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Research Article

Optimization of β-carotene production by an indigenous isolate of *Dunaliella salina* under salinity-gradient stress

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Abstract

Dunaliella salina has significant industrial aspects, especially for the accumulation of high amounts of β-carotene, which is the main natural source in aquatic food webs. The first step of commercial production is accurate identification owing to phenotypic plasticity in the genus *Dunaliella* and lack of rigid cell wall. In this study, *D. salina* was isolated from Sirjan Kafe Namak of Iran when algal bloom had occurred and then identified based on morphological and molecular properties. Morphological characteristics bore a close resemblance to the species *D. salina*, including cell size, cup-shaped chloroplast, large basal pyrenoid, and stigma position. Moreover, phylogenetic analysis of internal transcribed spacer (ITS) region (~700 bp) corroborated morphological features by confirming the isolate at the species level. Under five different salinity concentrations, this strain could accumulate β-carotene in the range of 0.51-2.78 μg/mL with optimum growth at 2 M NaCl comparable with reported data for *D. salina*.

Keywords: *Dunaliella*, Internal transcribed spacer, Salinity tolerance, Sirjan, Iran

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Introduction

Dunaliella is an extremophile microalga without a rigid cell wall that can tolerate 5 M NaCl (Borowitzka and Siva, 2007). *Dunaliella* is of great increasing interest to a wide range of researchers in nutraceutical, cosmetic and pharmaceutical industries (Marino *et al*., 2020; Roy *et al*., 2021). Some species of this genus accumulate natural β-carotene up to 10% of algal dry weight under stress conditions such as elevated light densities, high salinity and nutrient deficiency (Ben-Amotz and Avron, 1983; Gallego-Cartagena *et al*., 2019).

Additionally, the genus *Dunaliella* is recognized as a valuable source of proteins, carbohydrates, lipids, and vitamins for food and feed additives (Hosseinzadeh Gharajeh *et al*., 2020). Mainly in hypersaline habitats, *Dunaliella* is the dominant species of algal flora (Mohebbi, 2010) that forms the main natural food source for *Artemia* which is an excellent feed for fish and crustaceans, including shrimp, prawn, and larval stages of fish. Mohebbi *et al*. (2016) revealed that *Dunaliella* has a higher potential in creating better reproductive characteristics in *A. urmiana* than other algae including *Tetraselmis suecica, Nannochloropsis oculata, Chaetoceros* sp., *Chlorella* sp., *and Spirulina* sp. It also has higher efficiency of growth length, survival rates, and reproduction outcomes; therefore, it is reported as a desirable food for *A. urmiana*. Besides, Hannah *et al*. (2013) stated that *D. salina* improves the nutritional status of *Artemia* sp.

On the other hand, *D. salina* enhances the immunity of the shrimp *Penaeus monodon* against the white spot syndrome virus. Since β-carotene (source of pro-vitamin A) acts as an antioxidant, it is used as a natural feed additive in the aquaculture industry to enhance immune function and disease resistance (Madhumathi and Rengasamy, 2011). Thus *D. salina* not only promotes growth but also enhances health, immunity, and disease resistance in tiger shrimp, *P. monodon*, that is for accumulation of β-carotene and its ability to convert into astaxanthin. Moreover, food supplementation of *D. salina* enhances growth and body color in white shrimp P*enaeus indicus* to attain the desired flesh and skin pigmentation (Ahmad *et al*., 2015).

Identification of *Dunaliella* only by the morphological-based taxonomic study is quite challenging, thus many taxa are misidentified in works of literature due to the lack of rigid cell wall, which subsequently leads to high phenotypic plasticity (Oren, 2005; Borowitzka and Siva, 2007). Therefore, physiological, biochemical, and molecular characteristics were used to well identify species and even different strains of *D. salina* (Olmos *et al*., 2000; Olmos-Soto *et al*., 2002; Hejazi *et al*., 2010).

Iran is a suitable habitat for the green alga *Dunaliella*, for having several salt lakes such as Kafe Namak which is located 25 km west of Sirjan

desert area (29°26'50"N-55°22'57"E). Kafe Namak was an ephemeral lake that has changed to a salt pan, and there is no sign of life there due to high temperature, extreme evaporation, poor soil, and high salinity. According to the values of De Martone's aridity index, Sirjan has a semi-desert climate with an average of 135 mm rainfall annually.

In the present study, *D. salina* was isolated from Sirjan's Kafe Namak lake in Iran. It is described based on morphological features and molecular data of the ITS region. Physical and chemical properties, growth in different NaCl concentrations, andphotosynthetic pigments were also determined.

Materials and methods

Field collection, isolation and culture condition

The red color water sample was collected from Kafe Namak Lake (Fig. 1). It should be noted that the collected water was visibly red containing cubic salt crystals. The sample was kept at a low temperature while transferring to the laboratory (28±2ºC). Then various chemical parameters of the water sample, like the amount of phosphate and nitrate, electrical conductivity, salinity, and pH were determined.

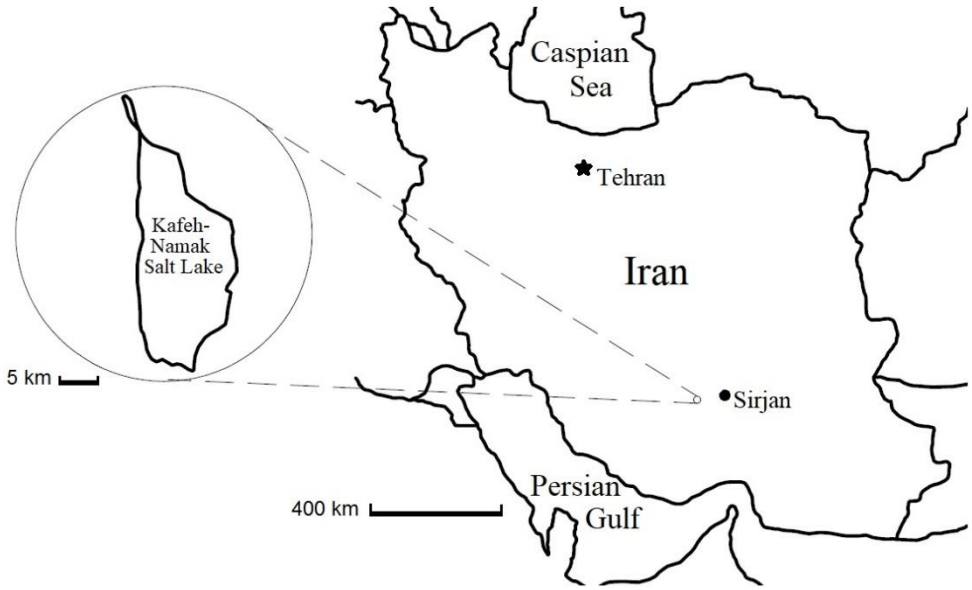

Figure 1: Map showing sampling location, 29° 26' 50" N- 55° 22' 57" E.

To avoid bacterial contamination, serial dilution was followed by plating (Droop, 1954), and increasing NaCl concentration was also implemented. After isolation, the colony was transferred to BG11 medium with 1.5 M NaCl concentration. The culture was shaken manually every day and incubated at 25±2°C under 16.8 h light/dark photoperiod provided by cool white fluorescent lamps at 52.84 µmol $m^{-2}s^{-1}$.

Morphological identification

Specimens were identified based on microscopic examination using an Olympus BH-2 microscope. All quantitative and qualitative characteristics were evaluated based on a minimum of 20 measurements for each character, including size, shape of the cell, and features of flagella, stigmas, and pyrenoids.

Salinity tolerance

To obtain the required salinity, purified alga was grown in BG11 medium containing five different NaCl concentrations (0.5, 1.0, 1.5, 2.0, and 2.5).

Growth and pigment analysis

Algal growth was monitored every 2 or 3 days by optical density (OD) that was determined at 730 nm using a Unico spectrophotometer model 2100 Vis. The number of photosynthetic pigments was evaluated according to the protocol provided by the method of Çelekli and Dönmez (2006). In brief, chlorophyll and total carotenoid were estimated via spectrophotometer in different salinity concentrations. Algal cells were harvested every week from each cultural medium by centrifugation and the cell contents were extracted with acetone 80% (v/v) under dim light, to avoid pigment bleaching at room temperature. Absorbance of chlorophyll a and carotenoid content were measured at appropriate wavelengths. Finally, concentrations of the pigments were calculated using the following equations (Jeffrey and Humphrey, 1975; Borowitzka and Siva, 2007):

1) $\text{Chlorophyll a}\ (\mu g/mL) = 11.93 \times A664 - 1.93 \times A647$
2) $\text{Total carotenoids}\ (\mu g/mL) = 3.86 \times A452 \times Vc/Vs$

Where A664, A647 and A452 = the absorbance at 664, 647, and 452 nm, respectively.
Vc=volume of culture sample (mL)
Vs=volume of extract (mL)

DNA extraction and PCR amplification of ITS Region

Genomic DNA of the strain was evaluated according to Hejazi *et al*. (2010). Full region of ITS was amplified using the two primers, AB1 and AB2. PCR reactions were evaluated in 50µl containing 20 ng genomic DNA in TE (Tris/EDTA) buffer, pH 8 and 50 ng of the primers using 1×PCR Master Kit (CinnaGen PCR Master Kit, Cat. No. PR8250C). PCR amplification was carried out under the following conditions: 5 min at 95°C as initial denaturing time, 30 cycles of 95°C for 1 min, 50°C for 50 sec and 72°C for 2 min followed by final extension step of 72°C for 10 min (Sathasivam *et al*., 2012). Phylogenetic analysis of sequences was done using PAUP* version 4.0b 10 (Swofford, 2002) with sequences obtained from GenBank databases (www.ncbi.nlm.nih.gov).

Results

Water analysis

Chemical properties of the water sample showed higher salinity (27.8% NaCl) but phosphate was at the lowest level <0.01 mg/L, and the amount of nitrate was 9.0 mg/L. Moreover, EC and pH of the collected water samples were 460000 µmhos/cm and 7.37, respectively.

Microscopic observation

The water sample was evaluated microscopically. According to Borowitzka and Siva (2007), the isolated strain was identified as *D. salina*, which belongs to the section Dunaliella with salinity optima of more than 6% NaCl and the capability of turning into red color due to accumulation of large amounts of β-carotene.

However, many phenotypic features of this genus depend on environmental conditions. The cell was oval to circular (7–15 µm) biflagellated, with the lack of cell wall, a cup-shaped chloroplast and green to dark red in color (Fig. 2, A-H).

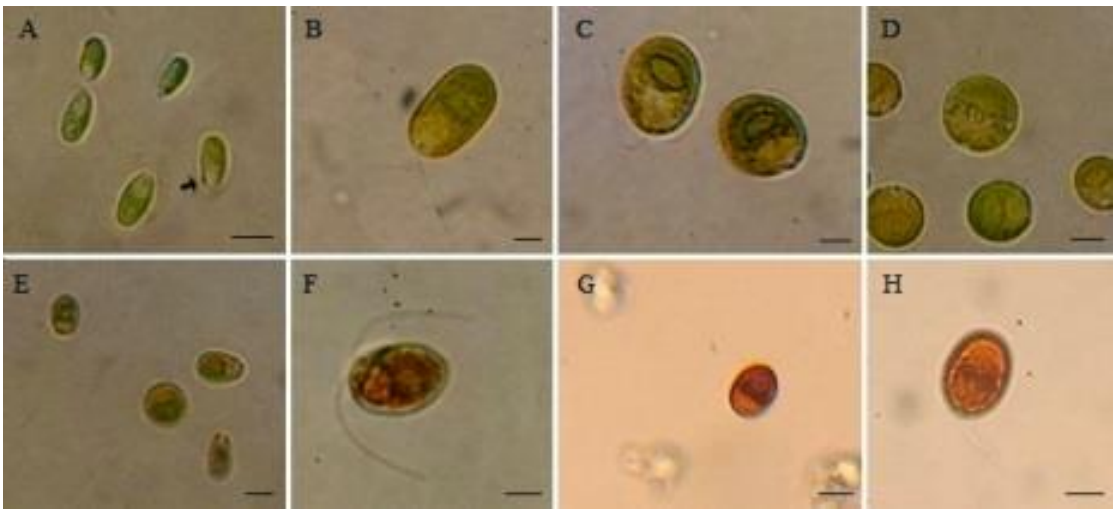

Figure 2: Light micrographs of *Dunaliella salina*. a) young cells (arrow indicates stigma), b) cylindrical cell, c) cells with a distinct pyrenoid, d) spherical cells without flagella and eyespot, e) ovoid and spherical cells, f) biflagellated cell in the early red phase, g and h) red phase. Scale = 5µm.

Effect of salinity on cell growth

Results of the grown taxon at different salinity concentrations are shown in Figure 3, which indicated absorbance to be at λ=730 nm. Biomass density of the cells gradually increased in all treatments, but the highest biomass density was obtained in 2.0 M NaCl concentration subsequently followed by 2.5, 1.5, 1.0, and 0.5 M NaCl concentrations, respectively. The number of cells was decreased after the 21st day of inoculation.

Effect of salinity on chlorophyll and β-carotene content

The effect of different salinities on chlorophyll and accumulated β-carotene concentration was evaluated. The results shown in Figure 4 indicated that on the 21th day after inoculation, the chlorophyll content (µg/mL) reached the maximum content in 2.5 M NaCl

concentration followed by 2.0, 1.5, 1.0 and 0.5 M salinity treatments.

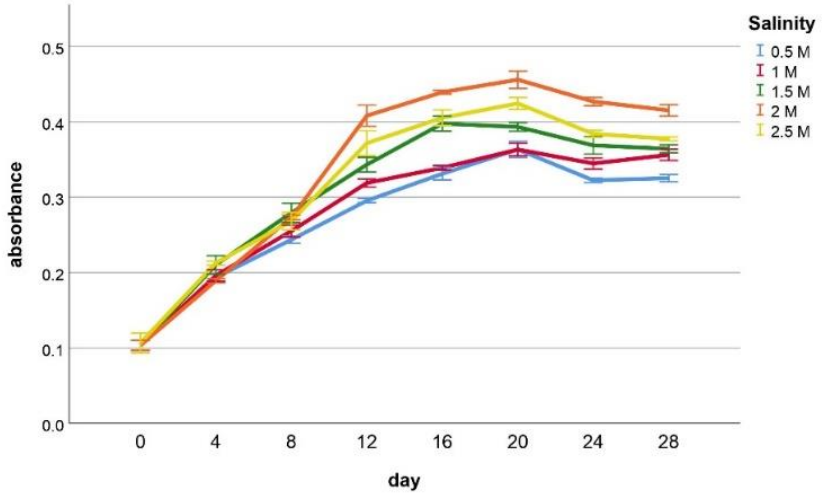

Figure 3: Growth of *Dunaliella salina* in different NaCl concentrations, according to absorbance at wavelength 730 nm. The data represent the means of three biological replicates. Error bars: 95% CI and ± 2 standard deviation.

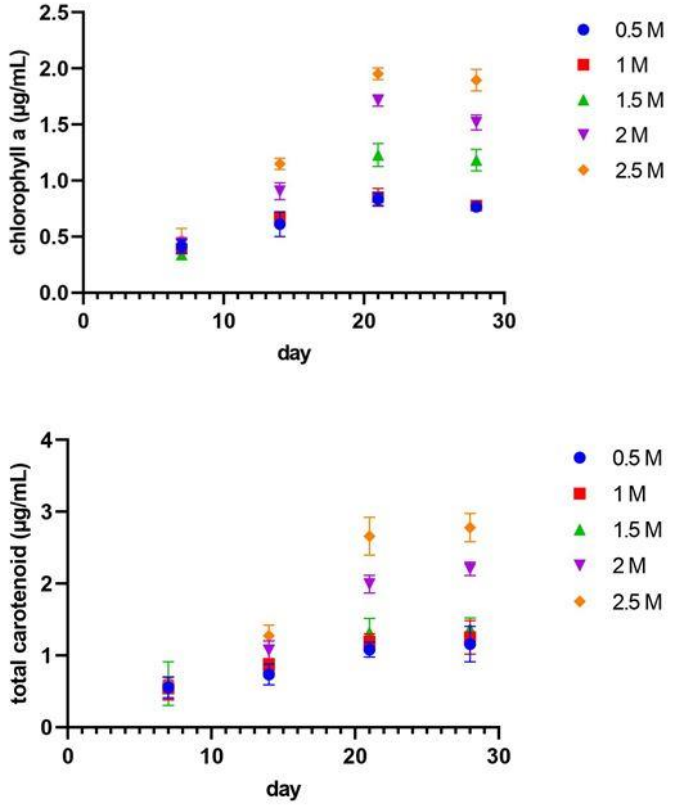

Figure 4: Chlorophyll a and total carotenoid content of *Dunaliella salina* in different NaCl concentrations. The data represent the means of three biological replicates. Error bars show standard deviation.

The β-carotene content ranged from 0.51 to 2.78 µg/mL during 28 days. By increasing NaCl concentration, a significant increase in β-carotenoid

amount was observed. Among the five different NaCl concentrations, the highest β-carotene production was obtained in 2.5 M NaCl, and the lowest was found in the 0.5 M NaCl medium treatment.

Phylogenetic analysis

The investigated species (accession number: MW286838) was compared to 18 reference strains in GenBank of closely related taxa belonging to the genus *Dunaliella*, and *Chlamydomonas reinhardti* was used as an outgroup. Based on the phylogenetic analysis, the isolated taxon fell into the section Dunaliella. It is also proved by morphological characteristics, especially the ability of turning color to red. In addition, the alga exhibited the highest similarity to *D. salina* (KJ094633) and *D. salina* (MN880875) isolated from England and Egypt, respectively (Fig. 5). Moreover, it was phylogenetically distant from two members of the genus *Dunaliella*, *D. pseudosalina* and *D. parva*, as it was verified by morphological differences.

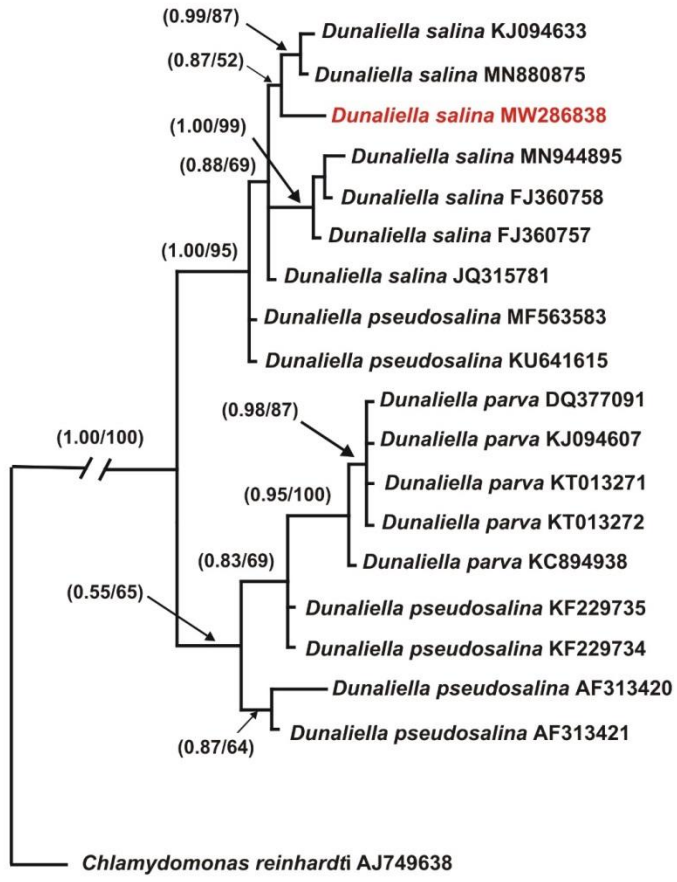

Figure 5: Fifty-percent majority rule consensus tree resulting from Bayesian analysis of the ITS dataset. Numbers above branches are posterior probability as well as parsimony bootstrap values, respectively. Values<45 % are not shown.

Discussion

Considering all results obtained in this study, it can be delineated that the taxon belongs to the section Dunaliella, including *D. parva*, *D. pseudosalina* and *D. salina*, as they can turn orange to red. Besides, the optimum salinity for growth is more than 6% NaCl. Under this section, the algae only matched in size to *D. salina* because it had larger cells than *D. parva* and *D. pseudosalina*. In addition to size, *D. salina* had diffuse rather than a distinct stigma, contrary to what is found in *D. parva* and *D. pseudosalina*. Furthermore, in our study cells were green to dark red, exactly comparable to *D. salina*. Nevertheless, *D. parva* cells are green to slightly orange and *D. pseudosalina* cells are green to orange (Borowitzka and Siva, 2007). Therefore, based on morphological and physiological characteristics (Table 1), the screened isolate proved to be *Dunaliella salina*.

Table 1: Comparison of three members of section Dunaliella (Borowitzka and Siva, 2007).

Section Dunaliella	***D. parva***	***D. pseudosalina***	***D. salina***
Cell color	green to slightly orange	green to orange	green to dark red
Cell size	9–16 μm long (mean 12 μm), 4–10 μm wide (mean 7 μm).	11–23 μm long (mean 18 μm), 6–16 μm wide (mean 11 μm).	5–29 μm long (mean 10.9–16.9 μm), 3.8–20.3 μm wide (mean 7.9–13.2 μm).
Stigma	Stigma small, red and distinctive	Stigma large and distinctive	Stigma anterior, diffuse and difficult to distinguish, especially in red cells

Moreover, in terms of morphology, the green color of *Dunaliella* cells turned red and the cell shape changed to spherical in high salinity. These results are similar to studies that had revealed some species change their shape under unfavorable conditions (Ben-Amotz *et al*., 2009). Further, as regards physiological behavior, the studied isolate of *Dunaliella* showed optimum growth at 2 M NaCl concentration on the 21st day of cultivation, which is similar to the growth results of *D. salina* isolated from the Gave-Khooni Salt Marsh (Hadi *et al*., 2008) while *D. tertiolecta* isolated from the same marsh did not show significant growth at this salinity (Hosseinzadeh Gharajeh *et al*., 2012). Similar results were reported by some studies that optimum growth was in about 2 M salinity (Loeblich, 1982; Sathasivam *et al*., 2014).

In this study, the salinity factor was optimized to increase carotenoid production due to the importance of *Dunaliella* and its commercial application in various industries. Many studies have tried to increase production efficiency by optimizing cultivation conditions (Gonabadi *et al*., 2021). One of the main environmental factors affecting the growth process and the production of economic metabolites in this genus is the concentration of salt in the culture medium (Ahuja *et al.*, 2020). Increased salinity enhances the accumulation of β-carotene and glycerol in this genus (Borowitzka, 2013). By optimizing the culture medium, we increased carotenoid production by about 2.5 fold (1.15 to 2.78 µg/mL), which is comparable with findings of previous studies (Borowitzka *et al*., 1990; Gonabadi *et al*., 2021).

The maximum carotenoid amount was 2.78 µg/mL, which occurred at 2.5 M NaCl on the last day of cultivation. This result was in accordance with the report of Hadi *et al*. (2008). Besides, the previous studies also asserted increasing salinity (here 2.5 M NaCl) favors carotenoid biosynthesis while it represses the growth rate (Gómez *et al*., 2003; Jahnke and White, 2003).

It should be noted that in 2015, the hypersaline species, *Dunaliella bioculata* Butcher, 1959 is reported from Sirjan Salt Lake (Soltani Nezhad and Mansouri, 2016). But in the present study, this species was not found. There are significant differences between the two species *D. bioculata* and *D. salina*. Firstly, *D. bioculata* belongs to the section Viridis, which is always green because it is not capable of turning red in culture. In addition, in terms of stigma, vegetative cells of *D. bioculata* usually have two stigmata while *D. salina* has only one diffuse stigma that is hard to see, especially in red cells. Furthermore, they vary in cell size and flagella length (Borowitzka and Siva, 2007).

In conclusion, based on the combination of morphological data with molecular characteristics of ITS regions the investigated isolate was identified as *Dunaliella salina*. It is properly validated by extra information on accumulation of high amounts of carotenoids and optimum growth at 2 M NaCl.

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