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INFLUENCE OF SALINITY ON THE GROWTH, PIGMENTATION AND ASCORBATE PEROXIDASE ACTIVITY OF *DUNALIELLA SALINA* ISOLATED FROM MAHARLU SALT LAKE IN SHIRAZ*

K. NIKOOKAR, A. MORADSHAHI** AND M. KHARATI

Department of Biology, Shiraz University, Shiraz, I. R. of Iran

Abstract – *Dunaliella salina* was isolated from Maharlu salt lake in Shiraz, Iran, and was analyzed for the effects of salinity stress on its growth, pigment content and ascorbate peroxidase activity. Growth, expressed as number of cells ml^{-1}, which was most rapid at 2 M NaCl, was reduced at salinity extremes. During the exponential phase of growth, increasing salinity from 0.5 to 4 M NaCl increased Chl a from 0.3 to 0.9 pg $cell^{-1}$, while β-carotene was decreased from 3.6 to 2.4 pg $cell^{-1}$ and Chl b remained unchanged. At stationary phase, increasing salinity increased the pigment content of cells. β-carotene content per cell reached its highest value of 10.1 pg at 4 M NaCl. Maximum β-carotene to total Chl ratio of 7.4 was obtained at 2 M NaCl. The TLC chromatogram of pigments extracted from cells at the exponential phase of growth resembled that of spinach leaves while the chromatogram of pigments extracted from cells at the stationary phase resembled that of carrot and commercial β-carotene. A significant increase in ascorbate peroxidase activity was observed with increasing salinity from 1 to 4 M NaCl. It is suggested that increased capacity to scavenge ROS may help to optimize algal biomass and β-carotene production.

Keywords – *Dunaliella salina*, isolation, salinity, β-carotene, ascorbate peroxidase

1. INTRODUCTION

The genus *Dunaliella* is widely distributed and has been recorded from an extremely wide range of habitats. Some species such as *D. lateralis* live in fresh water, while in hypersaline environments species like *D. salina* predominates [1]. *Dunaliella* has been reported in hypersaline lakes around the world including Maharlu salt lake in Shiraz, Iran [1-3]. The presence of *Dunaliella* in Maharlu salt lake was first reported in 1985 by M. Ginzburg and B. Z. Ginzburg and was called Iran-6 [2], but the isolated species was not identified.

When some species of *Dunaliella* are subjected to environmental stress conditions such as high salt concentrations, high light intensities or nutrient deprivation, they overproduce and accumulate very large amounts of β-carotene [4-8]. Studies using the halophilic *D. bardawil* have indicated a direct relationship between β-carotene content and salinity [9]. Carotenogenesis is also enhanced under the condition of nitrate limitation [5]. In *D. salina,* low nitrate concentration negatively affected growth, but enhanced carotenoid accumulation [3].

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**Corresponding author

Environmental stress conditions have been shown to promote the generation of reactive oxygen species (ROS) such as superoxide radicals (O_2^-), hydroxyl radicals ($^{\cdot}OH$) and singlet oxygen ($^1O_2$) [10]. In higher plants, tolerance to salinity stress is often manifested in an increased capacity to scavenge ROS [11]. A number of enzymatic and nonenzymatic antioxidative defense systems such as ascorbate peroxidase, glutathione reductase and β-carotene minimize the damage caused to lipids, proteins and nucleic acids by oxidative stress [10]. Rajguru *et al.* [12] reported higher constitutive levels of glutathione reductase and ascorbate peroxidase in salt-tolerant cultivars of cotton. An increase in the activities of antioxidant enzymes, monodehydroascorbate reductase, and ascorbate peroxidase in *D. tertiolecta,* which Butcher subjected to high salinities, was reported by Jahnke and White [13]. The activities of some other antioxidant enzymes such as catalase and superoxide dismutase were not altered by extreme salinities. Shiash *et al.* [14] proposed that high light intensities promote ROS production in *D. bardawil,* which in turn enhance synthesis and accumulation of β-carotene. The main protective role of β-carotene is to prevent the generation of singlet oxygen and scavenging peroxy radicals.

The present study addresses three issues: a) isolation and purification of *D. salina* from Maharlu salt lake, b) growth of isolated *D. salina* and pigment biosynthesis as affected by salinity c) effects of salt stress on the activity of antioxidant enzyme ascorbate peroxidase.

2. MATERIALS AND METHODS

a) Isolation and Identification of Dunaliella salina

Water samples were collected from Maharlu salt lake located 30 km southeast of Shiraz, Iran, and transferred to the laboratory of the biology department at Shiraz University. Single colonies were derived from individual cells grown on agar plates as described by Lers *et al.* [6] and Powtongsook *et al.* [15]. After 2 to 3 weeks, each colony was transferred to 10 ml liquid growth medium containing 2 M NaCl, 50 mM $NaHCO_3$, 5 mM $MgSO_4$, 0.75 mM KNO_3, 0.2 mM KH_2PO_4, 0.2 mM $CaCl_2$, 7 μM $MnCl_2$, 5 μM EDTA, 2 μM $FeCl_3$, 1 μM $CuCl_2$, 1 μM $CoCl_2$, 1 μM $(NH_4)_6Mo_7O_{24}$, and 1 μM $ZnCl_2$ (pH 7.5). The cultures were incubated in a growth chamber at 22°C under continuous illumination provided by cool white fluorescent lamps at an intensity of 4000 lux. *Dunaliella salina* was identified as described by Avron and Ben-Amotz [16].

b) Growth and Pigments Determination

1 ml of purified *D. salina* culture containing 10^5 cells was added to two 200 ml Erlenmeyer flasks containing 100 ml growth medium with indicated concentrations of NaCl. The flasks were maintained in the growth chamber under the same conditions as mentioned before. The samples for growth measurements and pigment determinations were taken at appropriate times during the incubation period. Three replications were made for each experiment.

For growth measurements, cell number was determined in a Coulter Counter Model ZBI with a 100 μm orifice. For chlorophylls and β-carotene determination, 3 ml of each culture was centrifuged at 2000g for 5 minutes and pigments were extracted from the algal pellet with 100% acetone. The extract was centrifuged at 5000g for 5 minutes and supernatant was reconstituted to 3 ml 80% (v/v) acetone with deionized water. Chlorophylls and β-carotene were determined as described by Eijckelhoff *et al.* [17].

c) Pigments analysis by TLC

About 5 ml of *D. salina* cultures at exponential and stationary phases were centrifuged at 2000g for 5 minutes and the pellets were extracted with 100% acetone. Pigments also were extracted from spinach leaf and carrot by homogenizing 1gr of each tissue in 100% acetone and centrifuging the homogenates at 5000g for 5 minutes. The extracted pigments were developed on silica gel with the solvent system hexane: diethylether: acetone [30: 15: 10] and the resolved pigments were compared with each other and with commercial β-carotene.

d) Determination of Ascorbate Peroxidase Activity

The algae was cultivated in the growth medium containing 1, 2, 3, or 4 M NaCl and incubated under the same conditions as before. During the incubation period, samples were taken from the cultures and centrifuged at 2000g for 5 minutes. 10 ml of grinding buffer containing 100 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA and 2.2 mM ascorbate [18] were added to the pellets. The cells were homogenized in a French press and the homogenates were centrifuged at 5000g for 5 minutes. The ascorbate peroxidase activity in the supernatant was measured by monitoring the decrease in A_{290} for one minute (extinction coefficient 2.8 mM^{-1} cm^{-1}). The 1-ml reaction mixture contained 100 mM potasium phosphate buffer (pH 7.0), 0.22 mM ascorbate, 0.3 mM H_2O_2 and enzyme extract [19].

3. RESULTS AND DISCUSSION

a) Identification of D. salina

Several species and strains of the genus *Dunaliella* grow in hypersaline Maharlu salt lake. On the basis of characteristics described by Avron and Ben-Amotz [16] such as two flagella of equal length, lack of contractile vacuoles, red appearance due to a large accumulation of β-carotene under extreme environmental conditions and a hardly visible lateral eye spot, *D. salina* was identified in natural *Dunaliella* populations in Maharlu salt lake (Fig.1).

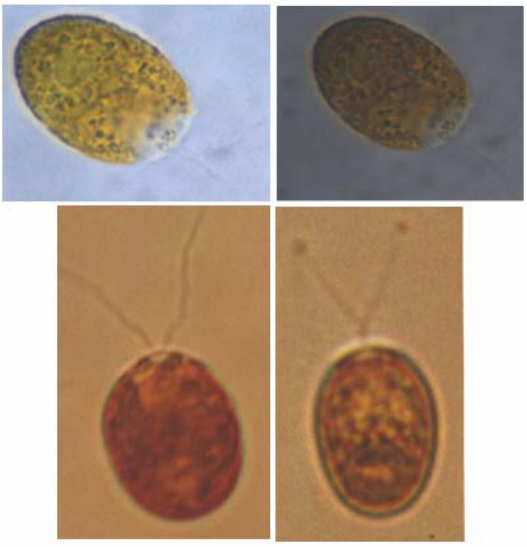

Fig. 1. *D. salina* at exponential (green) and stationary (orange-red) phases of growth

b) Growth and pigment content as affected by salinity

As illustrated in Fig. 2, growth on the basis of cell number was highest at 2 M NaCl and tended to decrease at lower salinities (0.5 and 1 M NaCl). Significant decrease in growth rates was observed at 3 and 4 M NaCl during the entire incubation period indicating the cultures were stressed.

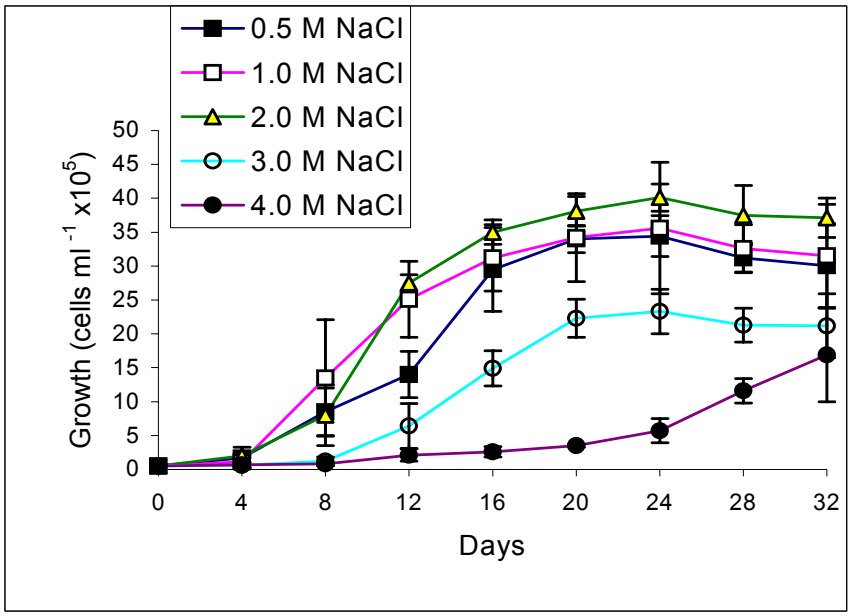

Fig. 2. Effects of salinity on growth of *D. salina.* Each bar represents mean±SE

Reduced growth at salinity extremes is also reported by other investigators [7, 13, 15]. Increasing salinity from 1.7 to 5.1 M reduced the specific growth rate in six clones of *D. salina* isolated from salt fields in Thailand [15]. Optimum NaCl concentrations for the growth of *D. salina* grown under continuous illumination at 4500 and 11000 lux were 2.6 and 1.8 M NaCl, respectively [20]. Marin *et al.* [3] reported the highest cell concentration when *D. salina* was grown at 14% (2.3 M) NaCl. In *D. tertiolecta,* cell division was most rapid between 0.1 and 0.5 M NaCl. Growth rates were reduced 30 to 55% at salinity extremes (0.05 and 3 M NaCl) [13].

At the exponential phase of growth (12 days after inoculation), the amounts of Chl a, Chl b and β-carotene, expressed in terms of μg per ml of culture, were highest at 2 M NaCl, compared to 0.5 and 4 M NaCl (Table 1). The same results were obtained at the stationary phase (28 days after inoculation) except for Chl b, which showed the highest amount at 0.5 M NaCl. The ratio of β-carotene to total Chl was inversely related to salinity at the exponential phase, while at the stationary phase a maximum ratio of 7.33 was observed at 2M NaCl (Table 2). When pigment content was expressed in terms of pg per cell, at the exponential phase of growth, Chl b remained unaffected while an increase in Chl a and decrease in β-carotene were observed with an increase in salinity. At the stationary phase, the highest pigment content was obtained at high salinity. The amount of β-carotene at 4 M NaCl was significantly higher compared to 0.5 and 2 M NaCl (Table 1). Again, at the exponential phase, β-carotene to total Chl ratio was inversely related to salinity, and at stationary phase a maximum ratio of 7.4 was obtained at 2 M NaCl (Table 2).

Table 1. Pigments content of *D. salina* as affected by salinity. Each value is mean ±SE

Pigments	Incubation (day)	NaCl (M) 0.5	2.0	4.0	0.5	2.0	4.0
		← g ml^{-1} →			← pg cell^{-1} →		
Chl a	12	0.5±0.12	1.3±0.1	0.2±0.06	0.3±0.08	0.4±0.03	0.9±0.2
	28	2.0±0.1	2.4±0.07	1.3±0.4	0.6±0.04	0.6±0.02	1.1±0.3
Chl b	12	0.2±0.04	0.6±0.12	0.1±0.04	0.2±0.03	0.2±0.04	0.2±0.1
	28	1.5±0.07	1.2±0.11	1.2±0.05	0.5±0.02	0.3±0.03	1.0±0.04
β-carotene	12	5.1±1.98	7.7±1.4	0.5±0.2	3.6±1.4	2.8±1.6	2.4±1.2
	28	14.9±0.92	25.8±1.6	1.7±0.6	4.8±0.3	6.9±0.4	10.0±0.5

Table 2. Effect of salinity on β-carotene to total Chl ratio in *D. salina*

Incubation Period (day)	NaCl (M) 0.5	2.0	4.0	0.5	2.0	4.0
	← Per ml of culture →			← per cell →		
12	6.8	4.2	2.0	7.0	4.3	2.1
28	4.4	7.33	4.7	4.4	7.4	4.8

Relationships between pigment content and salinity have been studied by several investigators [5, 13, 20, 21]. Analysis with Fisher's LSD test revealed an inverse relationship between carotenoid content of *D. salina* and salinity [21]. This is opposite to previous studies with *D. bardawil,* which indicated a direct relationship between β-carotene content and increasing salinity [9]. Studies using six clones of *D. salina* have also indicated a direct relationship between total carotenoid content and increasing salinity from 1.7 to 5.1 M. Among the isolated strains, clone DS91008 had the highest carotenoid content of 80.3 pg cell^{-1} at 5.1 M NaCl [15].Heidari *et al.* [20] reported that the amounts of β-carotene and Chl a per ml of *D. salina* culture grown under continuous illumination at 4500 and 11000 lux were highest at 2.6 and 1.8 M NaCl, respectively. Increasing salinity increased β-carotene to Chl ratio in *D. bardawil* and *D. salina* grown under a wide range of continuous light intensities [5]. During the exponential phase, a maximum ratio of 13.1 was observed in high salinity- and high light-grown *D. bardawil,* whereas *D. salina* cultivated under the same conditions had a ratio of 0.8. It was suggested that the increase in β-carotene to Chl ratio could be due to increased net synthesis of β-carotene, a decrease in Chl, or both. In several strains of *D. salina,* Liu *et al.* [21] reported an inverse linear relationship between Total Valuable Carotenoids (TVC) to Chl a ratio and salinity. The differences in pigment content as affected by salinity reported by various investigators might be due

to differences in environmental conditions employed, growth phase in which the algae were sampled, analytical procedures and equations used for pigment determination, the units that the pigments were expressed, and /or species and strain of *Dunaliella* used in the experiments. If, as is reported in the present work, β-carotene production is enhanced by high salinities, it is suggested that high salinity may promote ROS production, which in turn induce increased synthesis of antioxidants such as β-carotene. It has also been suggested that high salinity promotes abscisic acid metabolism, thereby possibly upregulating β-carotene production [22].

c) TLC analysis of pigments

Comparison of pigments from different sources by TLC is shown in Fig. 3. Chromatogram of pigments extracted from *D. salina* at the logarithmic phase of growth was similar to that of spinach leaves, while chromatogram of pigments extracted from cells at the stationary phase resembled commercial β-carotene and pigments extracted from carrots. TLC analysis confirms the results of others [4, 16] that at the stationary phase β-carotene is accumulated by *D. salina*. In addition, it clearly shows that the amounts of other pigments are reduced at the stationary phase.

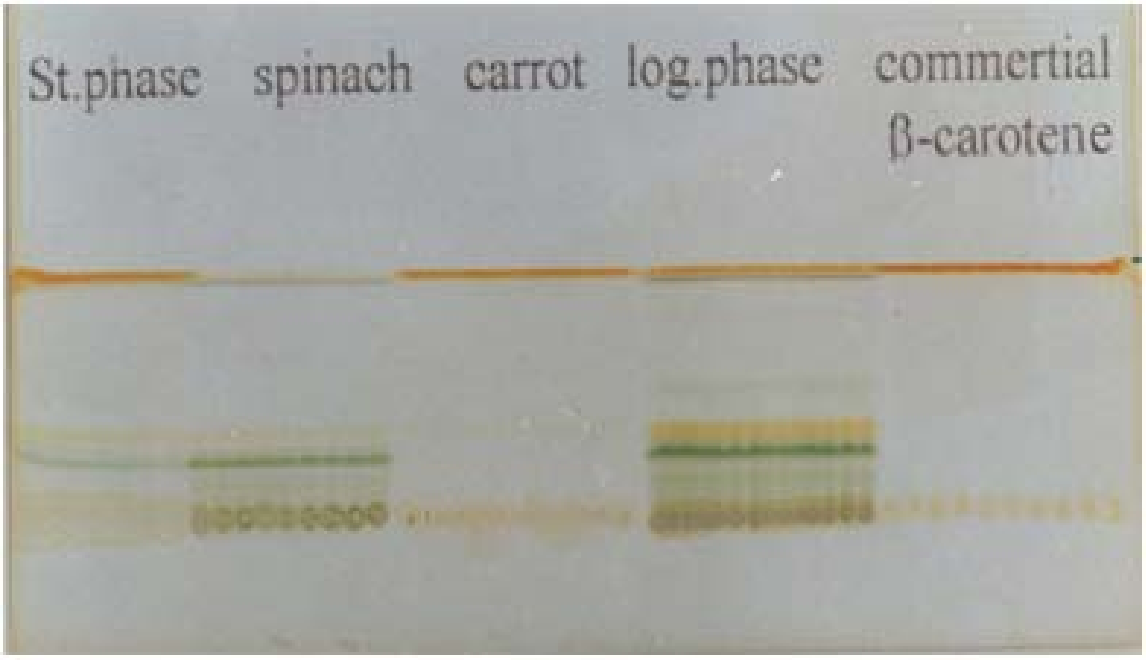

Fig. 3. Comparison of pigments from various sources by TLC. *D. salina* was cultivated at 2 M NaCl

d) Ascorbate peroxidase response to high salinity

Ascorbate peroxidase activity increased significantly with the increase in salinity (Fig. 4). A 171% increase in enzyme activity was observed at 4 M NaCl compared to 2 M NaCl. At each NaCl concentration, ascorbate peroxidase activity did not change greatly during the entire incubation period.

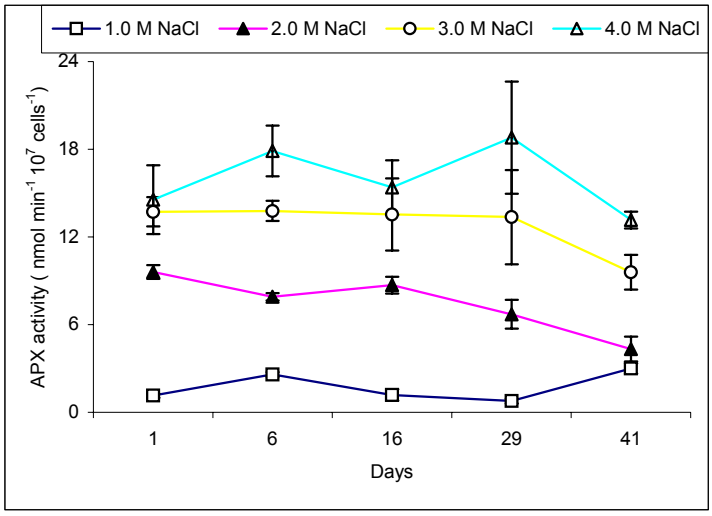

Fig. 4. Effect of growth salinity on ascorbate peroxidase activity in *D. salina*. Each value is mean±stdx t^2 0.025

A significant increase in ascorbate peroxidase activity at high salinities is consistent with the findings of other investigators in *D. tertiolecta* and *Chlamydomonas* [13, 23]. In salt tolerant plant species, higher constitutive levels and /or significant increases in ascorbate peroxidase and monodehydroascorbate reductase activities have been reported [24-26]. In microalgae an increase in the activities of these enzymes not only promotes ROS removal, but also may increase ATP synthesis via the Mehler-peroxidase reaction which is the source of additional ATP for Na^+ expulsion at high salinities [13].

In addition to environmental growth conditions, since salinity stress may promote ROS production which in turn limits photosynthesis and growth of *D. salina,* strains with increased capacity to scavenge ROS may help to optimize both algal biomass at the exponential phase of growth and overall carotenoid production.

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