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RESEARCH PAPER

The Effects of Salinity and Temperature on the Growth of *Dunaliella* sp. Isolated from the Salt Lake (Tuz Gölü), Turkey

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Abstract

In this study *Dunaliella* sp. was isolated from the hypersaline Lake Tuz and its cell density, dry matter, chlorophyll a, and β-carotene production were studied in a batch system. The aim of this research was to determine the optimal conditions for *Dunaliella sp*. growth. Therefore 3 different temperatures (18°C, 22°C, and 26°C) with 3 different salinities (25‰, 30‰, and 35‰) were tested over a 19-day study. Significant differences were found at the end of the cultivation period ($P<0.05$) for 18°C and 26°C. No statistically significance were reported for all tested salinity degrees ($P>0.05$). At the end of the experiment the maximum growth were noticed for 18°C 25‰ in which the culture reached 3.48 105 cell/ml. The lowest was reported for 26°C 35‰ (1.50 105 cell/ml). The culture of 18°C 25‰ had also the best chlorophyll a concentration and biomass amount. For the β-Carotene 22°C 30‰ (4.39 10 -6 mol/L) had the highest accumulation. The optimum temperature for *Dunaliella sp*. growth was 18°C at 25‰.

Keywords: *Dunaliella* sp, stress factors, pigment analysis, density.

Introduction

Dunaliella is a green, unicellular, biflagellate alga, belonging to the order Chlamydomonadales (Chlorophyceae, Chlorophyta). It is a halophilic genus that lacks a rigid cell wall. The alga was first described as *Haematococcus salinus* (Dunal, 1838), but it was not until 1905 that the name *Dunaliella* was given by Teodoresco (Teodoresco, 1905) who demonstrated that this genus was different from *Haematococcus* (Nguyen, Tran, Portilla and Vo, 2014). There are currently 23 recognized *Dunaliella* species (Oren, 2005). One of the best-known species is the halophile *Dunaliella salina* (Rad, Aksöz & Hejazi, 2011). *unaliella* cells turn from green to red due to carotenoid production (Tran, Doan, Louime, Giordano, & Portilla, 2014; Taha, Abo El Kheir, Hammouda, & Abd El-Hady, 2012). The genus can tolerate extreme variations in salinity ranging from 0.2-35%, but it is most frequently found naturally in habitats with salinities above 10%, such as oceans, brine lakes, salt marshes, and salt water ditches near the sea (Leach, Olivera, & Morais, 1998; Ben-Amotz & Avron, 1990). *Dunaliella* is a worldwide species found in numerous places including the Great Salt Lake in Utah (USA), the Dead Sea in Jordanie, and the West Bank, Pink Lake in Western Australia (Nguyen *et al*., 2014). Due to its halotolerance, *Dunaliella* have the ability to thrive under extreme salinities which constitute a selective criterion since it inhibits growth of other algae and predators (Park, Lee, & Jin, 2013). *Dunaliella* is also tolerant to a wide range of temperatures from below 0°C to above 40°C with the optimum begin between 21-40°C. The optimum growth temperature depends on the strain and light intensity. In case of high temperature, close to 40°C or above, carotenogenesis is stimulated. The optimum pH is between 7 and 9 (Borowitzka & Borowitzka, 1989). *D. salina* is able to accumulate high concentrations of β-carotene that can reach up to 10-14% of dry weight (Tafreshi & Shariati, 2009) especially when grown under environmental stresses such as intense irradiance, high salinity, nutrient starvation (nitrogen, phosphate, and sulphate) and extreme temperatures (El Baky & El Baroty, 2013; Tafreshi and Shariati, 2006).

β-carotene is the most common carotenoid and its main source is the genus *Dunaliella* (Emeish, 2012). β-carotene is a lipophilic high-value compound, accumulated as lipid globules in the interthylakoid spaces of the chloroplasts in *Dunaliella* (Raja, Hemaiswarya, & Rengasamy, 2007). It has a wide variety of market applications in food industry as a natural food coloring agent, in cosmetic and nutraceutical industries as an antioxidant additive, and in pharmaceutical industry as an anti-cancer compound. Additionally, β-carotene has been used in aquaculture feed industry as a natural colorant for fish

tissues and as pro-vitamin A for animal feed (Hejazi & Wijffels, 2003; Ben-Amotz & Avron, 1990; Garcia-Gonzales,Moreno, Manzano, Florencio, & Guerrero, 2005; García-Chavarría and Lara-Flores, 2013).

Material and Methods

Isolation, Identification, and Cultivation of *Dunaliella* sp.

Dunaliella sp. was isolated from the salt lake (T*uz Gölü*) (38°44′N 33°23′E), in Central Anatolia, Turkey. Samples were collected during summer (between July and August) and transferred to laboratory within 24h. The identification of the isolates (Figure 1) was established under microscope based on the morphological characters following Borowitzka and Siva (2007). The isolation of the required specie was done by agar plating methods. 1.5 % agar was added to 1 L of Conway Medium and this agar solution was sterilized in an autoclave for 15 min under 150 lbs pressure and 120°C temperature. Then this medium was poured in sterilized Petri dishes. *The inoculum was introduced using a* wire loop which was first sterilized in a flame, then the loop was loaded with a small amount of sample, and parallel streaks of the *Dunaliella* sp. suspension were made. The plates were covered and sealed with parafilm, and incubate under low light (40µmol/photon/s) at a constant temperature (18°C±1). The agar plates were incubated until colonies of cells appeared after 2 weeks approximately. Meanwhile the plates were regularly observed under microscope to make sure that a unialgal species (*Dunaliella* sp.) was isolated. Cells were picked up with a loop and then rinsed in a liquid culture medium in 50 ml Erlenmeyer flasks. After isolation, stock cultures were establish in a Conway Medium under controlled laboratory conditions, at room temperature (18°C±1), continuously illuminated by fluorescent lamps providing 40 µmol photons/m^2/s, and manually shaken three time per day. The stocks were maintained by monthly subculturing them (Figure 2).

The composition of Conway Medium was as following (Walne, 1974) (1 ml Conway Medium/liter of seawater): Stock enrichment solutions: $NaNO_3$ (100 g); NaH_2PO_4 (20 g); Na_2EDTA (45 g); H_3BO_3 (33.60 g); $FeCl_3.6H_2O$ (1.30 g); $MnC_2.4H_2O$ (0.36 g); Trace metal 1 ml*; Vitamin Mix 100 ml**, Distilled water 1 liter. *****Trace metal solution**:** $ZnCl_2$ (2.10 g); $CoC_2.6H_2O$ (2.00 g); $(NH_4)_6MO_7O_{24}.4H_2O$ (0. 90 g); $CuSO_4.5H_2O$ (2.00 g); Distilled water 100 ml. **Vitamin stock solution: B_{12} (10 mg); B_1 (thiamin) (20 mg); Distilled water (200 ml).

Experimental Culture Conditions

Dunaliella sp. was cultivated in a temperature controlled culture chambers at three different temperatures (18°C, 22°C, and 26°C), at three NaCl concentrations (25 ‰, 30 ‰, and 35 ‰), and at one

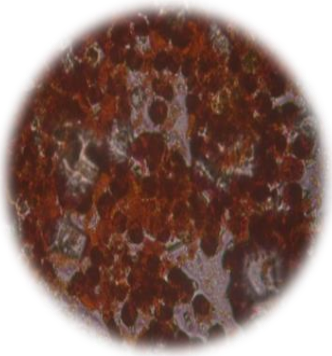

Figure 1. *Dunaliella* sp. after its isolation from Lake Tuz (40x objective lens).

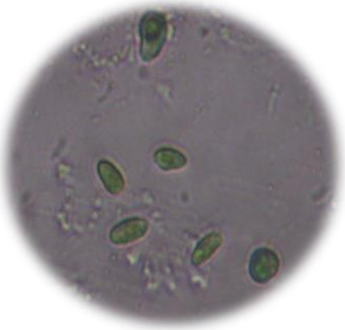

Figure 2. *Dunaliella* sp. few months after its cultivation in Lab conditions (40x objective lens).

light intensity (40 µmol photons/m^2/s). Three replicate cultures for each temperature per each NaCl concentration were done (18°C 25‰, 18°C 30‰, and 18°C35‰; 22°C 25 ‰, 22°C 30‰, and 22°C35‰ ; 26°C 25‰, 26°C 30‰, and 26°C35‰). Thus, 27 algal cultures were studied in batch systems and unshaken flasks (Figure 3). The isolates were grown in 500 ml Erlenmeyer flasks containing 250 ml of Conway Medium and inoculated with 50 ml samples from the stock cultures. At the beginning of the experiment pH was checked as 8.5.

This research was carried out over a 19-day period. These experimental conditions were selected to determine the cell density, the biomass dry weight, and the pigments amount (chlorophyll *a* and β-carotene) over time. The measurements were performed every two days and the means ± SD of the triplicates were presented. The cultures were harvested at the beginning of the stationary growth phase by centrifugation.

Cell Density

Cell density was checked by spectrophotometer UV-Vis SP-3000 nano and cell counting was made using a light microscope and a Sedgewick Rafter Cell S50 (Microliter). Samples were shacked to homogenize, then, 15 ml of each culture were transferred to 50 ml plastic centrifuge tubes. After reading the absorbance at 680nm, one drop of formalin solution was added to the remaining samples to fix the cells. Number of cells was calculated as follows:

$$\text{Algal cells per ml} = \frac{\textit{Number of algal cells counted}}{\textit{Number of squares observed}} \text{ x1000}$$

Biomass Analysis

Dry weight was determined by filtering 10 ml of algal culture through glass microfiber filters (Whatman GF/C™, 1.2 µm, UK). Algal biomass was dried at 105°C for two hours and weighed. The biomass was checked in the first day and the last day of experiment.

Chlorophyll *a* Analysis

Five ml from each culture were filtered using (Whatman GF/C™, 1.2 µm, UK). The filters were put in glass tubes and 10 ml of 90% acetone/water mixture was added for chlorophyll extraction. The tubes were put in the refrigerator for 24 h and analyzed the next day. The tubes were centrifuged (Hettich EBA-20) at 3500 rpm for 3 minutes at room temperature. The extracts were collected with Pasteur pipettes and chlorophyll *a* was determined spectrophotometrically using 4 wavelengths 630, 645, 665, and 750 nm. Chlorophyll *a* was calculated using the equation of Strickland and Parsons (1965).

β-Carotene Analysis

For the extraction of β-carotene, 2 ml of algal culture was taken at the end of the experiment from each flask. The cells were pelleted by centrifugation (Hettich EBA-20) at 4000 rpm for 10 min at room temperature. The supernatants were discarded and the pellets were resuspended in 5 ml of 80% acetone/water. β-carotene extraction from cellular debris was done by centrifuging at 4000 rpm for 10 min. A cuvette containing acetone was used as a blank to calibrate the spectrophotometer to the zero point. The concentration of β-carotene in the supernatant was determined spectrophotometrically at 455 nm and calculated according to Lambert-Beer equation (Javeria *et al*., 2013):

$$A = \varepsilon.c.d$$

A = Absorbance
ε = Molar extinction coefficient (l/mol.cm)
c = concentration (mol/L)
d = distance (1 cm)

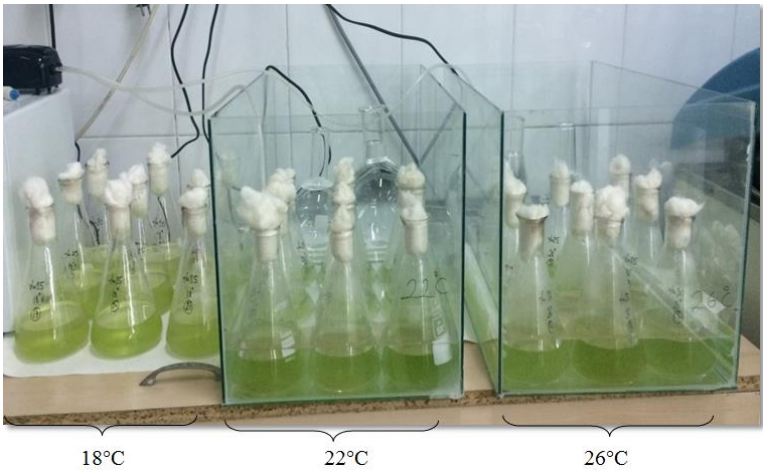

Figure 3. *Dunaliella* sp. cultured at different temperatures and salinities for 19 days.

The concentration of β-carotene was calculated as:

$c = A/\varepsilon.d$

ε for β-carotene = 134000 l/mol.cm

Statistical Analysis

Data were analyzed by Statistical analysis using IBM SPSS-24 and drawing graphs were performed by Microsoft excel (© 2007 Microsoft corporation, USA) programs. A one-way analysis of variance (ANOVA) was used to determine differences between groups. For multiple comparisons a Duncan test was used when significant differences were found. Each result shown was the mean of three replicated studies. Differences were considered to be significant at a probability of 5% ($P \leq 0.05$).

Results and Discussion

Dunaliella sp. cultures cultivated at the same temperature with different salinities have no statistically significant differences ($P>0.05$). Therefore the salinities 25‰, 30‰, and 35‰ were not effective and no differences were shown on the growth rate. However highly significant results ($P<0.05$) were found between *Dunaliella* sp. cultivated under 18°C and 26°C. No significance were found between 18°C, 22°C and 22°C, 26°C ($P>0.05$) at the end of the cultivation period. Growth of *Dunaliella* sp. lake isolate at different temperatures and salinities is shown in Figure 4. *Dunaliella* sp. cultivated under18°C 25‰ exhibited the highest cell density (3.48 10^5 cell/ml) than the other groups. The lowest concentration was reported for the 26°C 35‰ (1.50 10^5 cell/ml). There was a significant decrease ($P<0.05$) of the cell number with the increasing of the temperature. The increasing of the salinity for the 22°C and 26°C cultures seemed to improve slightly the density since it was better with 35‰ than 25‰ and 30‰. In the contrary for the 18°C the cell number was the most significant with 25‰ than 30‰ and 35‰. However, the differences were not significant ($P>0.05$) regarding the salinity.

The amounts of chlorophyll *a* increased during the 19-day period of study (Figure 5). At the end of the experiment the most significant chlorophyll concentrations were noticed for 18°C 25‰ followed by 22°C 35‰. Increasing temperatures caused a reduction in chlorophyll *a* content. The increasing of the salinity for the 22°C and 26°C cultures seemed to improve slightly the chlorophyll *a* content as the best concentrations were found for the 35‰ comparing to the 25‰ and the 30‰. However, the differences were not significant ($P>0.05$).

The biomass was checked on the first day and last day of the experiment (Figure 6). As expected the maximum biomass was reached by 18°C 25‰ (0.0252±0.00098 g) and the lowest was reported for 26°C 35‰ (0.0198 g±0.0018735). The β-carotene amounts were analyzed in the last day of the research. *Dunaliella* sp. cultivated at 22°C 30‰ yield the highest concentration (4.39 10^{-6} mol/L) followed by the 18°C cultures. The lowest β-carotene amounts were reported for the 26°C cultures with the smallest being for 25‰ (1.20 10^{-6} mol/L). Various studies investigated the effects of temperature, salinity and other parameters (nitrogen limitation, light intensity, and pH) on the growth of *Dunaliella* sp. Garcia, Freile-Pelegrin, & Robledo (2007) studied the effects of 6 different salinities (10%, 15%, 20%, 25%, 30% and 35%) and 6 different temperatures (18, 22, 26, 30, 34 and 38°C) on the growth of *Dunaliella* sp. isolated from Yucatan, Mexico. The species were identified as *D. salina* and *D. viridis* based on their morphological characteristics. The research was carried on over a 17-day period. Optimal growth temperature for *D. salina* was 22°C and 26°C for *D. viridis*. For both species a decreased in cell density was reported at 38°C. Maximum growth for *D. salina* was obtained at 10% NaCl and decreased as salinity increased. The highest concentration of *D. viridis* was at 15% NaCl with no significant differences between 20% and 25% NaCl concentrations. Ak, Cirik, and Göksan (2008) investigated the effects of light intensities (50 and 75 µmol photon/m^2/sec), salinities (1, 2, and 3M), and temperatures (25 and 28°C) on the growth of *D. viridis* over a 30-day period. The alga was isolated from *Çamaltı* Solar Saltern in Izmir, Turkey. The optimum conditions for *D. viridis* growth were obtained at 25°C, 50 µmol photon/m^2/sec, and 2M NaCl. Another study was carried out by Rad *et al*. (2011) in which they tested 3 salinity concentrations (1, 2, and 3M) on the growth of *Dunaliella* sp. isolated from Urmia Lake in Iran. The isolates were cultured under 25±2°C and 100 µmol photons.$m^{-2}.s^{-1}$ for 30 days. During their research they counted the cells and measured the carotenoid amount by microscopic counting and spectrophotometry. At the end of the experiment, the optimal salinities were 2 and 3M NaCl for the cell content (1.68 x 10^6 cell.ml^{-1}) and the β-carotene concentration (8.94 pg.$cell^{-1}$) means respectively. The effects of pH, light intensity, salt and nitrogen concentrations were tested on the growth of *Dunaliella* sp. isolated from Lake Tuz. The isolate was cultivated under 20±2°C during 39 days in batch culture experiments in unshaken flasks. Cell density and β-carotene content increased with light intensity and nitrogen limitation from 3.3 10^6 to 4.2 10^6 cell/ml at pH 7 and 20% NaCl concentration (Çelekli & Dönmez, 2006).

Conclusion

The present study shows that cell densities and pigment yields of *Dunaliella* sp. Lake Tuz isolate are strongly dependant on the temperature. Temperature clearly affected the cultures of *Dunaliella* sp. Increasing temperatures caused a reduction in cell

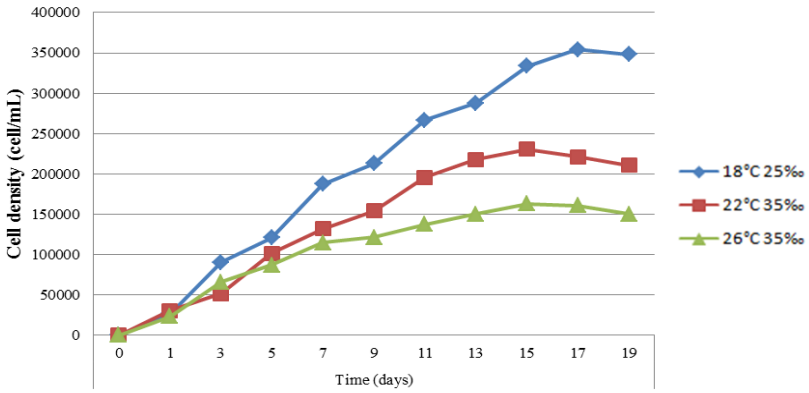

Figure 4. Cell density of *Dunaliella* sp. at different temperatures (18°C, 22°C and 26°C) and at different salinities (25‰, 30‰ and 35‰).

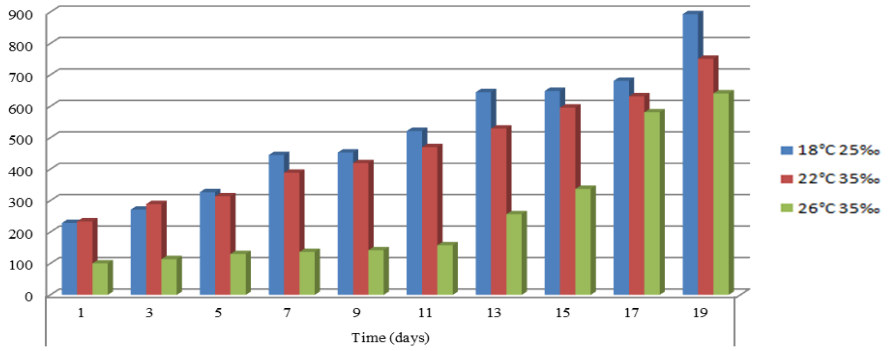

Figure 5. Effect of temperatures (18, 22, 26°C) and salinity (25‰, 30‰, and 35‰) on chlorophyll *a* amounts of *Dunaliella* sp.

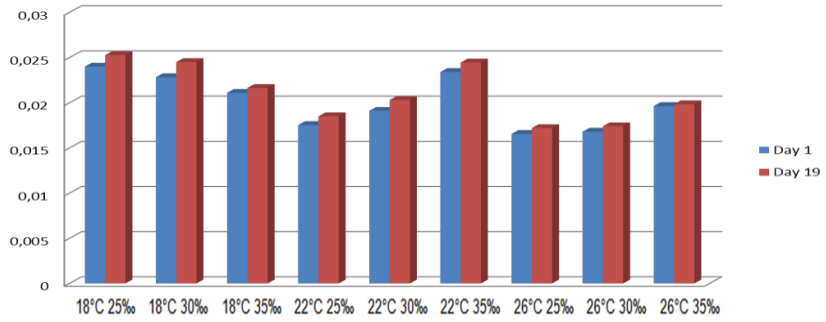

Figure 6: Biomass in the first day and last day of experiment

density and chlorophyll *a* contents. Variations of the salinity were no significant statistically. The evaluation of physiological attributes of this specie will be used to carry out cultivation in the lab. Therefore for the cultivation this isolate a temperature of 18°C and a salinity of 25‰ will be used.

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