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# The Effect of Temperature and Salinity on the Growth and Carotenogenesis of Three *Dunaliella* Species (*Dunaliella* sp. Lake Isolate, *D. salina* CCAP 19/18, and *D. bardawil* LB 2538) Cultivated under Laboratory Conditions

Imen Hamed, Burcu Ak, Oya Işık, Leyla Uslu, Kubilay Kazım Vursavuş

***Abstract*—**In this study, 3 species of *Dunaliella* (*Dunaliella* sp. Salt Lake isoalte (Tuz Gölü), *Dunaliella salina* CCAP19/18, and *Dunaliella bardawil* LB 2538) and their optical density, dry matter, chlorophyll *a,* total carotenoids, and β-carotene production were investigated in a batch system. The aim of this research was to compare carotenoids, and β-carotene production were investigated in a batch those 3 species. Therefore 2 stress factors were used: 2 different temperatures (20°C and 30°C) and 2 different salinities (30‰, and 60‰) were tested over a 17-day study. The highest growth and chlorophyll *a* was reported for *Dunaliella* sp. under 20°C/30‰ and 20°C/60‰ conditions respectively followed by *D. bardawil* and *D. salina*. Significant differences were noticed ($p<0.05$) for the other 3 species. The growth decreased as temperature and salinity increased since the lowest growth was noticed for the 30°C/60‰ group. The chlorophyll *a* content decreased also as temperature increased however when the NaCl concentration increased an augmentation of the content was noticed . In the 17$^{th}$ day of experiment the highest carotenoids concentration was reported for *D. bardawil* 20°C/30‰ (65,639±0,400 μg.mL$^{-1}$) and the most important β carotene concentration was for *D. salina* 20°C/60‰ (8,98E-07±0,013 mol/L).

***Keywords*—***Dunaliella* sp., *Dunaliella salina*, *Dunaliella bardawil*, stress factors, pigments, growth.

## I. Introduction

*DUNALIELLA* is a green, unicellular, biflagellate alga, belonging to the order Volvocales (Chlorophyceae, Chlorophyta). It is a halophilic genus that lacks a rigid cell wall. The alga was first described by Dunal in 1830 as *Haematococcus salinus* [1], but it was not until 1905 that the name *Dunaliella* was given by Teodoresco [2] who demonstrated that this genus was different from *Haematococcus* [3]. There are currently 23 recognized *Dunaliella* species [4]. One of the best-known specie is the halophile *Dunaliella salina* [5]. *Dunaliella* cells turn from green to red due to carotenoid production [6], [7].

*Dunaliella* is an halotolerant species which means that it can grow in various salinity levels from 0.2-35%. It is mostly found in natural habitats including oceans, brine lakes, and salt marshes with salinities above 10% [8], [9]. *Dunaliella* is a found in various regions including the Great Salt Lake in Utah (USA), the Dead Sea in Jordanie, and the West Bank, Pink Lake in Western Australia [3, 10]. *Dunaliella* is also resistance to a broad range of temperatures (0 °C to 40 °C) and the ideal ones are between 21-40°C. High temperature, close to 40°C or above, have been assosciated to carotenogenesis. The optimum pH is between 7 and 9 [11]. *D. salina* is able to accumulate high concentrations of β-carotene that can reach up to 10-14% of dry weight [12] especially when an environmental stress is applied such as high salinity, nutrient starvation (nitrogen, phosphate, and sulphate) and high temperatures [13, 14]. The differentiation between *Dunaliella salina* and *Dunaliella bardawil* has been made according to Olmos et al [15] on the basis of 18S rRNA gene sequences. It was determine that *D. salina* and *D. bardawil* contain one and two introns, respectively, within the 18S rRNA gene.

One of the most common carotenoid is β-carotene [16]. β-carotene is a lipophilic component, produced in the interthylakoid spaces of the chloroplasts in *Dunaliella* [17]. It is uded in numerous sectors such as food industry (natural food coloring agent), cosmetic and nutraceutical industries (antioxidant additive), pharmaceutical sector (anti-cancer compound). Besides, β-carotene has been utlized in aquaculture and feed animals feed industries since they can be used as natural colorant for fish tissues and as pro-vitamin A [9], [18]-[20]. In this study, the growth, total carotenoids, and β-carotene contents were compared between 3 *Dunaliella* species (*Dunaliella* sp., *D. salina*, and *D. bardawil*) under stress conditions of 2 temperatures (20 and 30 °C) and 2 salinities (30 and 60‰) over a 17-day period.

## II. Materials and Methods

### A. Algal Species

Three species (Fig. 1), from different geographic origins, were used in this experiment (as shown in Table I). *Dunaliella*

İmen Hamed is with Biotechnology Research and Application Centre, Cukurova University, Adana, Turkey (e-mail: imen_hh@yahoo.fr).
Burcu Ak, Oya Işık, and Leyla Uslu are with Department of Basic Sciences, Faculty of Fisheries, Cukurova University, Adana, Turkey (e-mail: bak@cu.edu.tr, oyaisik@cu.edu.tr, hizarcil@cu.edu.tr).
Kubilay Kazım Vursavuş is with Department of Agricultural Machinery and Technologies Engineering, Faculty of Agriculture, Çukurova University, Adana, Turkey (e-mail: kuvursa@cu.edu.tr).

sp. was isolated from the Salt Lake (Tuz Gölü) located in the central plateau of Turkey, 120 km south of Ankara. The identification of the isolates was established under microscope based on the morphological characters following Borowitzka and Siva [21]. The isolation of the required species was done by agar plating methods.

*Dunaliella salina* CCAP 19/18 was brought from the Culture Collection of Algae and Protozoa (CCAP) at Scotland, United Kingdom and *Dunaliella bardawil* LB 2538 was bought from UTEX Culture Collection of Algae at the University of Texas at Austin. The three species were established in a Conway medium under controlled laboratory conditions at a temperature of 20±1 and a continuous light intensity of 40 µmol/photon/s. The cultures were maintained by weekly subculturing them.

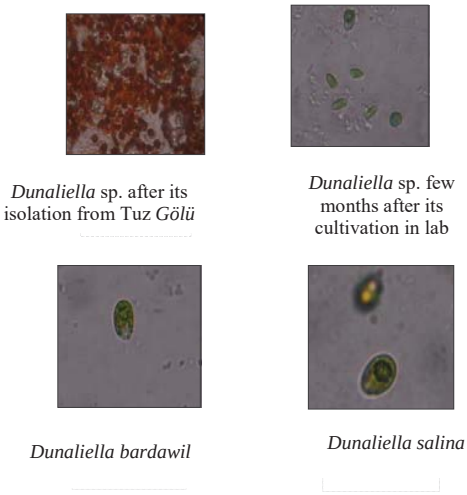


Fig. 1 *Dunaliella* species (x20 and 40 objective lens)

TABLE I
*DUNALIELLA* SPECIES USED IN THIS STUDY

| Strain | Source | Geographic origin |
|---|---|---|
| *Dunaliella* sp. | Lake Isolate | Tuz Gölü, Central Anatolia Region,Turkey |
| *Dunaliella salina* CCAP 19/18 | Culture Collection of Algae and Protozoa (CCAP) at Scotland, United Kingdom | Hutt Lagoon, Western Australia |
| *Dunaliella bardawil* LB 2538 | UTEX Culture Collection of Algae at the University of Texas at Austin | Lake Bardawil, North Sinai, Egypt |

### *B. Culture Conditions*

*Dunaliella* sp., *Dunaliella bardawil* and *Dunaliella salina* were cultivated in culture chambers at 2 different temperatures (20±1°C and 30±1°C) and 2 different salinities (30‰ and 60‰). Therefore, the following combined conditions were used 20°C/30‰ NaCl, 20°C/60‰ NaCl, 30°C/30‰ NaCl, and 30°C/60‰ NaCl. The species were grown in 500 ml Erlenmeyer flasks containing 250 ml of Conway medium and inoculated with 50 ml samples from the stock cultures (Figure 2). The flasks were continuously illuminated by fluorescent lamps providing 40 µmol photons/$m^2$/s and manually shaken three times per day. For each flask a duplicate was made, and each analysis was carried out in triplicate.

This research was carried out over a 17-day period. These parameters were chosen to identify the cell density, the biomass dry weight, and the pigments amount. The measurements were checked every two days and the means ±SD of the triplicates were given [22]. The *Dunaliella* cultures were harvested at the beginning of the stationary growth phase by centrifugation [22].


Fig. 2 *Dunaliella* species cultured at different temperatures and salinities for 17 days

### *C. Cell Density*

Density was monitored using a spectrophotometer (UV-Vis SP-3000 nano) at 680nm [22].

### *D. Biomass Test*

Dry biomass was define by filtering 10 ml of culture using glass microfiber filters (Whatman GF/C™, 1.2 µm, UK). A temperature of 105°C was used to dry the biomass for two hours [22].

### *E. Chlorophyll a Analysis*

Five ml from each erlenmeyer were filtered (Whatman GF/C™, 1.2 µm, UK). 10 ml of 90% acetone/water mixture was added the the filters that were put in tubes for chlorophyll extraction. The analysis was performed after 24h. Centrifugation (Hettich EBA-20) at 3500 rpm for 3 minutes at room temperature was performed. Chlorophyll *a* was establish with 4 wavelengths 630, 645, 665, and 750 nm. The equation of Strickland and Parsons was uesed to to calculate chlorophyll *a* (Chla) [22, 23].

$$Chla = 11.6 \cdot D_{665} - 1.31 \cdot D_{645} - 0.14 \cdot D_{630} \quad (1)$$

### *F. Total Carotenoids*

Carotenoids were extracted from *Dunaliella* species after 4, 12, and 17 days cultivation. Total carotenoid content was determined by filtering 10 mL of culture using Whatman GF/C™, 1.2 µm, UK. The carotenoids were extracted in 10 mL of ice cold 90% (v/v) acetone. After centrifugation, the absorbance of the clear supernatant was read in a

spectrophotometer at 452 nm. The total carotenoid concentration was calculated using the following formula [21]:

$$C = A_{452} \times 3.86 \times \frac{Vc}{Vs} \quad (2)$$

where C=total carotenoid concentration ($\mu g.mL^{-1}$); $V_c$=volume of culture sample (mL); $V_s$=volume of extract (mL).

*G. β-Carotene Analysis*

The β-carotene extraction was made in the last day of the experiment. For the extraction, 2 ml of culture was tested at the end of the experiment from each Erlenmeyer. To removed the supernatant, the algal cells were pelleted by centrifugation (Hettich EBA-20) at 4000 rpm for 10 min at room temperature. 5 ml of 80% acetone/water was added to the pellets to extract β-carotene. cellular debris were pelleted by centrifuging at 4000 rpm for 10 min. Lambert-Beer equation was used to calculate the β-carotene amount which was determined in the supernatant spectrophotometrically at 455 nm [22, 24]:

$$A = \varepsilon \cdot c \cdot d \quad (3)$$

β-carotene concentration: $c = \frac{A}{\varepsilon \cdot d}$; ε for β-carotene = 134000 (l/mol.cm)

*H. Statistical Analysis*

Results were examined using IBM SPSS-20. Graphs were drawn by Microsoft excel (©2007 Microsoft corporation, USA) programs. A one-way analysis of variance (ANOVA) and a Duncan test were used to determine differences between groups. Significant differences were considered at 5% ($p \leq 0.05$).

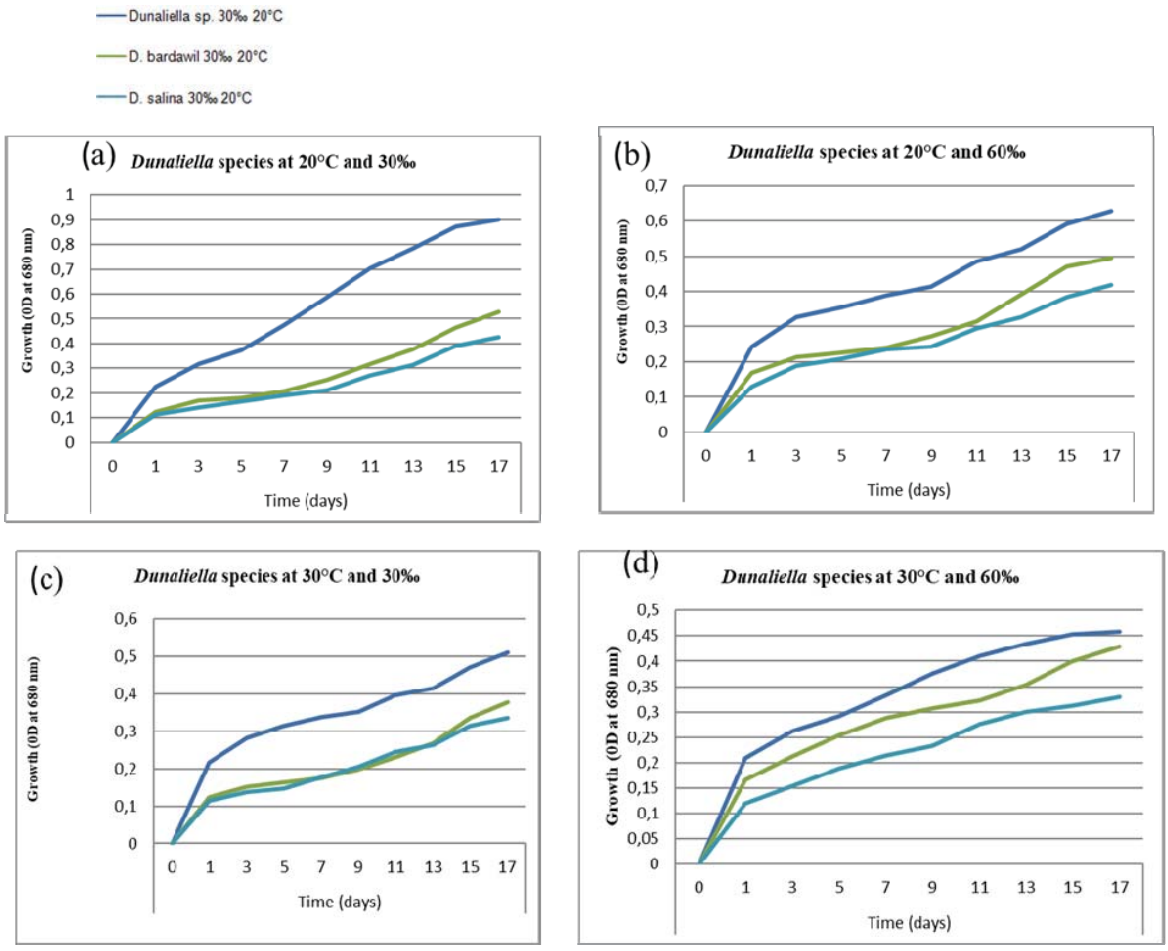


Fig. 3 Comparison of growth of three of *Dunaliella* species (*Dunaliella* sp., *Dunaliella bardawil*, and *Dunaliella salina*) in cultures maintained under four different treatment combinations of 2 temperatures and 2 salinities: (a) 20ºC/30‰; (b) 20°C/60‰; (c) 30ºC/30‰, and (d) 30ºC/ 60‰

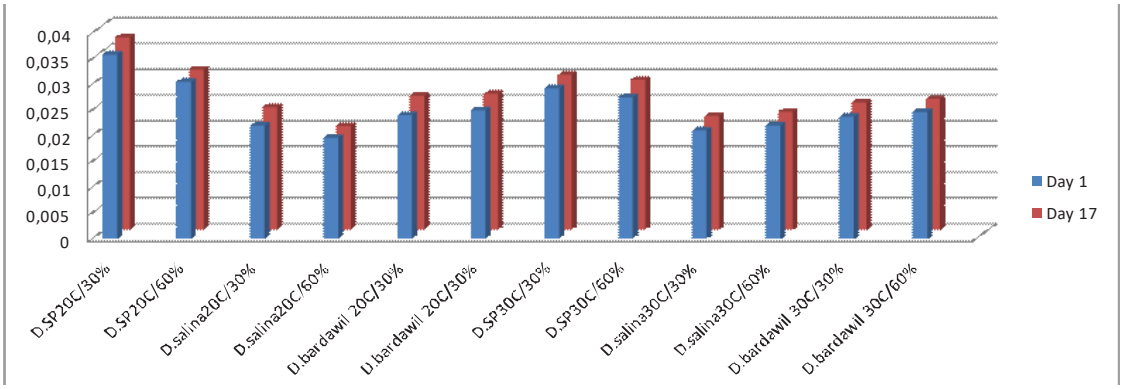


Fig. 4 Biomass (g) in the first day and the last day of the experiment

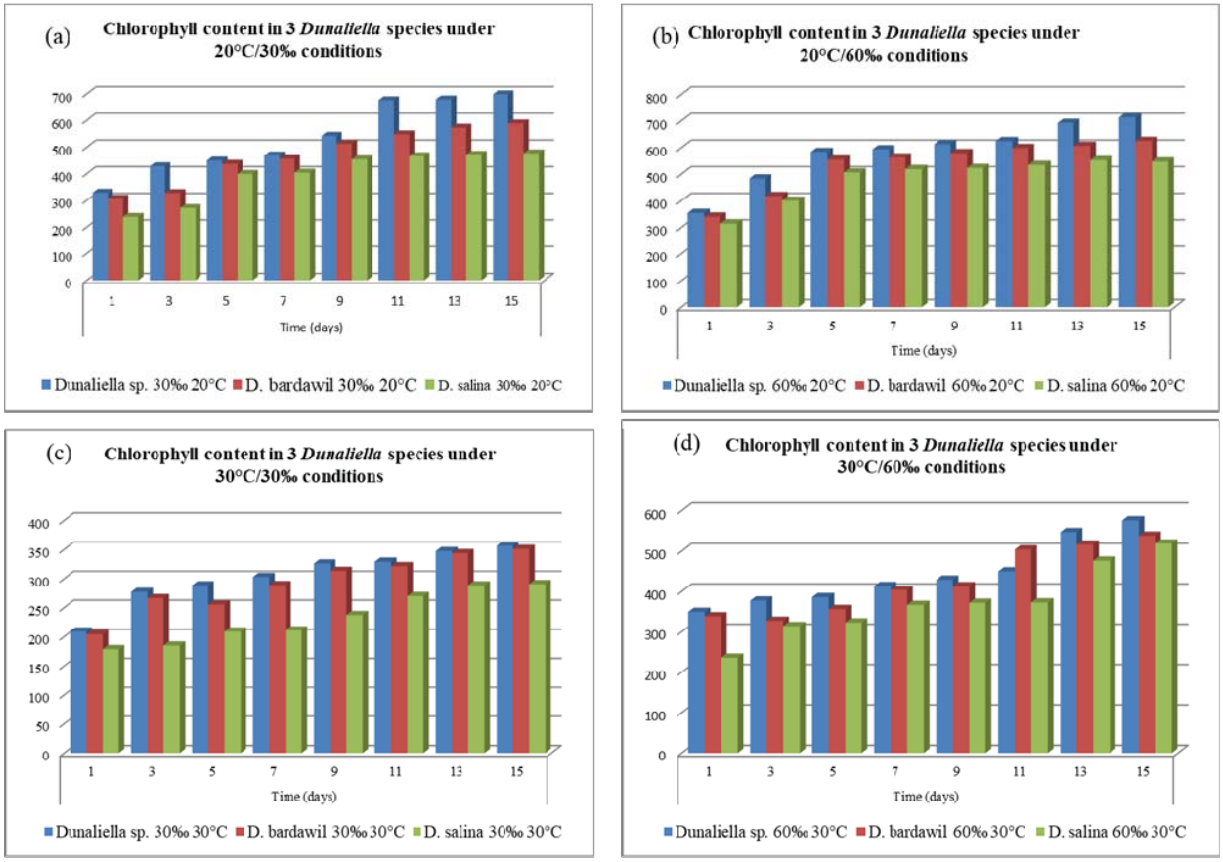


Fig. 5 Comparison of chlorophyll content (µg/L) of three of *Dunaliella* species (*Dunaliella* sp., *Dunaliella bardawil*, and *Dunaliella salina*) in cultures maintained under four different treatment combinations of 2 temperatures and 2 salinities: (a) 20ºC/30‰; (b) 20°C/60‰; (c) 30ºC/30‰, and (d) 30ºC/ 60‰

## III. Results and Discussion

### A. Growth and Biomass

*Dunaliella* sp. had the highest growth than *D. bardawil* and *D. salina* in all treatment conditions (Figure 3). The results were significant ($p<0.05$) between *Dunaliella* sp. and the other 2 species (*D. bardawil* and *D. salina*) for the 20°C/30‰ and 30°C/30‰ groups. No significance was found for the following conditions: 20°C/60‰ and 30°C/ 60‰. Maximum growth for *Dunaliella* sp. was obtained at 20°C/30‰ (0,902±0,012) followed by 20°C/60‰ (0,648±0,014) and decreased as temperature and salinity increased since the lowest growth was noticed for the 30°C/60‰ group (0,459±0,005). *D. bardawil* exhibited maximum growth at 20°C/30‰ (0,529±0,002) with no significant differences ($p>0.05$) between the other treatment groups.

The highest growth for D. salina was noticed at 20°C/30‰ (0,427±0,006). However, comparing to the other species D. salina had the lowest growth for all treatment groups. Between

D. bardawil and D. salina the results were not significant ($p>0.05$) for all treatment conditions. For the 3 Dunaliella species the best growth was obtained when a temperature of 20°C and a salinity of 30‰ NaCl were applied. The lowest growth was reported for the 30°C/60‰ treatment group for Dunaliella sp. and D. salina while for Dunaliella bardawil this result was noticed for the 30°C/30‰ group.

The biomass results were related to those of the growth (figure 4). The highest biomass was reported for Dunaliella sp. followed by Dunaliella bardawil and Dunaliella salina. The biomass was the most important for the 20°C/30‰ group.

*B. Chlorophyll a*

Similar to the precedent results, for the growth and the biomass, the chlorophyll *a* content was the highest for *Dunaliella* sp., followed by *D. bardawil* and *D. salina*. The content was the most important for the treatment condition of 20°C/60‰ in which 712,927 µg/L (*Dunaliella* sp.), 624,860µg/L (*D. bardawil*), and 547,233µg/L (*D. salina*) were reported. The lowest concentrations were for 30°C/30% group 356,553µg/L (*Dunaliella* sp.), 351,013µg/L (*D. bardawil*), and 290,633µg/L (*D. salina*). Clearly, the temperature affected the growth and chlorophyll *a* concentration. When the temperature increased the biomass, growth and chlorophyll *a* decreased. With the augmentation of the salinity, the growth of the 3 *Dunaliella* species decreased. However, regarding the chlorophyll *a* content an augmentation was noticed. There were no significant differences between all treatment groups except for *Dunaliella* sp. and *D. salina* ($p<0.05$) when 30°C/30‰ were tested.

*C. Total Carotenoids and β-Carotene*

The carotenoids content was not significant in the 4th and 12th of the experiment for all treatment conditions (Table 2). In the last day of the experiment significant differences ($p<0.05$) between all groups except for *Dunaliella* sp. 20°C/30‰ when compared with the other species and for all treatment conditions no statistically differences were noticed. In the 17th day of experiment the highest carotenoids concentration was for *D. bardawil* 20°C/30‰ (65,639±0,400 µg.mL$^{-1}$) followed by 20°C/60‰ (63,883±0,546 (µg.mL$^{-1}$). When, the temperature increased to 30°C the carotenoids content decreased for the 3 species. For instance the results found for *D. bardawil* earlier decreased to 18,875±0,117 µg.mL$^{-1}$ and 31,864±0,160 when 30°C/30‰ and 30°C/60‰ were applied respectively. The lowest concentrations of carotenoids were reported for *Dunaliella* sp. 20°C/30‰ and 20°C/60‰ (36,187±0,357 and 33,080±0,312 µg.mL$^{-1}$ respectively) which had the best growth and chloropyll *a* content (figure 3, 4, and 5). Thus, the growth and chlorophyll *a* content were inversely proportional to the total carotenoids.

Various researches investigated the effects of differents parameters such as temperature, salinity and nutirent starvation on the growth and carotenoids accumulation of *Dunaliella* species. Gómez et al. [25] studied the effect of 3 concentrations of NaCl (1M, 2M, and 3M) on the total carotenoids and β-carotene contents in 2 species: *D. salina* (isolated from the Atacama Desert, Chile) and in *D. bardawil* (isolated from the Bardawil Lake, Egypt). The highest carotenoid contents were obtained at 2M NaCl for both species. While the β-carotene was the most important at 3M for *D. bardawil* it was the highest for *D. salina* at 2M. In another study the effects of temperature (15 and 26ºC) and irradiance (40 and 110 µmol photons.m-2.s-$^{1}$) were investigated on the growth and the carotenogenesic capacity using 7 strains of *Dunaliella salina* three Chilean and four non-Chilean (Mexico, China, Australia, and Egypt). The Australian strain showed the highest accumulation of total carotenoids (40.7 mg. L$^{-1}$) at 15ºC and 110 µmol photons.m-2.s-1. In their research they also noticed that the temperature influenced the quality of β-carotene since 15ºC was the optimal temperature for the α-carotene accumulation, while 26ºC was the best for β-carotene accumulation. The production of β-carotene in all the strains was temperature dependent rather than photon flux dependent as higher accumulation of this pigments were found at 26ºC than at 15ºC. All of the non-Chilean strains exhibited the highest growth rates and the maximum cell densities, whereas 2 of the Chilean strains had the lowest values in both parameters [26]. Çelekli and Donmez, [27] isolated *Dunaliella* sp. from the saltlake (Tuz Gölü) and tested pH, light intensity, salt and nitrogen concentrations on growth and β-carotene accumulation. The isolate was cultivated under 20±2 °C during 39 days in batch cultures. Cell density and β-carotene content increased with light intensity and nitrogen limitation at pH 7 and 20% NaCl concentration. Garcia et al., [28] tested 6 salinity levels (10%, 15%, 20%, 25%, 30% and 35%) and 6 temperatures (18, 22, 26, 30, 34 and 38 °C) on 2 *Dunaliella* species isolated from Yucatan, Mexico. The species were identified as *D. salina* and *D. viridis*. The study was conducted over a 17-day period. *D. salina* had optimal growth at 22 °C and *D. viridis* at 26 °C. When the temprerature was incresed (38°C) for both species a decreased in cell density was reported. While the total carotenoid content in *D. salina* increased over time with temperature at 38 C, Chlorophyll *a* content in *D. salina* decreased with time at 18 and 22 °C.

Rad et al., [5] investigated for 1 month, at 25 ± 2°C 100 µmol photons.m$^{-2}$.s$^{-1}$, and 3 salinity degrees (1, 2, and 3M) the growth of *Dunaliella* sp. collected from Urmia Lake in Iran. They concluded that, the optimal salinity levels were 2M for the cell content (1.68 x 10$^{6}$ cell.ml$^{-1}$) and 3M for β-carotene accumulation (8.94 pg.cell$^{-1}$)

## IV. Conclusion

The present study shows that optical densities and pigment yields of the 3 *Dunaliella* species are strongly dependant on the temperature and salinity. The temperature and NaCl concentration clearly affected the cultures of *Dunaliella*. Increasing stress factors caused a reduction in optical density and pigments contents. The highest growth and chlorophyll *a* was reported for *Dunaliella* sp., followed by *D. bardawil* and *D. salina*. The highest results were associated with *D. bardawil* for the carotenoids and with *D. salina* for the β carotene. The evaluation of physiological characteristics of those species will be used to carry out cultivation in the lab.

TABLE II
THE TOTAL CAROTENOIDS CONTENT IN 3 *DUNALIELLA* SPECIES (MG.ML$^{-1}$)

| Day of the experiment | *Dunaliella sp.* | | | | *Dunaliella salina* | | | | *Dunaliella bardawil* | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 20°C/30‰ | 20°C/60‰ | 30°C/30‰ | 30°C/60‰ | 20°C/30‰ | 20°C/60‰ | 30°C/30‰ | 30°C/60‰ | 20°C/30‰ | 20°C/60‰ | 30°C/30‰ | 30°C/60‰ |
| 4th | 38,175± 0,131 | 52,882± 0,253 | 40,569± 0,154 | 39,140± 0,116 | 21,114± 0,049 | 23,006± 0,052 | 21,500± 0,057 | 35,975± 0,088 | 18,837± 0,033 | 40,337± 0,130 | 37,365± 0,202 | 55,237± 0,260 |
| 12th | 41,559± 0,334 | 35,589± 0,305 | 16,392± 0,132 | 23,803± 0,153 | 25,630± 0,165 | 31,086± 0,203 | 16,830± 0,076 | 15,594± 0,079 | 33,042± 0,156 | 44,519± 0,353 | 17,988± 0,092 | 36,233± 0,239 |
| 17th | 36,1875± 0,357 | 33,0802± 0,312 | 12,4871± 0,046 | 21,3072± 0,179 | 54,0593± 0,378 | 68,8624± 0,611 | 18,3929± 0,118 | 17,37± 0,074 | 65,6393± 0,400 | 63,883± 0,546 | 18,8754± 0,117 | 31,8643± 0,160 |

Means values, n=3

TABLE III
CONCENTRATION OF B-CAROTENE (MOL/L) FOR 3 *DUNALIELLA* SPECIES IN THE LAST DAY OF EXPERIMENT

| Day of the experiment | *Dunaliella sp.* | | | | *Dunaliella salina* | | | | *Dunaliella bardawil* | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 20°C/30‰ | 20°C/60‰ | 30°C/30‰ | 30°C/60‰ | 20°C/30‰ | 20°C/60‰ | 30°C/30‰ | 30°C/60‰ | 20°C/30‰ | 20°C/60‰ | 30°C/30‰ | 30°C/60‰ |
| 17th | 7,26E-07± 0,002 | 7,19E-07± 0,002 | 8,13E-07± 0,005 | 8,21E-07± 0,006 | 7,94E-07± 0,008 | 8,98E-07± 0,013 | 8,33E-07± 0,014 | 8,41E-07± 0,014 | 7,96E-07± 0,007 | 8,08E-07± 0,004 | 8,36E-07± 0,003 | 8,68E-07± 0,006 |

Means values, n=3

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