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Differential response of *Dunaliella salina* and *Dunaliella tertiolecta* isolated from brines of Sambhar Salt Lake of Rajasthan (India) to salinities: A study on growth, pigment and glycerol synthesis

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Original Article

Abstract
The physiological responses of *Dunaliella salina* and *Dunaliella tertiolecta* isolated from Sambhar Salt Lake of Rajasthan, India were studied at different salinities. Both Dunaliella species (*D. salina* and *D. tertiolecta*) were treated with a range of NaCl concentrations from 6 to 30% (w/v). Salinity more than 15% decreased the growth rate and chlorophyll level in *D. salina* while increased the total carotenoid and glycerol level in the cell. In case of *D. tertiolecta* salinity more than 6% adversely affect the growth rate, while glycerol level was increased as salinity increased but total carotenoid and chlorophyll level was not salt triggered. The highest cellular carotenoids level in case of *D. salina* was recorded at 25% NaCl concentration. The results showed that glycerol level in both the species was salt induced. In case of *D. salina* maximum level of glycerol (30.5±0.24 pg/cell) was at 30% salt concentration, while in case of *D. tertiolecta* (19.36±0.13 pg/cell) it was at 25% NaCl concentration. The highest specific growth rate in *D. salina* was observed at 15% NaCl (0.12 d^{-1}), while in case of *D. tertiolecta* it was observed at 6% NaCl (0.13 d^{-1}). These results indicated that *D. salina* was more salt tolerant than *D. tertiolecta*. Evaluation of physiological attributes of these species will be of help for carrying out mass cultivation.

Keywords: *Dunaliella, salinity, growth rate, glycerol, carotenoids.*

Introduction
Dunaliella is a type of halophilic biflagellated pink microalga. The genus *Dunaliella* is classified under the class Chlorophycea, order Volvocales, and the genus includes a variety of ill-defined unicellular species that thrive in hyper saline, marine and fresh water habitats. (Avron and Ben-Amotz,1992). *Dunaliella*, like other fresh water algae of the genus *Chlamydomonas*, is characterized by an ovoid cell volume usually in the shape of a pear, wider at the basal side and narrow at the anterior flagellated top. It is a unicellular, halotolerant photoautotrophic microalga, which lacks a rigid cell wall, but synthesizes various economically important organic compounds (Ghetti *et al.,* 1999).

It has been reported that some species of *Dunaliella* can produce large amount of ß- carotene in response to stress such as high light intensity, high concentration of NaCl, nutrient deficiency, etc. (Avron and Ben-Amotz, 1992). Carotenoids, especially ß-carotene, are the most important and the most abundant pigment in nature and only plants, algae and microorganisms can produce them naturally (Arun *et al.*, 2012). *D. salina* is capable of accumulating large amounts of carotenoids up to 14% of their dry weight (Borowitzka *et al.,* 1984).

These organisms are known to withstand sudden changes in salinity, irradiance and nutrient availability in their habitats (Oren, 2005). The genus *Dunaliella* has developed several physiological adaptations including the lack of a rigid cell wall, a variable intracellular concentration of glycerol (Kacka and Donmez, 2008), changes in photosynthetic pigments (Sanchez-Estudillo *et al.,* 2006) and structural modifications in the chloroplast (Stonynova-Bakal-ova and Toncheva-Panova, 2003). Mostly the *D. salina* has been widely employed for the production of valuable high quality chemicals such as carotenes and glycerol (Ben-Amotz *et al*., 1991).

A commercial application of other species of *Dunaliella* has been ignored perhaps due to lack of sufficient information about their physiological attributes. A deep understanding of the metabolic processes such as photosynthesis, photorespiration, osmotic adjustment and compartmentation, amino acid and carbohydrate synthesis of other species of *Dunaliella* in response to salinity stress is necessitated before their commercial exploitation (Ozturk *et al.,* 2002).

The present investigation is an attempt to study the response of *D. Salina* and *D. tertiolecta*, isolated from a salt brine of Sambhar Lake, Rajasthan (India), against a range of salinity stress. Efforts have been made to study the salinity induced production of ß-carotene and glycerol by these isolated species. Evaluation of physiological attributes will play an important role in further development of biotechnology for mass cultivation of these species.

Material and methods

Source of alga and isolation of organisms: *Dunaliella* used for the present study was isolated from Sambhar salt Lake of Rajasthan, India. Single colonies were derived from individual cells by repeated sub-culturing on agar plates as described by Lers *et. al.* (1990) and Powtongsook *et. al.* (1995). Each colony was transferred to liquid nutrient medium as described by Ben-Amotz and Avron (1983). The cells were identified on the basis of morphology by using phase contrast microscope (Preisig, 1992) and they were designated as *D. salina* and *D. tertiolecta*.

Morphological identification: Several species of the genus *Dunaliella* grows in hypersaline Sambhar salt Lake of Rajasthan, India. Two species of *Dunaliella* were successfully isolated and maintained in the basal medium under laboratory conditions. The two species were segregated based on their morphological characteristics viz., cell shape, cell colour, cell length, width, flagella length chloroplast arrangement, and growth conditions (Preisig, 1992; Leonardi and Caceres, 1997). The mean cell length and breadth of the cells were calculated from the measurements of 100 cells. They were also subjected to different NaCl concentration ranging from 6% to 30% (w/v) and studied for their growth.

Growth condition: *Dunaliella* species were maintained in Bold Basal medium as cell suspension in 250 ml Erlenmeyer flasks containing 100 ml of liquid medium with 6% salt (NaCl) concentration (w/v), and pH of the medium was adjusted to 7.5 by adding 40 mM of tris buffer. Phosphate and bicarbonate nutrient stock solutions were sterilized separately and were added in the medium after autoclaving so as to avoid precipitation. Cultures were kept at 25°C under continuous light with an intensity of 10 Wm^{-2}.

Experimental setup: To study the effect of salinity on the growth, pigments, protein and glycerol synthesis in both the species of *Dunaliella* (*D. salina* and *D. tertiolecta*), experiment was performed in 250 ml conical flask containing 100 ml of growth medium (Bold Basal medium) with varying salt concentrations (6, 10, 15, 20, 25, 30%). The entire flasks were inoculated with the 1% *Dunaliella* culture containing 105 cells/ml. All the experiments were performed in triplicates.

Growth measurements: Growth was measured in terms of optical density at 680 nm by using UV-visible Spectrophotometer (Schimadzu, Japan) (Lichtenthaler, 1987). Cell counts were also monitored by direct counting, using a light microscope (magnification × 40) with a 1 mm deep counting chamber (Neubauer improved).At least 10 replicates were taken and the mean number of cells/ml in culture was calculated from the recorded values. The growth rate and specific growth rate were determined as described by Garcia *et. al.* (2007).

Pigment extraction and analysis: A homogeneous suspension of 4 ml was taken from each culture flask after thorough mixing. Cells were centrifuged at 5000 rpm for 5 minutes and the washed cell pellet was mixed with 4 ml of acetone/water (80:20, v/v). The mixture was left for 1-2 min at room temperature to ensure complete extraction. The extract was centrifuged for 5 min at 5000 rpm and the colourless biomass was discarded (Jin *et al.,* 2003). The amount of extracted pigments in the solvent phase was quantified as per the method described by Lichtenthaler (1987).

Total protein determination: Protein content was determined using the method of Lowry *et. al.* (1951).

Glycerol determination: The cells were washed twice in isotonic media and filtered through miracloth paper. To 200 µl of cell suspension, 1 ml of periodate reagent (65 mg sodium metaperiodate, 10 ml acetic acid, 7.7 g ammonium acetate), and 2.5 ml of acetylacetone reagent (2.5 ml acetyl-acetone, 247.5 ml isopropanol) were added. The mixture was incubated at 45°C for

20 min. Thereafter, optical density was determined at 410 nm. The values were compared with the standard curve prepared by using known amount of glycerol (Chitlaru and Pick, 1989).

Results

Isolation and identification of Dunaliella species

D. salina possessed two flagella of equal length, contractile vacuole was not clearly visible, it turned orange/red due to a large accumulation of ß-carotene under high salt concentration. Lateral eye spot was clearly visible (Fig. 5(a) and Fig. 5(b)). While in case of *D. tertiolecta* cells were ellipsoidal, oval, pyriforms, apically broader and posterior narrow regions, they were always green, chloroplast located at the basal region; stigma is not clearly visible (Jayappriyan *et al.,* 2010) (Fig. 6(a) and Fig. 6(b)).

Effect of different salt concentration on the growth

To monitor the effect of NaCl concentration on cell growth of *D. salina* and *D. tertiolecta*, cells were grown in the flask with several NaCl concentrations (from 6% to 30%). Growth was monitored in terms of cell count/ml. Growth was significantly reduced in response to increasing NaCl concentration from 6% to 30% in case of *D. tertiolecta* (Fig. 1(b)), whereas in case of *D. salina*, the maximum growth was observed at 15% NaCl concentration(Fig. 1(a)). The results showed that *D. salina* requires high NaCl concentration for its growth and it is obligate halophile, while *D. tertiolecta* did not depend upon NaCl for its growth and it was therefore a facultative halophile.

Specific growth rate were also calculated and it was found that both the species showed slow growth rate. In the case of *D. salina* maximum growth rate was at 15% salt concentration (0.12 μ/day) while in the case of *D. tertiolecta* it was the maximum at 6% salt concentration (0.13 μ/day). A further increase in the NaCl concentrations for respective species resulted into sharp decline in the specific growth rate. Unlike the *D. salina*, there was complete inhibition of growth at 30% salt concentration in the case of *D. tertiolecta*.

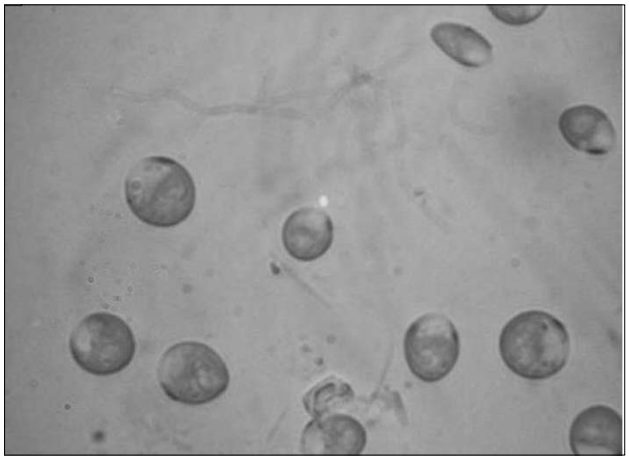


Fig.5(a)

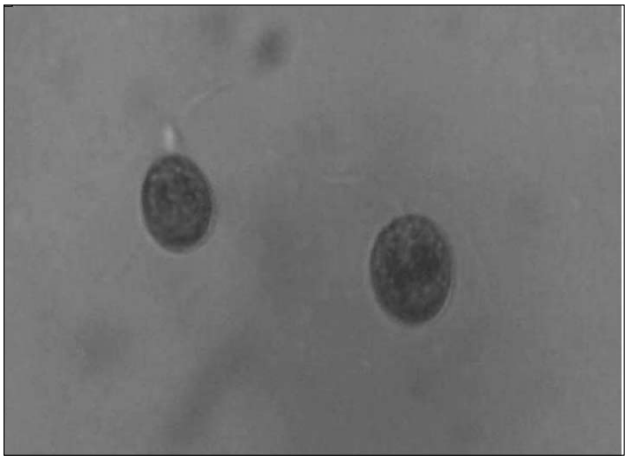
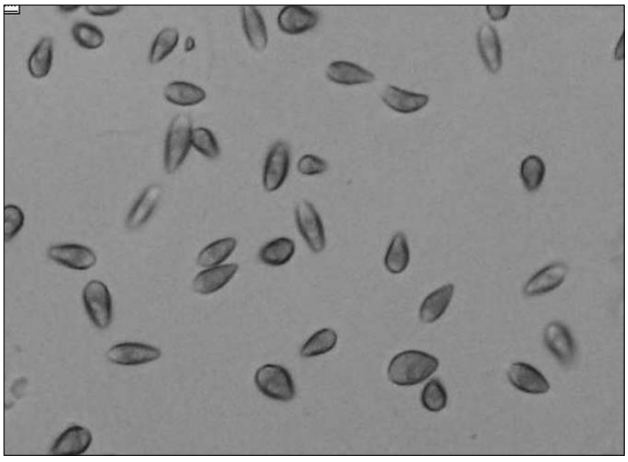
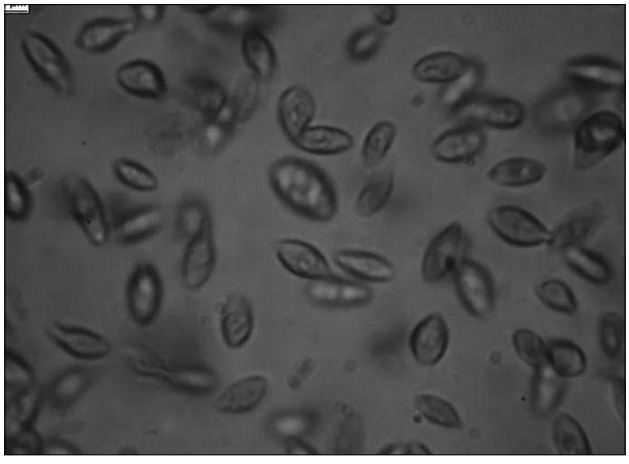


Fig.5(a) & 5(b) *D. salina* at exponential and stationary phases of growth (Under 400X magnification)



6(a)



6(a) & 6(b). *D. tertiolecta* at exponential and stationary phases of growth (Under 400X magnification)

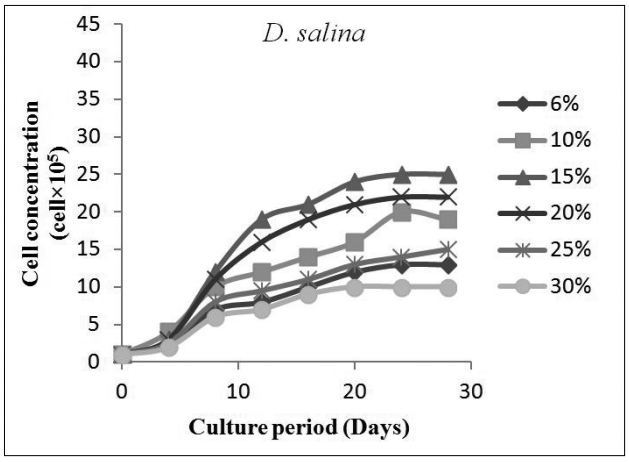


Fig.1(a). Effect of salinity on cell growth of *D. salina* (all data presented are the mean of three replicate)

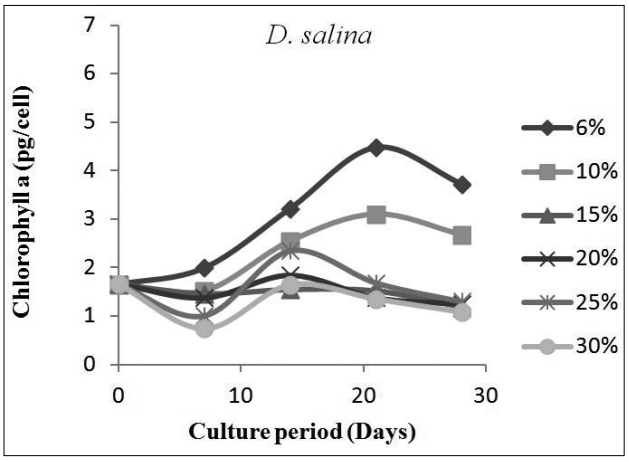


Fig.2(a). Effect of salinity on chlorophyll a production by *D. salina* (all data presented are the mean of three replicate)

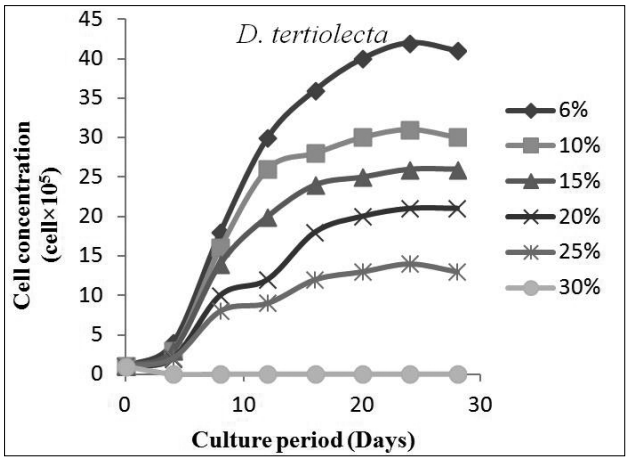


Fig.1(b). Effect of salinity on cell growth of *D. tertiolecta* (all data presented are the mean of three replicate)

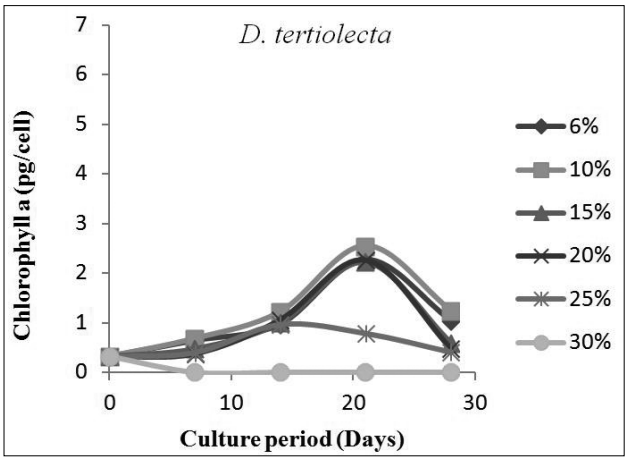


Fig.2(b). Effect of salinity on chlorophyll a production by *D. tertiolecta* (all data presented are the mean of three replicate)

Effect of NaCl concentration on the pigmentation

A range of salinity conditions (6-30% NaCl) were used to study the pigmentation in both the species of *Dunaliella*. An increase in the salinity regime up to 15% showed concentration dependent increase in the level of chlorophyll a content of *D. salina* and *D. tertiolecta*. The level of chlorophyll a was originally higher in *D. salina* (4.48 pg/cell) at 6% NaCl concentration(Fig. 2(a)) than that observed in the *D. tertiolecta* (2.55 pg/cell) at 10%(Fig. 2(b)). But higher range salinity (15-30% salinity) showed a faster decline in the chlorophyll a content of *D. tertiolecta* than the *D. salina*.

Similarly, the salinity stress has an effect on carotenogenesis. The *D. salina* and *D. tertiolecta* were tested for carotenogenesis in the range of 6% to 30% NaCl. In case of *D. salina*, the carotenogenesis showed concentration dependent increase as the salt concentration was increased. The highest cellular carotenoid level in case of *D. salina* was recorded at 25% NaCl concentration. On the other hand, an increase in the carotenoid level of *D. tertiolecta* was observed under lower salinity regime up to 10% NaCl concentration (Fig. 3(b)). At higher concentrations of NaCl (beyond 20% NaCl), there was sharp decline in the carotenoid content of *D. tertiolecta*. Results on the ratio of carotenoid/chlorophyll showed that carotenoids synthesis was several fold enhanced in *D. salina* with increasing salinity, whereas *D. tertiolecta* exhibited slightly higher synthesis of carotenoids than the chlorophyll, especially at lower range of salinity (Fig. 4).

Effect of NaCl concentration on the Protein and glycerol content

If we consider 6% salt concentration (w/v) as a control, the results showed that protein in control was 120.64±0.39 and

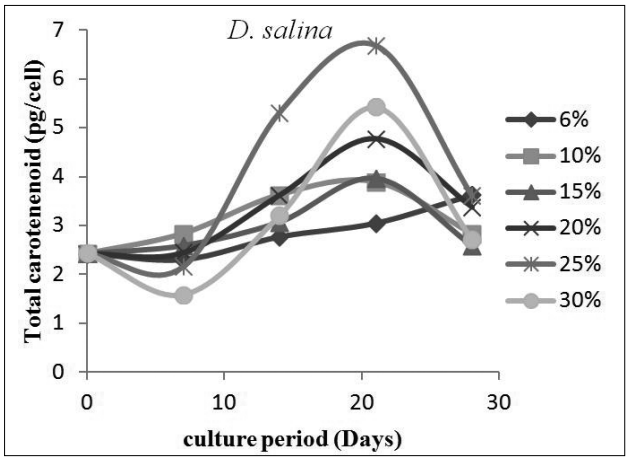


Fig.3(a). Effect of salinity on total carotenoid production by *D. salina* (all data presented are the mean of three replicate)

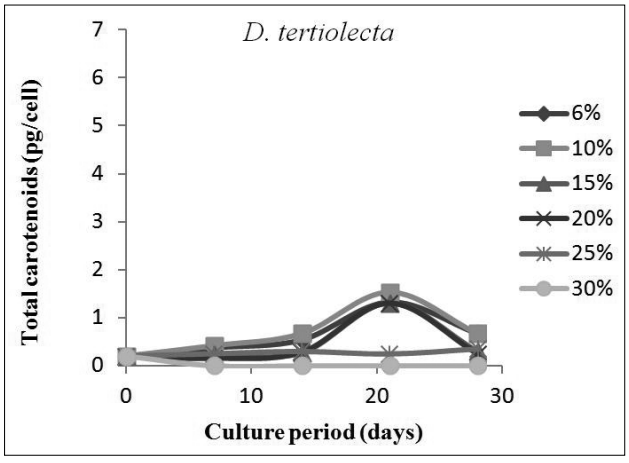


Fig.3(b). Effect of salinity on total carotenoid production by *D. tertiolecta* (all data presented are the mean of three replicate)

57.62±0.25 pg/cell for *D. salina* and *D. tertiolecta*, respectively (Table 1).In the case of *D. salina* maximum protein content was observed at 25% salt concentration, while in the case of *D. tertiolecta* it was the maximum at 20% salt concentration.

The level of glycerol was measured in the selected *Dunaliella* species. After the exposure to salt stress condition, a massive change in the glycerol concentrations was observed. The maximum level of glycerol was achieved at 30% salt concentration in case of *D. salina* (30.5±0.24 pg/cell), while

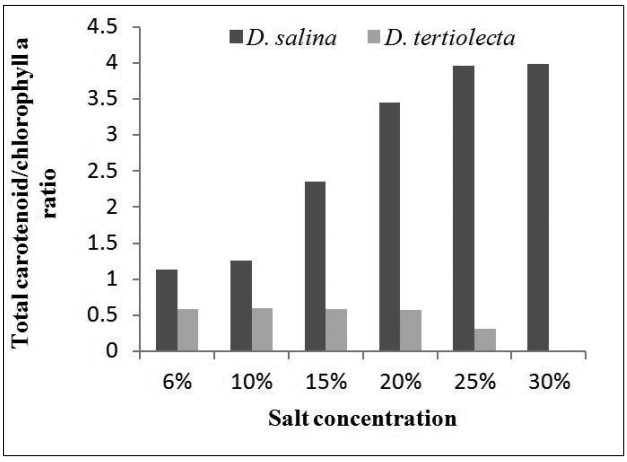


Fig.4 Total carotenoid to chlorophyll a ratio in 21 days old culture of *D. salina* and *D. tertiolecta* in BB medium containing different salt concentration.(all data presented are the mean of three replicate)

in the case of *D. tertiolecta* (19.36±0.13 pg/cell) it was at 25% NaCl concentration (Table 1). Low concentration of NaCl in growth medium especially for *D. salina* showed significant reduction in the glycerol content. It was observed that glycerol level in both the species of *Dunaliella* was salt induced.

Discussion

An Increase in the initial NaCl concentration from 6% to 25% significantly retards the growth of *D. tertiolecta* and concentration above 30% completely inhibited the growth. On the other hand, growth of *D. salina* showed concentration dependent increase in the growth up to 15% NaCl concentration, perhaps due to obligate halotolerant character of this species (Peeler *et al.*, 1989).

In the present study, it has been observed that there was relatively an enhanced synthesis of ß-carotenoid as compared to chlorophyll pigment in both the species. However, salinity induced carotenoid synthesis in *D. salina* is several fold higher than that in the *D. tertiolecta*. A higher rate of carotenoid synthesis in both the species of *Dunaliella* in response to salinity stress may be on account of protective role of carotenoid against the NaCl induced oxidative damage (Singh and Kshatriya, 2002). Earlier, it has been reported that carotenoids play an effective role in protecting the

Table-1: Effect of NaCl concentration on the Protein and glycerol level

Salt conc. (in percent w/v)	*D. salina* Protein level (pg/cell)	*D. tertiolecta* Protein level (pg/cell)	*D. salina* Glycerol level (pg/cell)	*D. tertiolecta* Glycerol level (pg/cell)
6%	120.64±0.39	57.62±0.25	4.39±0.24	1.05±0.08
10%	139.51±0.12	99.26±0.26	5.46±0.09	2.85±0.14
15%	145.89±0.16	113.89±0.38	7.18±0.23	5.97±0.10
20%	161.61±0.52	128.67±0.26	12.44±0.13	12.22±0.06
25%	172.42±0.26	121.8±0.14	26.70±0.17	19.36±0.13
30%	155.17±0.21	-	30.5±0.24	-

photosynthetic pigments against photoxidative damage (Gotz *et al.,* 1999). El Baz *et. al.* (2002) also reported that *Dunaliella* can accumulate large amount of carotenoids per cell reaching up to 14% of their dry weight when grown under stress conditions such as high intensity irradiation, high salt concentration and nutrient limitation. (Bar *et al.,* 1995).

Several workers have demonstrated that *Dunaliella* is capable of accumulating ß carotene and glycerol under conditions of high irradiance and other stress conditions (Arun and Singh 2012). Under the low salinity regime, Ben-Amotz *et. al.* (1985) have observed reduction in the level of protein, carbohydrates and pigment contents. Salinity stress leads to a series of changes in photosynthesis, photorespiration, osmotic adjustment and compartmentation, amino acid and carbohydrate synthesis (Ozturk *et al.,* 2002). In the present study it was observed that, in both the species of *Dunaliella* the level of glycerol in the cell was salt induced. The level of glycerol was more in case of *D. salina* than in *D. tertiolecta*; this was due to the higher salt tolerance in *D. salina* than in *D. tertiolecta*. (Pick, 2002) studied that *Dunaliella* responds to salt stress by massive accumulation of glycerol, enhanced elimination of Na^+ ions and accumulation of specific proteins. Protein levels were also high in both the species at high salinity condition. It seems that glycerol serves as an osmo-regulator of cell cytoplasm under the salinity stress (Hadi *et al.,* 2008). However, at low external salinities, the existing glycerol gets converted into starch in order to maintain the intracellular osmotic potential (Chitlaru and Pick, 1991).

In the present investigation, two isolated *Dunaliella* species, *D. salina* and *D. tertiolecta* exhibited differential response to salinity stress (6-30%). Understanding the physiological response of these species tounique environmental conditions may help in choosingthe right salt concentration as well as in selecting species to improve biological process efficiency. These data will help in understanding the right culture condition for the mass production of these species.

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