

Characterization of a new *Dunaliella salina* strain MSI-1 based on nuclear rDNA ITS sequences and its physiological response to changes in composition of growth media

Hajar Zamani · Ali Moradshahi ·
Hamid Reza Karbalaee-Heidari

Received: 5 March 2010 / Revised: 15 August 2010 / Accepted: 23 August 2010 / Published online: 8 September 2010
© Springer Science+Business Media B.V. 2010

Abstract Since the first formal description of the unicellular green algae *Dunaliella salina*, its presence in hypersaline environments worldwide and its physiological responses to different environmental conditions have been studied extensively. Moreover, due to massive carotenoid accumulation by some strains under specific growth conditions, its biotechnological applications have attracted a great deal of scientific interest. In this study, the phylogenetic relationship, growth, and carotenogenesis of a new strain of *Dunaliella salina* isolated from Maharlu salt lake in Shiraz (latitude 29.26°N, longitude 52.48°E), Iran were investigated. First, a phylogram based on neighbor-joining analysis of the nuclear rDNA ITS (ITS-1 + 5.8 rDNA + ITS-2) sequence data was constructed. The phylogenetic tree showed that the new isolate is part of a major clade containing several strains of *D. salina* and was designated as *D. salina* MSI-1. Then, the responses of the new isolate to the initial pH of the culture media and different concentrations of nitrate, NH_4^+ , and citrate were examined.

As with other strains of *D. salina*, growth and carotenogenesis were controlled by the levels of nitrate and NH_4^+ in the growth media. Low available nitrogen negatively affected growth but enhanced carotenoid accumulation. Insensitivity of carotenogenesis to citrate indicates a minor contribution of cytosolic IPP synthesis to the overall carotenoid production. Despite changes in the initial pH of the culture media over the experimental period, the initial pH had marked effects on the growth and carotenogenesis of the new isolate. These effects, together with the higher cell carotenoid content observed at pH 11.0, await further research. The results confirm that the analysis of the ITS sequences is a reliable basis for determination of the genetic relatedness among strains of the genus *Dunaliella*, and the search for strains with novel characteristics may have valuable biotechnological applications.

Keywords *Dunaliella salina* strain MSI-1 · ITS sequence · Nitrogen source · pH · Carotenogenesis

Introduction

Dunaliella salina is a halophilic unicellular green algae with a wide geographical distribution (Borowitzka & Borowitzka, 1988a; Javor, 1989). This species of the genus *Dunaliella* is the major primary producer in the hypersaline environment and is capable of producing large amounts of carotenoids

Handling editor: Luigi Naselli-Flores

H. Zamani · A. Moradshahi (✉) ·
H. R. Karbalaee-Heidari
Department of Biology, Faculty of Sciences,
Shiraz University, 71454 Shiraz, Iran
e-mail: amoradshahi81@yahoo.com

under inductive conditions (Ben-Amotz & Avron, 1990).

Although the annual global demand for beta-carotene is around 1430 tons, less than 3% is natural carotene and the balance is met through synthetic carotene (Gomez & Gonzalez, 2004). Many studies have shown that carotenoid accumulation by *D. salina* is enhanced by suboptimal growth conditions such as high salinity or nutrient limitation (Loeblich, 1982; Borowitzka et al., 1990). Some strains of *D. salina* can accumulate large amounts of carotenoids which may consist of more than 10% of their dry weights (Borowitzka & Borowitzka, 1988a; Borowitzka et al., 1984). Growing *D. salina* ARL5 in a salt solution complex comprising 0.35 M KCl, 0.6 M MgSO₄, 2.0 M NaCl, and 0.5 mM KNO₃ produced not less than 12% beta-carotene on dry weight basis in a single stage of active growth (Venkatesh et al., 2005). The addition of 3 μM citric acid and 10 mM MgSO₄ to the culture media improved dry weight and beta-carotene production by *D. salina* (Hai-Zhu, 1998). Cultivation of *D. salina* as a natural source of beta-carotene on a mass scale still needs to be developed to satisfy the global demand. Biochemical, physiological, and genetic variability has been reported at an intraspecific level in *D. salina* with different capacities for growth and carotenogenesis (Gomez & Gonzalez, 2004). Genetic studies for strain selection together with the optimization of growth and carotenogenesis conditions are of prime importance to achieving this goal.

The genetic relationship among several strains of *Dunaliella* from geographically very distant locations, such as the USA, England, Chile, Mexico, and China, has been reported by several investigators (Gonzalez et al., 2001; Gomez & Gonzalez, 2004). To the best of our knowledge, no report has been provided for the phylogenetic relationship of Iranian *D. salina* strains with those in other countries.

In this study, *D. salina* was isolated from Maharlu salt lake in Shiraz (latitude 29.26 N, longitude 52.48 E), Iran and by using the sequence of internal transcribed spacers of the 45S nuclear ribosomal cistron (ITS-1 and ITS-2 plus 5.8S rRNA gene), its phylogenetic relationship with reported *D. salina* strains was established. The aim was then to investigate the growth and carotenogenesis of the isolated strain as affected by pH and different concentrations of KNO₃, NH₄Cl, and citrate of the culture media.

Materials and methods

Microalgae source

Dunaliella salina was isolated from Maharlu salt lake in Shiraz (latitude 29.26 N, longitude 52.48 E), Iran and purified as described previously (Nikookar et al., 2004).

Growth conditions

Dunaliella salina was cultured in 250-ml flasks with 100 ml modified growth medium as described by Ben-Amotz & Avron (1990). To investigate the effects of pH on growth and carotenogenesis, the initial pH of the culture medium was adjusted at 7, 8, 9, 10, or 11 using 0.1 M NaOH or HCl. As the nitrogen source, different concentrations of KNO₃ (0.25, 0.5, 1.0, 2.5, and 5.0 mM) or NH₄Cl (0.05, 0.1, 0.5, and 1.0 mM) were added to the culture media. In another set of experiments, sodium citrate at 1, 5, and 25 μM was added to each flask. The flasks were kept in a growth chamber set at 22 ± 2°C and continuously illuminated with cool white fluorescent lamps at an intensity of 50 μmol m⁻² s⁻¹. At specified time intervals, triplicate samples were taken for various measurements.

Determination of cell concentration

Cell concentration was measured by cell count using a hemacytometer and reported as the number of cells ml⁻¹ of the culture medium.

Total carotenoids determination

The algal cells in 1 ml of culture medium were harvested by centrifugation at 1500g for 15 min, and pigments were extracted by ultrasonication of the cells in 80% acetone. The extract was centrifuged at 5000g for 10 min, and total carotenoids were determined as described by Eijkelhoff & Dekker (1997).

Nitrate determination

The concentration of nitrate in the culture media was measured spectrophotometrically as reported by Coesel et al. (2008).

Total DNA extraction and ITS PCR amplification

Fifty millilitre of 21-day growing batch culture cells were harvested by centrifugation at 5000g for 10 min, and the genomic DNA extraction was performed as described by Gomez & Gonzalez (2001). The ITS region was amplified using the universal primer AB28 (5'-GGGATCCATATGCTTAAGTTCAGCG GGT-3') and TW81 (5'-GGGATCCGTTTCCGTAG GTGAACCTGC). The amplification was done by initial denaturation at 95°C for 4 min followed by 35 cycles of 93°C for 1 min, 55°C for 45 s, and 72°C for 1 min with a final extension step of 10 min at 72°C. The amplified DNA was analyzed by 1% agarose gel electrophoresis, and the ITS fragment was purified using the Roche agarose gel DNA extraction kit. The purified PCR product was sequenced in both directions using an automated sequencer by SeqLab Company, Germany.

Data analysis

The ITS region sequences obtained in this study were aligned with the sequences in the GenBank database using the alignment tool of the MEGA software package. A phylogram was constructed with MEGA 4.0 software programs (Tamura et al., 2007) using the different methods integrated in the software, including maximum-likelihood, maximum parsimony, UPGMA, and neighbor-joining procedures.

Statistical analysis: Statistical analysis was carried out using SPSS version 13.0 and reported as mean \pm standard error (SE).

Results

Characteristics of the isolated green microalga

Few species of the genus *Dunaliella* grow in the hypersaline Maharlu salt lake in Iran. Among these, one strain that was capable of producing large amounts of carotenoid compounds was isolated and subjected to further characterization. On the basis of morphological descriptions presented by Borowitzka & Siva (2007) and also by Oren (2005), the isolate was named *Dunaliella salina*, being green to orange at the logarithmic phase and orange to red at the exponential phase of growth. To confirm the identity

of the isolate, nuclear rDNA ITS (ITS-1 + 5.8 rDNA + ITS-2) sequences were amplified by PCR, which resulted in a single band on the electrophoresis gel with a 686-bp length and was submitted to the GenBank under accession no. GQ337903.

Sequence comparison of the ITS region by BLAST search related it to other *Dunaliella salina* strains with 99% sequence identity, and therefore, the new isolate was designated *Dunaliella salina* strain MSI-1. The phylogenetic tree (Fig. 1) constructed by the neighbor-joining method indicated that the strain MSI-1 is part of the cluster within the genus *Dunaliella*. Among the described strains, the closest relative of the isolate MSI-1 is *Dunaliella salina* Mexican (AF546093, AF546094) and the most distant were strains of *Dunaliella viridis* and *D. parva* UTEX 1983.

Effect of initial pH of the media on cell concentration and carotenogenesis

The number of cell ml^{-1} of the growth medium augmented with time at all initial pHs tested, being highest at pH 9 and lowest at pH 11 (Fig. 2). Carotenoid content in terms of $\mu\text{g ml}^{-1}$ of growth media followed nearly the same pattern as cell concentration (Fig. 3A). In terms of pg cell^{-1} , carotenoid content was highest at pH 11 and lowest at pH 9 (Fig. 3B). When the pH of the growth media was monitored over the entire experimental period, pHs 10 and 11 approached pH 9 and pHs 7 and 8 approached and then exceeded pH 9 (Fig. 4). In a weakly buffered medium initial pH and its subsequent changes over time may affect nutrient availability and cellular metabolism, which is evident by the affected cell number and carotenogenesis.

Growth and carotenogenesis as affected by nitrate, ammonium, and citrate

Different concentrations of nitrate affected both growth and carotenogenesis. Based on nitrogen availability, the cell number augmented with the increase in nitrate concentration, being highest at 2.5 mM nitrate (Fig. 5). The rate of increase in cell number was highest between 4 and 8 days after the start of the experiment. The pattern of carotenoid accumulation as affected by nitrate was different from the changes in cell number with time; after 4 weeks, the carotenoid contents ml^{-1} of the cultures

Fig. 1 Neighbor-joining tree based on ITS sequences showing the position of strain MSI-1 relative to some other strains of *D. salina*. The optimal tree with the sum of branch length = 0.463 is shown. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). Bootstrap values greater than 50% are indicated

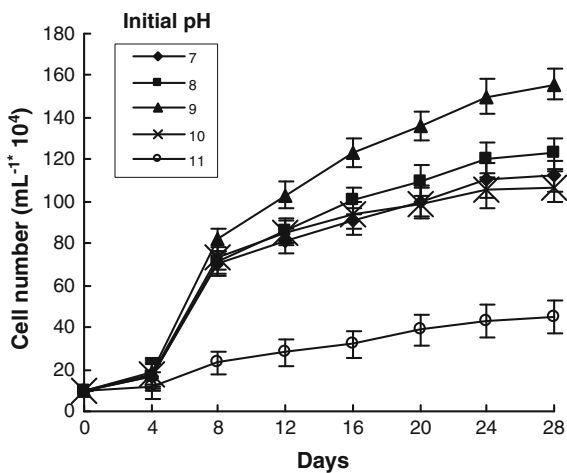
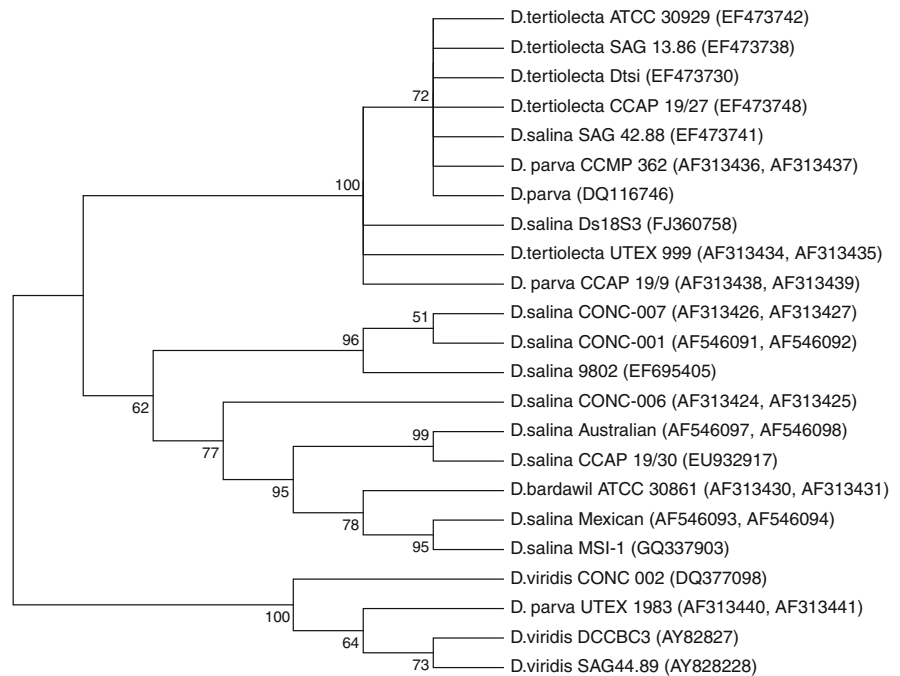


Fig. 2 Cell concentration as affected by initial pH of the growth media. The pHs were adjusted at the start of the experiment, and changes in cell number were followed at 4-day intervals. Each value is mean \pm SE

were only slightly affected by the initial nitrate concentration (Fig. 6A). As a matter of fact, at low nitrate concentrations higher carotenoid content per cell (Fig. 6B) compensated the reduced cell number (Fig. 5), so the pigment contents mL^{-1} of the cultures were essentially unaffected at the late stationary phase of growth (Fig. 6A). At 0.25 and 0.5 mM initial nitrate, carotenogenesis began much earlier

than other concentrations (Fig. 6B). At 2.5 and 5 mM nitrate, due to the presence of a relatively high nitrate concentration in the growth medium (Table 1), carotenogenesis was reduced and delayed until the late stationary phase of growth.

When ammonium was used as the nitrogen source, the number of cell mL^{-1} of medium at 0.1, 0.5, and 1.0 mM ammonium chloride, respectively, were 1.7-, 4.96-, and 3.5-fold greater compared to 0.05 mM. The carotenoid content expressed as $\mu\text{g mL}^{-1}$ was the highest at 0.5 mM, whereas in terms of pg cell^{-1} it decreased progressively with the increase in ammonium concentration (Table 2).

The addition of different concentrations of citrate to the culture medium had a slight effect on cell number and carotenogenesis. Only at 25 μM citrate did carotenoid contents, in terms of $\mu\text{g mL}^{-1}$ and pg cell^{-1} , increase by 28 and 23%, respectively (Table 3).

Discussion

Genetic relatedness at the genus, species, and strain levels can be analyzed by comparison of a nuclear rDNA internal transcribed spacer. These sequences are less conserved than rDNA genes and are used to

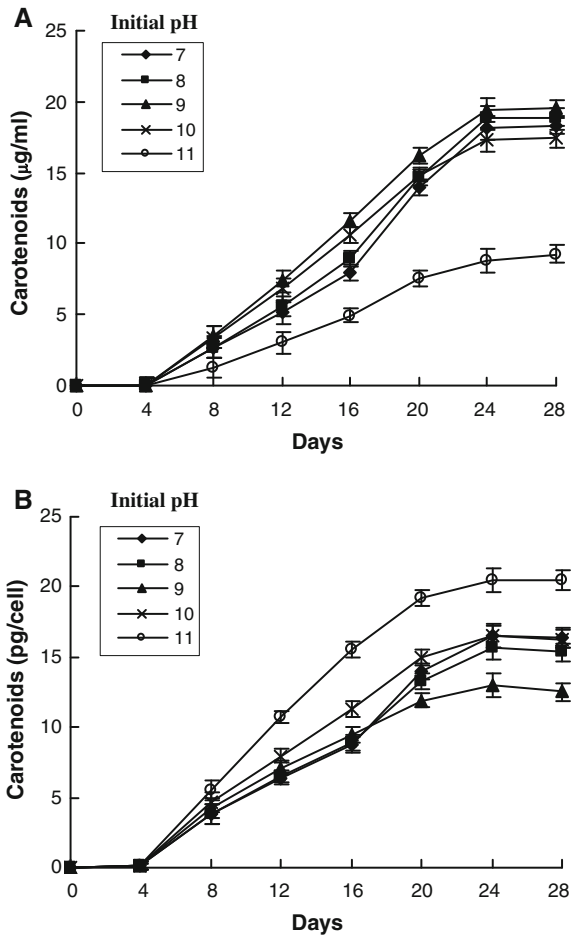


Fig. 3 Carotenoid content as affected by initial pH of the growth media expressed as **A** $\mu\text{g ml}^{-1}$ and **B** pg cell^{-1} . Each value is mean \pm SE

examine the genetic variation in various algal groups (Gonzalez et al., 1999; Coleman & Mai, 1997). The size of the ITS region amplified in this study was very similar to those reported for other *Dunaliella* strains, and the phylogram revealed that the strain can be classified as a new isolate in the *Dunaliella salina* species. The strain *D. salina* CONC-006 was more divergent than *D. salina* Australian and CCAP 19/30 with regard to MSI-1. The tree shows that all *D. salina* strains fall into one major clade except *D. salina* Ds 18S3 and *D. salina* SAG 42.88. The presence of *D. bardawil* ATCC 30861 in this major clade confirms its reidentification as *D. salina* by Borowitzka & Siva (2007). Four strains of the halophilic species *D. parva*, a carotenogenic species which can accumulate β -carotene up to 4% of the dry

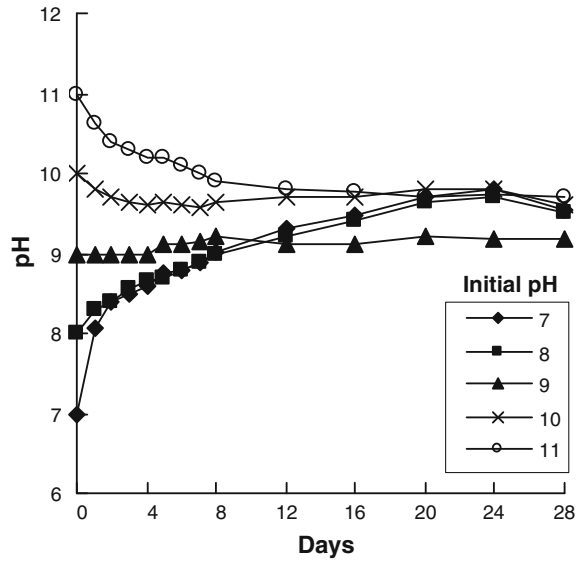


Fig. 4 Changes in the initial pH of the growth media over time. The pHs of the growth media were adjusted at the indicated values, and changes in pHs were measured at specified time intervals during the experimental period. Each value is mean \pm SE

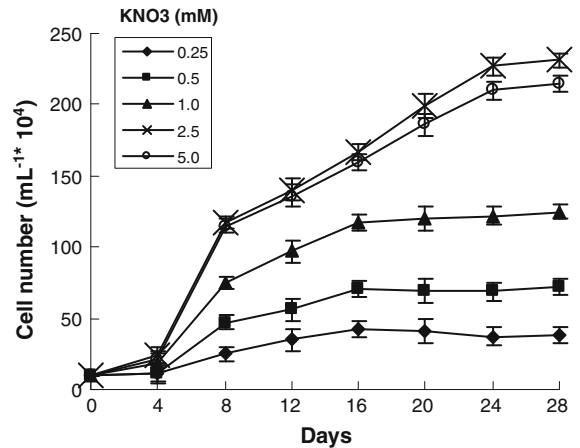


Fig. 5 Effect of different concentrations of NO_3^- on cell number. Indicated NO_3^- concentrations were added at the onset of the experiment, and changes in cell number were followed at 4-day intervals. Each value is mean \pm SE

weight, fell into different clades containing other *Dunaliella* species. *D. parva* CCMP 362 and DQ 116746 grouped with *D. tertiolecta*, and *D. parva* UTEX 1983 grouped with *D. viridis*. It has been suggested that *D. parva* UTEX 1983 should be renamed *D. viridis* and strain CCMP 362 should be renamed *D. tertiolecta* (Gonzalez et al., 2001).

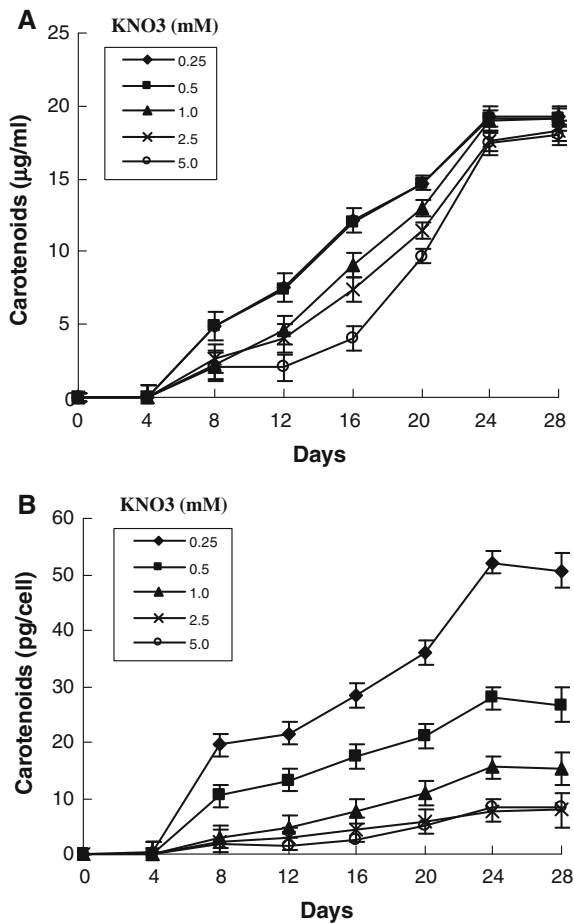


Fig. 6 Effect of different concentrations of NO_3^- on carotenoid content **A** ml^{-1} of growth medium **B** cell^{-1} . Each value is mean \pm SE

Table 1 Nitrate concentration in the media as a function of incubation time

Incubation time (day)	KNO ₃ concentration in the media (mM)				
	0.25	0.5	1.0	2.5	5.0
1	0.25 \pm 0.030	0.50 \pm 0.110	1.00 \pm 0.120	2.50 \pm 0.160	5.00 \pm 0.180
2	0.22 \pm 0.030	0.51 \pm 0.120	1.03 \pm 0.110	2.61 \pm 0.180	5.06 \pm 0.170
3	0.19 \pm 0.020	0.51 \pm 0.110	1.07 \pm 0.140	2.73 \pm 0.180	5.12 \pm 0.190
4	0.11 \pm 0.010	0.39 \pm 0.007	0.98 \pm 0.130	2.62 \pm 0.160	4.98 \pm 0.190
5	0.02 \pm 0.003	0.27 \pm 0.050	0.88 \pm 0.100	2.50 \pm 0.150	4.84 \pm 0.170
6	0.00 \pm 0.000	0.11 \pm 0.100	0.67 \pm 0.110	2.22 \pm 0.130	4.62 \pm 0.150
7	0.00 \pm 0.000	0.00 \pm 0.000	0.49 \pm 0.090	1.92 \pm 0.110	4.48 \pm 0.150
8	0.00 \pm 0.000	0.00 \pm 0.000	0.23 \pm 0.040	1.77 \pm 0.160	4.39 \pm 0.140
12	0.00 \pm 0.000	0.00 \pm 0.000	0.00 \pm 0.000	1.23 \pm 0.100	3.89 \pm 0.160
16	0.00 \pm 0.000	0.00 \pm 0.000	0.00 \pm 0.000	0.03 \pm 0.006	3.34 \pm 0.180
20	0.00 \pm 0.000	0.01 \pm 0.005	0.00 \pm 0.000	0.05 \pm 0.003	2.55 \pm 0.170
24	0.02 \pm 0.002	0.04 \pm 0.010	0.07 \pm 0.020	0.12 \pm 0.040	2.07 \pm 0.210
28	0.17 \pm 0.041	0.16 \pm 0.010	0.12 \pm 0.050	0.28 \pm 0.050	1.32 \pm 0.120

Day 1 represents the initial nitrate concentration of the culture media. Each value is mean \pm SE

Growth and carotenogenesis by *D. salina* are largely controlled by environmental factors such as salinity, light intensity, nutrient availability, and the pH of the culture medium (Nikookar et al., 2004; Fazeli et al., 2006; Abd EL-Baky et al., 2004).

Dunaliella salina is usually cultured at pH values of 7.5–8.6. Although in this study the optimum growth of *D. salina* strain MSI-1 and carotenoid production in terms of $\mu\text{g ml}^{-1}$ of the culture medium occurred at pH 9, the cell carotenoid content was favored by alkaline pH 11. Therefore, due to the alkalophilic nature of the strain, growing *D. salina* MSI-1 at pH 9 and increasing the culture pH to 11 at the late logarithmic phase of growth will possibly enhance carotenoid production by the algal cells.

The effect of pH on the metabolic activities of microorganisms and growth and carotenoid production by *Blakeslea trispora* have been reported by several investigators (Kim et al., 1996; Forage et al., 1985). When *Blakeslea trispora* was cultivated over an initial pH range of 2–12, cell growth and β -carotene production were favored by a strong alkaline culture condition (pH 10). Although in the presence of a nonionic surfactant, Span 20, cell growth was higher at acidic pHs, the β -carotene production again considerably increased at pHs 10 and 11. Using other strains of *B. trispora*, Choudhari & Singhal (2008) reported a higher yield of β -carotene at pH 7–8 as compared to other pH values. Strain dependence on pH for optimum carotenoid production awaits further research.

Table 2 Effects of different concentrations of NH_4^+ on cell number and carotenoid content of *D. salina*

	NH_4Cl (mM)			
	0.05	0.1	0.5	1
Cell number ($\text{ml}^{-1} \times 10^4$)	24 ± 1.12 (100)	41 ± 1.25 (171)	119 ± 1.73 (496)	84 ± 1.40 (350)
Carotenoids ($\mu\text{g/ml}$)	11.0 ± 0.54 (100)	14.9 ± 0.41 (135)	25.4 ± 0.56 (231)	15.6 ± 0.32 (142)
Carotenoids (pg/cell)	45.7 ± 0.64 (100)	36.3 ± 0.61 (80)	21.3 ± 0.46 (47)	18.6 ± 0.84 (41)

Figures in parenthesis are percent relative to 0.05 mM NH_4^+ in the culture medium. Each value is mean \pm SE

Table 3 Cell number and carotenoid contents in *D. salina* as affected by different concentrations of citrate

	Citrate (μM)			
	0	1	5	25
Cell number ($\text{ml}^{-1} \times 10^4$)	128 ± 0.93 (100)	130 ± 1.42 (102)	132 ± 1.61 (103)	134 ± 1.50 (105)
Carotenoids ($\mu\text{g/ml}$)	20.4 ± 0.27 (100)	20.8 ± 0.31 (102)	22.2 ± 0.54 (109)	26.1 ± 0.61 (128)
Carotenoids (pg/cell)	15.9 ± 0.26 (100)	16.0 ± 0.42 (101)	16.8 ± 0.51 (106)	19.5 ± 0.31 (123)

Figures in parenthesis are percent relative to the control (0.0 μM citrate). Each value is mean \pm SE

Augmentation in the cell number with an increase in nitrate concentration is well documented (Marin et al., 1998; Jimenez & Niell, 1991). In contrast, carotenoid content in terms of pg cell^{-1} is favored by low nitrate. As is clearly evident, carotenogenesis is partly controlled by the availability of nitrogen in the growth medium, which may act as a sink for partitioning fixed carbon when amino acid biosynthesis is reduced. The relatively unaffected carotenoid content per ml of cultures at the late stationary phase of growth is due to the augmentation in the cell number with an elevated nitrate concentration. Marin et al. (1998) reported that low nitrate negatively affected growth, but enhanced carotenoid accumulation per cell. The steady-state levels of transcripts encoding the first two enzymes committed to the carotenoid biosynthetic pathway, i.e., phytoene synthase and phytoene desaturase, increased substantially under nutrient-limiting conditions (Coesel et al., 2008). The same results were found for lycopene beta-cyclase, which is the third enzyme in the carotenoid biosynthetic pathway (Ramos et al., 2008).

Similar trends in the growth and carotenogenesis of the strain MSI-1 were obtained when NH_4^+ was used as the nitrogen source, except that both growth and carotenoids per ml of culture increased with the increase in NH_4^+ up to 0.5 mM and then declined.

Carotenogenesis by individual cell was highest at the lowest NH_4^+ concentration tested.

When the growths in the presence of NH_4^+ and NO_3^- are compared, a higher cell number was obtained at 2.5 mM NO_3^- compared to 0.5 mM NH_4^+ . The nitrate limitation (0.25 mM nitrate, lowest concentration tested) was more inductive to carotenogenesis than the NH_4^+ limitation (0.05 mM NH_4^+). Working on cyanobacteria *Microcystis viridis*, Ruckert & Giani (2004) showed that, although the intrinsic growth rate was higher in the presence of NH_4^+ , the maximum cell number was obtained when nitrate or a combination of nitrate plus ammonium was used as the nitrogen source. This was explained by higher rates of uptake and the assimilation of ammonium rather than nitrate. Experiments carried out by Borowitzka & Borowitzka (1988b) showed that ammonium nitrate inhibited β -carotene formation and also, due to the acidification of the medium, eventually resulted in cell death.

In experiments carried out by Hai-Zhu et al. (1998) on *D. salina*, the addition of 3 μM citrate to the culture medium increased both the growth and carotenogenesis. The results were explained on the basis of activation of acetyl CoA carboxylase by citrate during lipid biosynthesis. Our results do not confirm the enhancement of the growth and carotenogenesis by citrate. Since the pathway for

isopentenylpyrophosphate (IPP) formation in chloroplast is different from IPP formation by the mevalonic acid pathway in cytosol and acetyl CoA carboxylase is not involved in cytosolic IPP synthesis (Lichtenthaler et al., 1997), the relative insensitivity of growth and carotenogenesis to citrate observed in this study seems reasonable.

In conclusion, it is evident that the amplified ITS sequence is suitable to show the phylogenetic relationships among strains of *D. salina* and the responses of the new isolate from Maharlu salt lake toward nitrate concentration in the growth media is similar to those reported for other strains of *D. salina*. The insensitivity of carotenogenesis to citrate indicates the minor contribution of cytosolic IPP synthesis to the overall carotenoid production. pH dependence of carotenogenesis and how, despite changes toward pH 9, the initial pH of the media affects growth and carotenogenesis await further research.

Acknowledgments The authors would like to thank the Shiraz University Research Council for supporting this research.

References

- Abd EL-Baky, H. H., F. K. El-Baz & G. S. El-Baroty, 2004. Production of antioxidant by the green alga *Dunaliella salina*. *International Journal of Agriculture and Biology* 6: 49–57.
- Ben-Amotz, A. & M. Avron, 1990. The biotechnology of cultivating the halotolerant alga *Dunaliella*. *Trends in Biotechnology* 8: 121–126.
- Borowitzka, M. A. & L. J. Borowitzka, 1988a. *Dunaliella*. In Borowitzka, M. A. & L. J. Borowitzka (eds), *Micro-algal Biotechnology*. Cambridge University Press, Cambridge, DC: 27–58.
- Borowitzka, M. A. & L. J. Borowitzka, 1988b. Limits to growth and carotenogenesis in laboratory and large-scale outdoor cultures of *D. salina*. In Stadler, T., J. Mollion, M. C. Berdus, Y. Karamanos, H. Morvan & D. Christiane (eds), *Alga Biotechnology*. Elsevier Applied Science, Barking DC: 139–150.
- Borowitzka, M. A. & C. J. Siva, 2007. The taxonomy of the genus *Dunaliella* (Chlorophyta, Dunaliellales) with emphasis on the marine and halophilic species. *Journal of Applied Phycology* 19: 567–590.
- Borowitzka, L. J., M. A. Borowitzka & T. Moulton, 1984. Mass culture of *Dunaliella*: from laboratory to pilot plant. *Hydrobiologia* 116(117): 115–121.
- Borowitzka, M. A., L. J. Borowitzka & D. Kessly, 1990. Effects of salinity increase on carotenoid accumulation in the green alga *Dunaliella salina*. *Journal of Applied Phycology* 2: 11–119.
- Choudhari, S. & R. Singhal, 2008. Media optimization for the production of β -carotene by *Blakeslea trispora*: a statistical approach. *Bioresource Technology* 99: 722–730.
- Coesel, S. N., A. C. Baumgartner, L. M. Teles, A. A. Ramos, N. M. Henriques, L. Cancela & J. C. S. Varela, 2008. Nutrient limitation is the main regulatory factor for carotenoid accumulation and for Psy and Pds steady state transcript levels in *Dunaliella salina* (Chlorophyta) exposed to high light and salt stress. *Marine Biotechnology* 10: 602–611.
- Coleman, A. W. & J. C. Mai, 1997. Ribosomal DNA ITS-1 and ITS-2 sequence comparison as a tool for predicting genetic relatedness. *Journal of Molecular Evolution* 44: 258–271.
- Eijkelhoff, C. & J. P. Dekker, 1997. A routine method to determine the chlorophyll a, pheophytin a, and β -carotene contents of isolated photosystem II reaction center complexes. *Photosynthesis Research* 52: 69–73.
- Fazeli, M. R., H. Tofighi, N. Samadi, H. Jamalifar & A. Fazel, 2006. Carotenoid accumulation by *Dunaliella tertiolecta* (Lake Urmia isolate) and *Dunaliella salina* (CCAP 19/18 & WT) under stress conditions. *Daru* 14(3): 146–150.
- Forage, R. G., D. E. F. Harrisen & D. E. Pitt, 1985. Effect of environment on microbial activity. In Moo-yong M., (ed.), *Comprehensive Biotechnology*. Pergamon Press, DC: 263–270.
- Gomez, P. I. & M. A. Gonzalez, 2001. Genetic polymorphism in eight Chilean of the carotenogenic microalga *Dunaliella salina* Teodoresco (Chlorophyta). *Biological Research* 34: 23–30.
- Gomez, I. P. & M. A. Gonzalez, 2004. Genetic variation among seven strains of *Dunaliella salina* (Chlorophyta) with industrial potential, based on RAPD banding patterns and on nuclear ITS rDNA sequences. *Aquaculture* 233: 149–162.
- Gonzalez, M. A., P. I. Gomez & R. Montoya, 1999. Comparison of PCR-RFLP analysis of the ITS region with morphological criteria of various strains of *Dunaliella*. *Journal of Applied Phycology* 10: 573–580.
- Gonzalez, M. A., A. W. Coleman, P. I. Gomez & R. Montoya, 2001. Phylogenetic relationship among various strains of *Dunaliella* (Chlorophyceae) based on nuclear ITS rDNA sequences. *Journal of Phycology* 37: 604–611.
- Hai-Zhu, J., G. Cheng-Hua, H. Jun, W. Qing-Hau, J. Zhu-Mao & S. Fu-Zhang, 1998. Effects of Mg^{2+} , NaCl, citric acid and other factors on synthesis and accumulation of β -carotene in *Dunaliella salina*. *Chinese Journal of Oceanology and Limnology* 16: 364–368.
- Javor, B., 1989. *Hypersaline Environments*. Microbiology and Biogeochemistry. Springer-Verlag, Berlin DC: 328–335.
- Jimenez, C. & F. X. Niell, 1991. Growth of *Dunaliella viridis* Teodoresco: effect of salinity, temperature and nitrogen concentration. *Journal of Applied Phycology* 3: 319–327.
- Kim, S. W., W. T. Seo & Y. H. Park, 1996. Increased β -carotene synthesis in *Blakeslea trispora* under strong alkaline culture condition. *Biotechnology Letters* 18: 1287–1290.
- Lichtenthaler, H. K., M. Rohmer & J. Schwender, 1997. Two independent biochemical pathways for isopentenyl diphosphate (IPP) and isoprenoid biosynthesis in higher plants. *Physiologia Plantarum* 101: 643–652.

- Loeblich, L. A., 1982. Photosynthetic and pigments influenced by light intensity and salinity in the halophilic *Dunaliella salina* (Chlorophyta). *Journal of Marine Biological Association of the United Kingdom* 62: 493–508.
- Marin, N., F. Morales, C. Lodeiros & E. Tamigneaux, 1998. Effect of nitrate concentration on growth and pigment synthesis of *Dunaliella salina* cultivated under low illumination and preadapted to different salinities. *Journal of Applied Phycology* 10: 405–411.
- Nikookar, K., A. Moradshahi & M. Kharati, 2004. Influence of salinity on the growth, pigmentation and ascorbate peroxidase activity of *Dunaliella salina* isolated from Maharlu salt lake in Shiraz. *Iranian Journal of Science and Technology* 28: 117–125.
- Oren, A., 2005. A hundred years of *Dunaliella* research: 1905–2005. *Saline systems* 1: 2.
- Ramos, A., S. Coesel, A. Marques, M. Rodrigues, A. Baumgartner, J. Noronha, A. Rauter, B. Brenig & J. Varela, 2008. Isolation and characterization of a stress-inducible *Dunaliella salina* Lcy- β gene encoding a functional lycopene β -cyclase. *Applied Microbiology and Biotechnology* 79: 819–828.
- Ruckert, G. V. & A. Giani, 2004. Effect of nitrate and ammonium on the growth and protein concentration of *Microcystis viridis* Lemmermann (Cyanobacteria). *Revista Brasileira de Botanica* 27: 325–331.
- Tamura, K., J. Dudley, M. Nei & S. Kumar, 2007. MEGA 4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Molecular Biology Evolution* 24(8): 1596–1599.
- Venkatesh, N. S., R. Balaji & P. Satnyamurthy, 2005. Medium for the production of betacarotene and other carotenoids from *Dunaliella salina* (ARL 5) and a strain of *Dunaliella salina* for production of carotenes using the novel media. U.S. Patent 6936459 B1.