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Growth and pigment development of *Dunaliella salina* Teod. in response to ammonium nitrate nutrition

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A B S T R A C T

The microalgae, *Dunaliella salina* was isolated from Maharlu Salt Lake, south east of Shiraz, Iran. The isolated strain was identified by both morphological and physiological markers. The complete ITS region (ITS1 + ITS2) including the 5.8S rDNA gene used as molecular marker confirmed our identification. Growth and cell proliferation, total chlorophyll and carotenoid contents were determined in the presence of 0.125, 0.25, 0.50, 1.0 and 2.0 mM ammonium nitrate. After five weeks, a maximum cell density of about $(4.4 \pm 0.21)\times10^6$ mL^{-1} was observed in the growth medium containing 1mM NH_4NO_3. Increasing NH_4NO_3 concentrations up to 1mM, resulted in an increase in the cells total chlorophyll contents. The highest amount of cell carotenoid contents was produced in media containing the least amount of NH_4NO_3 (0.125 mM). Manipulating the type and amount of external nitrogen sources to induce the synthesis of the highest amounts of carotenoid compounds in this microalgae strain can be of great commercial values to food industries.

Key words: *Dunaliella salina*; ITS; Ammonium nitrate; Carotenoid synthesis

INTRODUCTION

Lipid soluble pigment β-carotene has several commercial values as a food coloring agent, pro-vitamin A (retinol) in human and animal diets, an additive in cosmetics, as a healthy food product under antioxidant claims and in the preparation of multivitamins [1]. *Dunaliella salina* which is a halophillic unicellular biflagellate microalga, belongs to chlorophyta and is known as the most efficient overproducer of natural β-carotene. Deficiencies in inorganic nutrients such as nitrate, sulfate and phosphate all induce β-carotene accumulation in *Dunaliella* [2]. Similar to higher plants, *Dunaliella* grows best in cultures where balanced concentrations of nitrogen as a macronutrient are present. This essential element can be supplied either in inorganic (nitrate and ammonium salts) or in organic (urea) forms [3].

In intensive growth culture systems where *Dunaliella* is grown for the production of highly valued β-carotene, the type and concentrations of nutrients and also the internal conditions of the cultures are defined and regulated by growers in such a way as to ensure the maximum amount of

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cell growth and β-carotene production. In carotenogenic strains of *Dunaliella*, N-limitation is the most potent inducer of β-carotene production [2].

In the biotechnology of β-carotene biosynthesis, inorganic nitrogen salts may act as a double edged sword. Proper nitrogen concentrations result in high biomass production; and at the same time, nitrogen deficiency leads to carotenogenesis induction [4-6]. Also, chemical forms of nitrogen control the carotenogenesis process. In *D. salina* cultures, nitrate supplied as the source of nitrogen supports higher growth rates [3] while ammonium salts increase β-carotene biosynthesis [7]. To be assimilated, ammonium does not need to be reduced like nitrate, hence causing the cell's energy saving process. However, ammonium toxicity at high concentrations must be considered as a limiting factor for β-carotene biosynthesis. High ammonium concentrations are lethal for *Dunaliella* species [8]. Therefore, the present study was undertaken to find out whether ammonium nitrate used as the nitrogen source for the Iranian strain of *D. salina* isolated from Maharlu Salt Lake can cause both high biomass and high β-carotene productions, and if it can, what would the optimum ammonium nitrate concentration for the highest β-carotene production be?

MATERIALS AND METHODS

Microalgae, growth conditions and pigments analysis: *D. salina* alga collected from Maharlou Salt Lake, south east of Shiraz, Iran, was identified as described previously [9]. Modified culture media [9] in three replicates containing 250 mL of 0.125, 0.250, 0.500, 1.000 and 2.000 mM NH_4NO_3 were prepared as nitrogen sources. First, each flask was inoculated with the proper number of alga to contain about 10^4 cells mL^{-1}. The culture solutions were kept in an incubator under light/dark photoperiods of 16 hrs of light (72 µmol photons m^{-2} s^{-1}) at 25°C and 8 hrs of dark at 17°C, for five weeks. Haemocytometer was used to calculate cell numbers.

Growth rate was calculated at the onset of the logarithmic growth phase, the time between first and second weeks of growth, using the following formula:

$$K' = \text{Ln}\ (N_2/N_1)/(T_2 - T_1)$$

where N_1 and N_2 represent cell numbers at time T_1 and T_2 respectively [2].

The amounts of chlorophyll a, b, total chlorophyll and β-carotene were determined by spectrophotometeric method [10, 11].

DNA extraction, amplification and sequencing: Total DNA was extracted by the Gomez and Gonzales method [12]. Double stranded DNA from the complete ITS region, including the 5.8 S rDNA gene, was amplified by 35 cycles of symmetric PCR using primers initially designed by White et al., [13] and modified by Mummenhoff [14]. The primers were N-nc: F 5´-AGGAG AAGTCGTAACAAG-3´and C29A: R 5´-AGTTTCTTTTCCTCCGCT-3´.

The PCR profile consisted of a hot start at 94°C for 5 min, 35 cycles of amplification (1min 94°C, 1 min 38°C, 1 min 72°C) and a final elongation step for 10 min at 72°C. PCR products were purified using the Boehringer PCR product purification kit (Roche Molecular Biochemical). The purified PCR product was sequenced by MWG Company, Germany.

Data analysis: The ITS sequences were aligned with the GenBank database sequences and a phylogram was created. The experiment was conducted as a completely randomized design with three replicates. Each value represents the mean ± standard error at $p<0.05$ calculated using a one way ANOVA. The data collected were imported to Microsoft Excel software for calculations and for graphical representations. SPSS software (17.0 version) was used for the analysis of variance.

RESULTS

Both morphological characters observed by light microscope and the pattern of change in the culture color to orange as the algal physiological response [2], were used to identify *D. salina.* Our identification was confirmed by ITS rDNA analysis used as a molecular tool.

A fragment of ITS sequences was amplified and a fraction of the PCR product was sequenced. Figure 1 shows the banding pattern of an amplified 720 bp sequence. The NCBI blast analysis of the amplified sequence obtained by the phylogenetic tree is shown in figure 2.

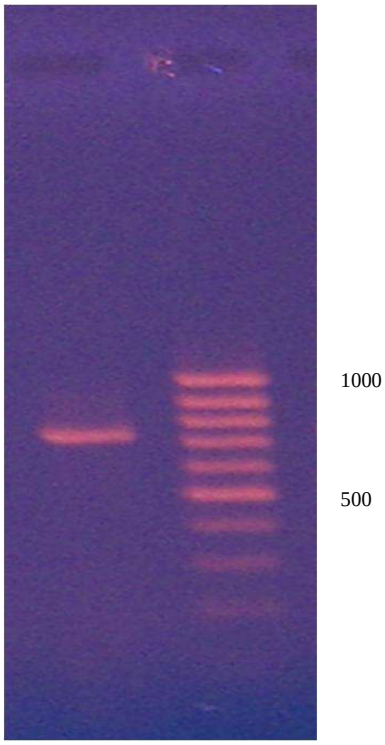

Figure 1. Agarose gel electrophoresis of amplified sequences from isolated *D. salina*. The left lane represents 720 bp amplified ITS sequence (ITS1 + ITS2 including 5.8S rDNA) and the right lane is a 100 bp ladder.

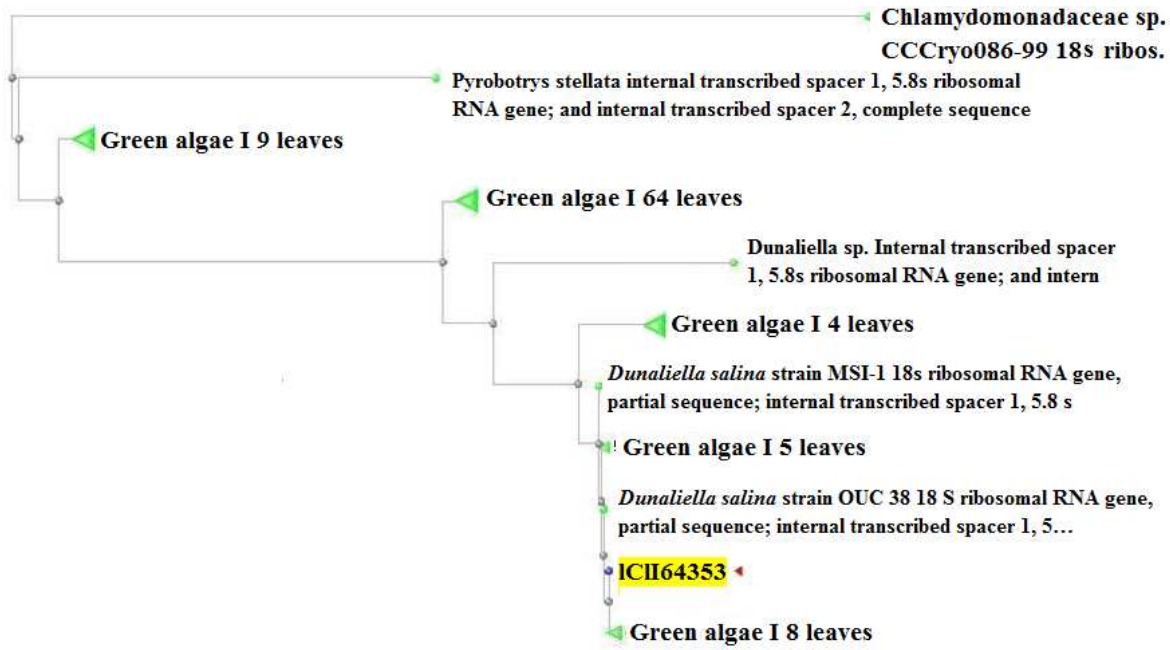

Figure 2. Phylogenetic relationship of isolated *D. salina* from Maharlu Salt Lake. Arrowed sequence inferred by 720 bp amplified sequences (ITS1 + ITS2 including 5.8S rDNA). The tree shown resulted from a neighbor joining analysis using NCBI BLAST.

Results presented in table 1 show that the highest culture growth rate occurred in the presence of 0.5 mM ammonium nitrate in the logarithmic phase. However, maximum cell density occurred in the stationary phase in the presence of 1 mM ammonium nitrate. Although the amounts of cell chlorophyll a and b and total chlorophyll contents increased with the increase in ammonium nitrate up to 1 mM, maximum carotenoid production occurred in the presence of 0.125 mM ammonium nitrate (Table 2).

Table 1. Cell density and total carotene content at stationary phase

NH_4NO_3 (mM)	Cell Numbers ($\times 10^6 mL^{-1}$)	Growth rate (%)	Yield of carotene (µg/mL)
0.125	0.929 ± 0.04	21.4 ± 3.7	6.74
0.25	1.870 ± 0.24	23.4 ± 2.4	12.79
0.5	3.412 ± 0.07	29.6 ± 1.2	17.29
1	4.408 ± 0.21	28.0 ± 3.3	21.24
2	2.558 ± 0.29	NC	13.01

NOTES: Growth rate was determined at early logarithmic phase (NC=not computable by haemocytometer).
Values of cell number and growth rate are mean ± SE.
Yield is the product of cell number by cell carotene content.

Table 2. Cells pigments content (pg $cell^{-1}$) as affected by ammonium nitrate concentrations after 5 weeks at stationary phase

NH_4NO_3 (mM)	Chlorophyll a	Chlorophyll b	Total Chlorophyll	Carotene
0.125	1.70 ± 0.17	0.37 ± 0.04	2.07 ± 0.21	7.25 ± 0.32
0.250	2.08 ± 0.31	0.46 ± 0.06	2.54 ± 0.38	6.83 ± 0.60
0.500	2.43 ± 0.11	0.73 ± 0.04	3.17 ± 0.16	5.06 ± 0.35
1.000	2.61 ± 0.16	1.15 ± 0.09	3.76 ± 0.26	4.81 ± 0.24
2.000	2.82 ± 0.16	0.97 ± 0.09	3.79 ± 0.24	5.08 ± 0.12

NOTE: Each value is mean ± SE

DISCUSSION

D. salina is a worldwide hypersaline microalgae species [2]. There are only few reports of the presence of this species in Maharlou Salt Lake [9, 15]. Besides morphological and physiological markers, the addition of molecular marker (ITS) confirms the identification and gives better insight of the phylogenic relations among the taxa within the genus *Dunaliella*. As shown in figure 1, the 720 bp spacer regions (ITS1 + ITS2) have been amplified. Such regions have been used by others for the identification of the genus *Dunaliella* in the past [15-18]. In figure 2, the region shown by the arrow in the phylogenic tree indicates the neighboring of the isolated strain with higher plants, green algae, *D. salina* and also with another strain of the same algae previously identified from this habitat [15].

Growth and pigment development: Ammonium nutrition causes the acidification of the media and high ammonium concentration could be lethal [8]. Ammonium supplied as ammonium nitrate at relatively high concentrations may also have the same toxic effects on cultured cells. Therefore, when 2 mM NH_4 NO_3 was supplied, cell growth was delayed and due to low cell density at the early logarithmic phase, cell numbers were low and could not be measured by the haemocytometer (Table 1). High ammonium concentrations also impaired chlorophyll biosynthesis, causing no significant increase in chlorophyll at 2 mM NH_4NO_3. The highest β-carotene biosynthesis occurred when the concentration of NH_4NO_3 in the media was the lowest (Table 2). From these results it can be deduced that low nitrogen supply in the form of ammonium nitrate is an effective inducer of β-carotene biosynthesis in *D. salina* (Table 2). Using the product of cell numbers in each treatment by the amount of cells β-carotene, an estimate can be made of the quantity of this pigment present in the culture (Table 1). Applying this calculation to estimate the maximum amount of β-carotene production by the isolated *Dunaliella* strain, it is proposed that in order to produce both the optimum amount of biomass and the highest amount of β-carotene, this strain be grown in a culture media containing 1 mM NH_4NO_3.

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Conflict of Interest: Authors have no financial or any non-financial competing interests.

REFERENCES

1. Garcia-Gonzalez M, Moreno J, Manzano JC, Florencio FJ, Guerrero MG. Production of *Dunaliella salina* biomass rich in 9-cis-β-carotene and lutein in a closed tubular photobioreactor. J Biotec 2005;115:81-90.
2. Ben-Amotz A, Polle JEW, Rao DVS. The Alga Dunaliella: Biodiversity, Physiology, Genomics and Biotechnology, 2009, Science Publisher, Enfield, NH, USA
3. Thakur A, Kumar HD. Effect of different nitrogen sources on growth and glycerol production by *Dunaliella salina*. Cytobios 1999;97:79-83.
4. Sciandra A, Gostan J, Collos Y, Descolas-Gros C, Lebouulanger C, Martin Jezequel V, Denis M, Lefevre D, Copin-Montegut C, Avril B. Growth-compensating phenomena in continuous cultures of *Dunaliella tertiolecta* limited simultaneously by light and nitrate. Limnol Oceanogr 1997;42:1325-1339.
5. Borowitzka MA. The mass culture of *Dunaliella salina*. http://www.fao.org. 2013.
6. Ben-Amotz A, Shaish A. β-carotene biosynthesis. pp 205-216. In *Dunaliella*: Physiology, Biochemistry and Biotechnology. M. Avron and A. Ben-Amotz (eds). CRC, 1992. Boca Raton, USA
7. Giordano M, Bowes G. Gas exchange and C allocation in *Dunaliella salina* cells in response to the N source and CO_2 concentration used for growth. Plant Physiol 1997;115:1049-1056.
8. Grant BR. Effect of carbon dioxide concentration and buffer system on nitrate and nitrite assimilation by *Dunaliella tertiolecta*. J Gen Microbiol 1968;54:327-336.
9. Nikookar K, Moradshahi A, Kharati M. Influence of salinity on the growth, pigmentation and ascorbate peroxidase activity of *D. salina* isolated from Maharlu Salt Lake in Shiraz. Iranian J Sci Technol 2004;28:117-125.
10. Porra R. The chequered history of the development and use of simultaneous equations for the accurate determination of chlorophylls a and b. Photosynth Res 2002;73:149-156.
11. Shaish A, Ben-Amotz A, Avron M. Biosynthesis of β-Carotene in *Dunaliella*. Method Enzymol 1992;213:439-444.
12. Gomez PI, Gonzalez MA. Genetic polymorphism in eight Chilean of the carotenogenetic microalga *Dunaliella salina* Teodoresco (Chlorophyta). Biol Res 2001;34:23-30.
13. White TJ, Burns T, Lee S, Taylor J, Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics: 315 –322. In: V. Innis, D. Gelfand, JJ. Sninsky, TJ. White (eds.) PCR protocols: a guide to methods and applications. 1990, San Diego.
14. Mummenhoff K. Molecular phylogenetics of Thlaspi (Brassicaceae) based on chloroplast DNA restriction site variation and sequences of the internal transcribed spacers of nuclear ribosomal DNA. Can J Bot 1997;75:469-482.
15. Zamani H, Moradshahi A, Karbalaei-Heidari HR. Characterization of new *Dunaliella salina* strain MSI-1 based on nuclear rDNA ITS sequences and its physiological response to changes in composition of growth media. Hyrobiologia 2011;658:67-75.
16. Gonzalez MA, Gomez P, Montoya R. Comparison of PCR-RFLP analysis of the ITS region with morphological criteria of various strains of *Dunaliella*. J Appl Phycol 1999;10: 573-580.

17. Gonzalez MA, Coleman AW, Gomez P, Montoya R. Phylogenetic relationship among various strains of *Dunaliella* (Chlorophyceae) based on nuclear ITS rDNA sequences. J Phycol 2001;37:604-611.
18. Kharati-Koupaei M, Zamani H, Moradshahi A. Molecular identification of *Dunaliella Viridis* Teod. strain MSV-1 utilizing rDNA ITS sequences and its growth responses to salinity and copper toxicity. Mol Biol Res Commun 2012;1:8-15.