben-Amots and Averon (12), Gomez et al. [24] and Fazeli et al. (22). Despite the lower cell density at 3M NaCl concentration, the isolate produced more  $\beta$ -carotene, which could be noteworthy for biotech companies.

## CONCLUSION

Different salinity levels might affect the quantity and composition of carotenoids in *Dunaliella* sp. isolates. Increasing the culture medium's NaCl concentration to 3M

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