# CAROTENOIDS ACCUMULATION BY *DUNALIELLA TERTIOLECTA* (LAKE URMIA ISOLATE) AND *DUNALIELLA SALINA* (CCAP 19/18 & WT) UNDER STRESS CONDITIONS

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**ABSTRACT**

Carotenoids are widely used in nutraceutical, additives and cosmetics as colorants and antioxidants. *Dunaliella* is the main natural source which accumulates massive amount of carotenoids. This study examines the effect of different concentrations of NaCl (0.3M to 2M) and light intensities of 50 and 150 µmol/m²s on total carotenoids accumulated by *Dunaliella tertiolecta* (DCCBC26), from Urmia lake (North West of Iran) compared to those obtained from *Dunaliella salina* CCAP 19/18 and also the wild type *Dunaliella salina* WT strains. In all microalgaes production of carotenoids were triggered by increase in light intensities. Changes of intensity from 50 µmol/m²s to 150 µmol/m²s led to 2.4, 2.1 and 1.4 folds of carotenoids production by *D. salina* CCAP, *D. salina* WT and *D. tertiolecta* respectively. In both salina strains carotenoids production improved with higher salinity picking at salt concentration of 2M, while *D. tertiolecta* showed optimum carotenoids production at 0.7M.

**Keywords:** *Dunaliella salina; Dunaliella tertiolecta*; Carotenoids; salinity ; light intensity

## INTRODUCTION

Carotenoids are chemicals with significant commercial interest which are used as coloring agents in neutraceuticals, pharmaceuticals, cosmetics and foods (1). These compounds have antioxidant properties and have attracted attention as potential agents in chemoprevention of cancers (2). They have beneficial role as dietaries in cataract and also in age-related macular degeneration (3). Of about 1000 carotenoids found in nature only a few of them occur in abundance in fruits and vegetables. These include β-carotene (carrots), lycopene (tomatoes) and lutein (spinach) (4). Although some carotenoids e.g. β-carotene and zeaxanthin are available in synthetic forms, there is growing interest on natural microalgal as well as bacterial and yeast sources of carotenoids driven by the world public opinion on synthetic additives (5). Recently bacteria have been investigated for possible accumulation of carotenoids. The nonfastidious and nonpathogenic *Flavobacterium multivorum* have been considered as an important microbial source for production of zeaxanthin (5,6,7). *Rhodotorula glutinis* and *Phafia rhodozyma* are among yeasts with capabilities of carotenoid accumulation (8,9). Microalgae are eucaryotic photosynthetic microorganisms which are used to produce highly valuable compounds such as carotenoids (10). Increasing attention in recent years has been paid to *Dunaliella* a microalgae of which *Dunaliella bardawil* and *D. salina* have shown potential sources for large amounts of β-carotene and glycerol (11,12). The halotolerant green algae are able to grow in a wide salt range of 50 mM to 5.5 M (close to the saturation limit of NaCl). Although high salinity favours carotenoid production by the microalgae but cell density is usually depressed at elevated salt concentrations (12,13). Carotenoid production and accumulation are reported to be positively affected by white-light irradiation in algae, fungi, and bacteria. However one should not expect a unique response of organsisms to illumination. It is also well documented that extent of carotenoid production by cells of microalgae is influenced by light densities and increasing photon flux densities result in higher carotenoids accumulation (14). The present study aimed to clarify the significance of salt and light intensity on carotenoids accumulation by a *Dunaliella tertiolecta* isolated from the salty Urmia Lake in North-west of Iran compared to those by two strains of *Dunaliella salina* (CCAP 19/18 and WT).

## MATERIALS AND METHODS

*The microalgae*

The microalgae used included a wild type *Dunaliella salina* assigned WT which was

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generously donated by Prof. Anastasios Melis from Department of Plant and Microbial Biology, University of California, Berkeley, USA and *Dunaliella salina* CCAP 19/18 was a gift from Mohammad Amin Hejazi from Food and Bioprocess Engineering Group, Wageningen University, Wageningen, Netherland. *Dunaliella tertiolecta* was isolated from the Urmia Lake (a salty lake in North-West of Iran) and was identified by morphological and genetic analyses. The identity of the isolate was confirmed by comparing the internal transcribed spacer region 2 (ITS2) sequences of the isolate to those in Dunaliella Culture Collection at Brooklyn College (DCCBC) stock where the isolate were stored as *Dunaliella tertiolecta* DCCBC26.

*Culture condition*

The artificial seawater medium (ASW) which was used to cultivate the Dunaliella strains contained: 5 mM $KNO_3$, 4.5 mM $MgCl_2.6\ H_2O$, 0.5 mM $MgSO_4$. 7 H2O, 3 mM $CaCl_2$. 2 $H_2O$, 0.13 mM $K_2\ HPO_4$, 0.02 mM $FeCl_3$, 0.02 mM EDTA, 25 mM $NaHCO_3$, 1 mg $l^{-1}$ of trace elements stock with 50 mM $H_3BO_3$, 10 mM $MnCl_2$ . $4H_2O$, 0.8 mM $ZnSO_4$ .7 $H_2O$, $CuSO_4$. $5H_2$ O, 2 mM $NaMoO_4$ . $2H_2O$, 1.5 mM $NaVO_3$ , 0.2 mM $CoCl_2$. $6H_2O$. Different concentrations of NaCl (0.05, 0.1, 0.5, 1, 2, 3 M) were added to the medium and the pH were adjusted to 7.5 by addition of 40 mM of Tris-buffer. In order to avoid precipitation of certain compounds, all stock solutions were sterilized separately and pooled aseptically. Sterilization was accomplished by autoclaving at 121 ºC. Sodium bicarbonate stock was heat-sterilized at 130 ºC (15). Shake flasks each containing 200 ml of ASW medium were inoculated with vegetative cells of microalgae to achieve an initial cell density of 6000 cell $ml^{-1}$. Flasks were incubated at 34˚C under continuous illumination of light of intensities of 50 µmol/m²s (LL) or 150 µmol/m²s (HL) and were shaken maually twice a day to ensure a uniform illumination of the cells. Irradiance intensities were measured using a Delta OHM (Model DO 9721) radiometer.

*Pigment extraction and analysis*

A sample of 4 ml was taken from each culture medium after mixing thoroughly. Cells were spinned at 5000 rpm for 5 minutes and the pelleted biomass were mixed with 4 ml of acetone/water (80:20 v/v). The mixtures were vortexed for 1-2 min to ensure complete extraction. Tubes were centrifuged again for 5 min at 5000 rpm and the colorless biomass were discarded (1). The amount of extracted pigments in the solvent phase were quantified by a spectrophotometric method described by Lichtenthaler (16).

*Growth estimation*

Growth rates of microalgae were estimated spectrophotometrically by measurement of culture turbidity at 687 nm (16). Cells were also determined by direct counting, using a light microscope (magnification × 40) with a %1 mm deep counting chamber (Neubauer improved).

*Statistical analysis*

Data were statistically analyzed by the one-way ANOVA method and were expressed as means ± SE ($p<0.05$ was considered as significant).

## RESULTS AND DISCUSSION

Carotenoids accumulation, mainly in the form of β-carotene in *D. salina* are triggered by suboptimal growth conditions e.g. light intensity (15), salinity or temperature (18) and nutrient limitation (19). The kinetics of growth, total carotenoids and chlorophyll production by *D. tertiolecta* DCCBC26, *D. salina* CCAP and *D. salina* WT in ASW media containing different NaCl concentrations under irradiances of 50 and 150 µmol/m²s are depicted in Tables 1 and 2. Lower salinities (0.3M and 0.7M at irradiances of 50 and 150 µmol/m²s respectively) favoured growth of *D. tertiolecta* DCCBC26 while *D. salina* CCAP had its optimal growth at high salt concentration of 2M. These results are in accordance with the report in which it is indicated *D. tertiolecta* grows better at the lower salt concentrations (20) in which growth of *D. salina* growth at 0.1 M NaCl was not observed, but did so at 5.1 M while maximum growth occurred at intermediate salinities (20). Total carotenoids accumulated by the three strain of *Dunaliella* were affected by light intensity. *D. salina* CCAP 19/18 was a better producer of carotenoids under higher light illumination. Maximum carotenoids production by *D. tertiolecta* DCCBC26 happened at salt concentration of 0.7M, while those for *D. salina* CCAP 19/18 was at 2M. Taking into account the content of carotenoids per cell, *D. salina* WT was unaffected by the salinities which were used but the other strains responded differently. Productivity of *D. salina* CCAP 19/18 cells for carotenoids was positively as well as synergistically regulated by both salinity and light stresses. The highest amount of carotenoids obtained by *D. salina* CCAP 19/18 strain at light intensity of 50 µmol/m²s was 8.5 (pg $cell^{-1}$) at 1.5M NaCl while those at illumination of 150 µmol/m²s reached the peak of 19.11 (pg $cell^{-1}$) at 2M salinity. Investigation on the photoautotrophic microalgae *Dunaliella salina* Teorodesco CCAP 19/30, a major reported producer of β-carotene have also shown that high light-intensity could improve carotenoid biosynthesis by the microalgael cells (21). Other investigators have

**Table 1.** The kinetics of growth, total carotenoids and chlorophyll a production by *D. tertiolecta* DCCBC26, *D. salina* CCAP and *D. salina* WT in ASW media containing different NaCl concentrations under irradiance of 50 µmol/m²s in 21 days.

| | Chl a (mg/l) | | | | | Chl a (pg cell$^{-1}$) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| NaCl (M) | 0.3 | 0.7 | 1 | 1.5 | 2 | 0.3 | 0.7 | 1 | 1.5 | 2 |
| Strain | | | | | | | | | | |
| *D.tertiolectaD*CCBC26 | 9.84 | 8.06 | 7.37 | 6.69 | 6.02 | 3.02 | 3.42 | 3.50 | 3.34 | 3.25 |
| *D. salina* CCAP19/18 | 0.52 | 1.50 | 2.07 | 4.16 | 4.75 | 5.20 | 6.25 | 5.75 | 9.90 | 9.13 |
| *D.salina* WT | 7.88 | 9.55 | 11.74 | 11.93 | 12.40 | 3.50 | 3.82 | 4.26 | 3.97 | 3.35 |
| | Car (mg/l) | | | | | Car (pg cell$^{-1}$) | | | | |
| NaCl (M) | 0.3 | 0.7 | 1 | 1.5 | 2 | 0.3 | 0.7 | 1 | 1.5 | 2 |
| *D.tertiolectaD*CCBC26 | 3.75 | 3.06 | 2.96 | 2.70 | 2.38 | 1.15 | 1.30 | 1.40 | 1.35 | 1.28 |
| *D. salina* CCAP19/18 | 0.17 | 0.26 | 2.47 | 3.57 | 3.00 | 1.70 | 6.50 | 6.80 | 8.50 | 5.50 |
| *D.salina* WT | 1.81 | 2.32 | 2.52 | 2.38 | 2.22 | 0.88 | 0.73 | 0.90 | 0.95 | 0.80 |
| | Cell count(× $10^6$ /ml) | | | | | $OD_{687nm}$ | | | | |
| NaCl (M) | 0.3 | 0.7 | 1 | 1.5 | 2 | 0.3 | 0.7 | 1 | 1.5 | 2 |
| *D.tertiolectaD*CCBC26 | 3.25 | 2.35 | 2.10 | 2.00 | 1.85 | 0.630 | 0.492 | 0.486 | 0.455 | 0.400 |
| *D. salina* CCAP19/18 | 0.10 | 0.24 | 0.36 | 0.42 | 0.52 | 0.004 | 0.034 | 0.163 | 0.202 | 0.267 |
| *D.salina* WT | 2.25 | 2.50 | 2.75 | 3.00 | 3.70 | 0.510 | 0.535 | 0.630 | 0.620 | 0.652 |

Microalgael growth were expressed as cells per ml and also by measuring the $OD_{687\ nm}$. Chlorophyll a (Chl a) and total carotenoids (Car) contents were calculated as per volume (mg/l) and also per cell (pg cell$^{-1}$). Data are expressed as means of three replicates.

**Table 2.** The kinetics of growth, total carotenoids and chlorophyll a production by *D. tertiolecta* DCCBC26, *D. salina* CCAP 19/18 and *D. salina* WT in ASW media containing different NaCl concentrations under irradiance of 150 µmol/m²s in 21 days.

| | Chl a (mg/l) | | | | | Chl a (pg cell$^{-1}$) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| NaCl (M) | 0.3 | 0.7 | 1 | 1.5 | 2 | 0.3 | 0.7 | 1 | 1.5 | 2 |
| Strain | | | | | | | | | | |
| *D.tertiolectaD*CCBC26 | 10.47 | 11.06 | 3.28 | 2.17 | 1.87 | 1.74 | 1.63 | 1.09 | 0.96 | 0.76 |
| *D.salina* CCAP19/18 | 0.33 | 0.91 | 1.50 | 1.93 | 2.65 | 3.00 | 3.95 | 4.83 | 3.93 | 4.81 |
| *D.salina* WT | 3.38 | 5.22 | 3.48 | 4.78 | 4.50 | 1.64 | 1.51 | 1.74 | 1.91 | 1.63 |
| | Car (mg/l) | | | | | Car (pg cell$^{-1}$) | | | | |
| NaCl (M) | 0.3 | 0.7 | 1 | 1.5 | 2 | 0.3 | 0.7 | 1 | 1.5 | 2 |
| *D.tertiolectaD*CCBC26 | 4.78 | 5.10 | 1.91 | 1.24 | 1.19 | 0.79 | 0.75 | 0.63 | 0.55 | 0.48 |
| *D.salina* CCAP19/18 | 0.82 | 1.85 | 3.50 | 4.73 | 8.60 | 7.45 | 8.04 | 11.29 | 12.12 | 19.11 |
| *D. salina* WT | 3.10 | 3.65 | 4.53 | 4.44 | 4.70 | 1.37 | 1.46 | 1.47 | 1.48 | 1.27 |
| | Cell count(× $10^6$ /ml) | | | | | $OD_{687nm}$ | | | | |
| NaCl (M) | 0.3 | 0.7 | 1 | 1.5 | 2 | 0.3 | 0.7 | 1 | 1.5 | 2 |
| *D.tertiolectaD*CCBC26 | 6.00 | 6.75 | 3.00 | 2.25 | 2.45 | 1.100 | 1.240 | 0.554 | 0.335 | 0.339 |
| *D.salina* CCAP19/18 | 0.11 | 0.23 | 0.31 | 0.49 | 0.55 | 0.033 | 0.047 | 0.096 | 0.186 | 0.233 |
| *D.salina* WT | 2.05 | 3.45 | 2.00 | 2.50 | 2.75 | 0.440 | 0.600 | 0.493 | 0.578 | 0.510 |

Microalgael growth were expressed as cells per ml and also by measuring the $OD_{687\ nm}$. Chlorophyll a (Chl a) and total carotenoids (Car) contents were calculated as per volume (mg/l) and also per cell (pg cell$^{-1}$).

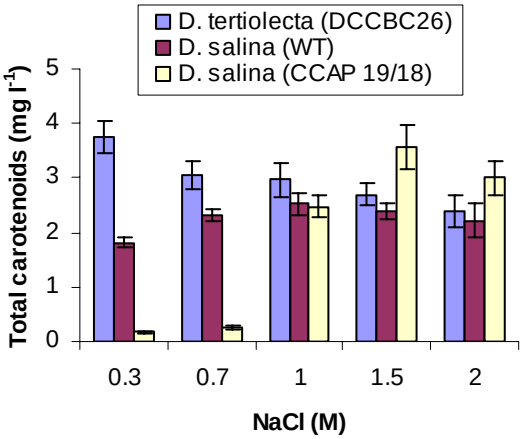


**Figure. 1**. Total carotenoids production by 21 days-old cultures of *D. tertiolecta* DCCBC26, *D. salina* CCAP 19/18 and *D. salina* WT in ASW media containing different NaCl concentrations under illumination of 50 µmol/m²s at 34 °C.

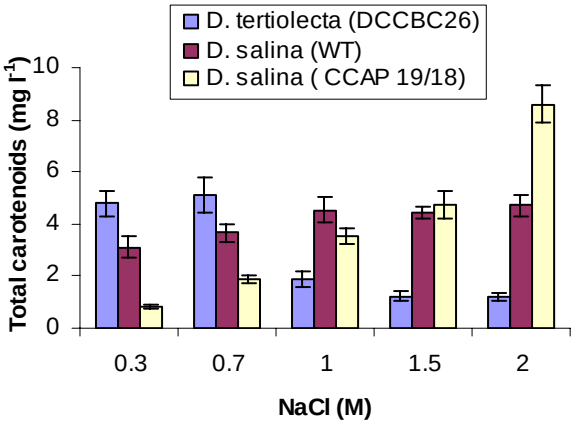


**Figure. 2**. Total carotenoids production by 21 days-old cultures of *D. tertiolecta* DCCBC26, *D. salina* CCAP 19/18 and *D. salina* WT in ASW media containing different NaCl concentrations under illumination of 150 µmol/m²s at 34 °C.

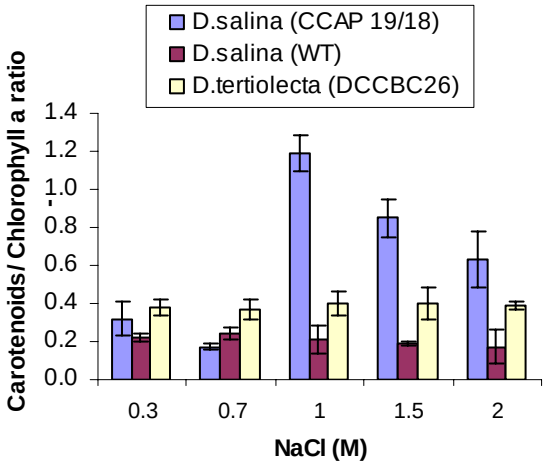


**Figure. 3**. Total carotenoids to chlorophyll a ratios in 21 days-old cultures of *D. tertiolecta* DCCBC26, *D. salina* CCAP 19/18 and *D. salina* WT in ASW media containing different NaCl concentrations under illumination of 50 µmol/m²s at 34 °C.

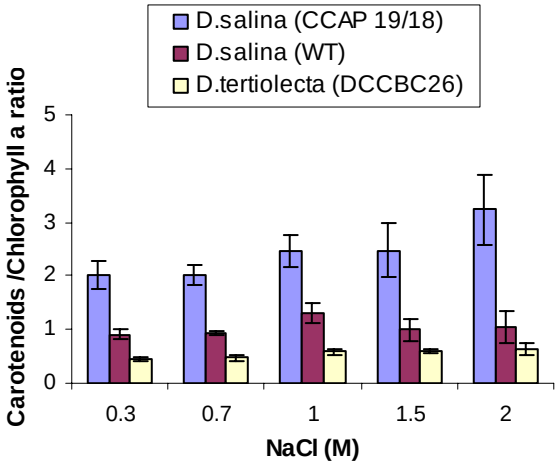


**Figure. 4**. Total carotenoids to chlorophyll a ratios in 21 days-old cultures of *D. tertiolecta* DCCBC26, *D. salina* CCAP 19/18 and *D. salina* WT in ASW media containing different NaCl concentrations under illumination of 150 µmol/m²s at 34 °C.

also suggested that adjustment of light and salinity are the best methods to achieve optimal carotene production in commercial cultures of *D. salina* (22). Total carotenoids to chlorophyll a ratios in *D. tertiolecta* DCCBC26 cells at light intensity of 50 µmol/m²s was not affected by salinity. Carotenoids to chlorophyll a ratio increased by all the strains with light intensity but the increase was more significant in *D. salina* CCAP 19/18 (5 fold increase at 2M salinity) while it was only 1.6 in *D. tertiolecta* DCCBC26 (Figures 3 and 4).

## CONCLUSIONS

In the present study, physiological factors affecting growth and carotenoids production by a microalgael isolate (*D. tertiolecta* DCCBC26) from the Urmia salt lake, north of Iran in comparison with two well documentated strains of *D. salina* CCAP 19/18 and *D. salina* WT were investigated. The lake is famous for its Artemia, a zooplankton also known as brine shrimp which is fed by the microalgae, *D. tertiolecta* as a supplement of vitamin E and carotenoids (23). *D. tertiolecta* DCCBC26 did not act as a halophyl and showed better growth and carotenoids accumulation at low salinities, a trend which is not usually observed with other microalgae such as *Dunaliella salina*. These results could be of value for enrichment attempts of the local Artemia prodution fields.

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## REFERENCES

1. Jin ES, Feth B, Melis A. A mutant of the green algae *Dunaliella salina* constitutively accumulates zeaxanthin under all growth conditions. Biotechnol Bioeng 2003; 81:115-124.
2. Nishino H, Murakosh M, Ii T, Takemura M, Kuchide M, Kanazawa M, Mou XY, Wada S. Carotenoids in cancer chemoprevention. Cancer Metastasis Rev 2002; 21:257-264.
3. Moeller SM, Jacques PF, Blumberg JB. The potential role of dietary xanthophylls in cataract and age-related macular degeneration. J Am Coll Nutr 2000;92: 55-58.
4. Prasad KN, Kumar A, Kochupillai V, Cole WC. High doses of multiple antioxidant vitamins: Essential ingredients in improving the efficacy of standard cancer therapy. J Am Coll Nutr 1999;18:13-25.
5. Bhosale P, Larson AJ, Bernstein PS. Factorial analysis of tricarboxylic acid cycle intermediates for optimization of zeaxanthin production from Flavobacterium multivorum. J Appl Microbiol 2004;96:623-629.
6. Masetto A, flores-Cotera LB, Diaz C, Langley E, Sanchez S. Application of a complete factorial design for production of zeaxanthin by Flavobacterium sp. J Biosci Bioeng 2001;92: 55-58.
7. Alcantara S, Sanchez S. Influence of carbon and nitrogen sources on Flavobacterium growth and zeaxanthin biosynthesis. J Ind Microbiol Biotechnol 1999;23:697-700.
8. Nelis hJ, Deleenheer AP. Microbial sources of carotenoid pigments used in food and feeds. J Appl Bacteriol 1991;70:181-191.
9. Bhosale P, Gadre RV. Production of β-carotene by a Rhodotorula glutinis mutant in sea water medium. Bioresource Technol 2001;76:53-55.
10. Barbosa M, Janssen MGJ, Ham N, Tramper J, Wijffels RH. Microalgae cultivation in air-lift reactors: modelling biomass yield and growth rate as a function of mixing frequency. Biotechnol Bioeng 2003; 82:170-179.
11. Phadwal K, Singh PK. Effect of nutrient depletion on β-carotene and glycerol accumulation in two strain of Dunaliella sp. Bioresource Technol 2003;90:55-58.
12. Gomez PI, Barriga A, Cifuentes AS, Gonzalez MA. Effect of salinity on the quantity and quality of carotenoids accumulated by *Dunaliella salina* (strain CONC-007) and *Dunaliella bardawil* (strain ATCC 30861) Chlorophyta. Biol Res 2003;36:185-192.
13. Jahnke LS, White LA. Long-term hyposaline and hypersaline stresses produce distinct antioxidant responses in the marine alga *Dunaliella tertiolecta*. J Plant Physiol 2003;160:1193-1202.
14. Ben-Amotz A, Lers A, Avron M. Stereoisomers of β-carotene and phytone in the alga Dunaliella bardawil (Chlorophyceae). Plant Physiol. 1988; 86: 1286-1291.
15. Hejazi MA, Wijffels RH. Effect of light intensity on β-carotene production and extraction by *Dunaliella salina* in two-phase bioreactors. Biomol Eng 2003;20:171-175.
16. Lichtenthaler HK. Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. Methods Enzymol 1987;148:350-382.
17. Jahnke LS. Massive carotenoid accumulation in *Dunaliella bardawil* induced by ultraviolet-A radiation. J Photochem. Photobiol 1999;48:68-74.
18. Ben-Amotz A, Avron M. On the factors which determine the massive β-carotene accumulation in the halotolerant alga *Dunaliella bardawil*. Plant Physiol 1983;72: 593–597.
19. Ben-Amotz A. Effect of irradiance and nutrient deficiency on the chemical composition of *Dunaliella bardawil* (Ben-Amotz and Avron) (Volvocales, Chlorophyceae). J Plant Physiol 1987;131: 479–487.
20. Cifuentes AS, Conzales MA, Inosroza I, Alguilera A. Reappraisal of physiological attributes of nine strains of Dunaliella (Chlorophyceae): Growth and pigment content across a salinity gradient. J phycol 2001;37: 334-344.
21. Orset SC, Young AJ. Exposure to low irradiances favors the synthesis of 9-cis β,β-carotene in *Dunaliella salina* (Teod.). Plant Physiol 2000;122:609–618
22. Marin N, Morales F, Lodeiros C, Tamigneaux E. Effect of nitrate concentration on growth and pigment synthesis of *Dunaliella salina* cultivated under low illumination and preadapted to different salinities. J Appl Phycol 1998;10**:**405–411.
23. Carballo-Cardena EC, Tuan Pham, M, Janssen M, Wijffels RH. Vitamin E (α-tocopherol) production by the marin microalgae *Dunaliella tertiolecta* and *Tetraselmis suecica* in batch cultivation. Biomol Eng 2003; 20:139-147.