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Expression analysis of photosynthesis genes in *Dunaliella salina* grown at different NaCl concentrations

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ABSTRACT

The expression of five genes involved in photosynthesis was investigated under different salinity conditions in *Dunaliella salina*. These genes encodes ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCo) large and small subunits, major light-harvesting chlorophyll (chl) a/b protein of photosystem II, carbonic anhydrase and adenosine tri phosphate synthase alpha subunit. As *D. salina* grows in hypersaline conditions, it is important to study expression of these genes in relation to different salinity levels. Changes in the mRNA level under different NaCl conditions were determined using the comparative C_T method. Cell number and chl a content were estimated to determine optimal growth at different NaCl concentrations. Real-time PCR results showed enhanced expression of photosynthesis genes upon exposure of *D. salina* cells from Sambhar Lake to 1.7 M NaCl containing medium. This salinity corresponds to only one-third of the salinity of the Sambhar Lake habitat. In another salt condition, the expression level of all genes was found to be down-regulated at 1.0 M (sub-optimal concentration for growth of *D. salina*), 2.5 M and 3.0 M NaCl (supra-optimal concentration for growth) when compared with 1.7 M NaCl containing medium as a control. The present study will help in understanding the salinity tolerance in *D. salina* as a model microalga.

1. INTRODUCTION

External salinity is a major abiotic stress that affects growth and physiological processes in terrestrial and aquatic organisms [1]. Most plant species will not survive in high salt concentrations as growth is severely limited above 200 mM NaCl [2] and only the halo-tolerant organism are known to survive under such conditions. Various species of microalgae have been used as model organisms to study the effect of salinity on their different physiology processes [3, 4]. The halophytes show a number of physiological and biochemical reaction mechanisms like sequestration of salt ions in vacuoles and synthesis of organic osmolytes such as sugars, proline and glycerol in the cytoplasm to balance the osmotic pressure [5]. *Dunaliella salina* is a convenient model organism [6] to study photosynthesis associated proteins (i.e. RuBisCo, carbonic anhydrase *etc*.) under extreme environmental conditions because it has the capacity to tolerate a wide range of salinity [7, 8], light [9] and nutrient concentrations [10]. It is a unicellular, ovoid to spherically shaped, biflagellate, cell wall less, halotolerant (50 mM to 5.0 M NaCl) and high β-carotene producing microalga. There are several reports on growth requirements [11, 12], glycerol [13] and β-carotene production [14] by *D. salina* but work on photosynthesis under different extreme growth conditions is quite limited.

Five important photosynthesis related proteins are ribulose-1,5-bisphosphate carboxylase/oxygenase large (RbcL) and small (RbcS) subunit, major light-harvesting chlorophyll a/b protein of photosystem II (LHCIIb), carbonic anhydrase (CA) and ATP synthase alpha subunit (AtpA). RuBisCo has a pivotal role in carbon fixation by catalyzing the carboxylation of ribulose bisphosphate. The RuBisCo of oxygenic phototrophs generally consists of eight large subunits and eight small subunits. In land plants and green algae, the large subunits are encoded by *rbcL* in the chloroplast genome. The smaller subunits are produced by *rbcS* in the nuclear genome. Solar energy is collected by the major light-harvesting chlorophyll a/b protein of photosystem II (LHCIIb) which remain associated with light harvesting complex of photosystem II (LHC-II) [15]. The function of LHC-II is to transfer light energy to the reaction center of photosystem-II [16]. The carbonic anhydrase plays a key role in photosynthesis [17].

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In various eukaryotic algae and cyanobacteria, it indirectly enhances the supply of CO_2 from the outside of cells to RuBisCo under CO_2-limiting conditions [18]. Adenosine tri phosphate synthase is important in synthesizing ATP for energy in cells. It contains two regions- F_o and F_1. Both regions contain several subunits like alpha, beta, gamma *etc*. *Dunaliella* is adapted to grow under high salt medium. Decreasing the external salinity has been reported to inhibit photosynthesis initially [19] whereas in most other plants and algae, salt stress inhibits photosynthesis. Responses of *Dunaliella* upon exposure to high salinities include enhanced CO_2 assimilation during photosynthesis, glycerol synthesis (organic osmolyte), elimination of Na^+ ions and accumulation of specific proteins [20]. The complete mechanisms allowing the proliferation of *D. salina* in high and even saturating NaCl concentration is only partially understood. The aim of this study was to determine the expression of photosynthesis associated genes of *D. salina* at various salinity conditions. It is important to study the responses of photosynthesis associated genes at high salinities because such genes were not expressed in non-halotolerent species whereas expressed in *Dunaliella* at high salinity condition (this salinity could be lethal for non-halotolerent species and *Dunaliella* can easily survive in high salinity). The present study is very useful to compare *in vivo* and *in vitro* expression of photosynthesis associated genes. To find out optimum growth condition at different NaCl concentrations, cell number and chlorophyll-a content were also investigated.

2. MATERIAL AND METHODS

2.1 Algal strain and culture conditions

Dunaliella salina, isolated from Sambhar Lake (pH ~9.5), India (26°58'N 75°05'E), was cultured on medium (pH= 7.5) containing 1.7 M NaCl, 7.3 mM $MgCl_2.6H_2O$, 2.0 mM $MgSO_4.7H_2O$, 2.6 mM KCl, 2.3 mM $CaCl_2$, 4.9 mM KNO_3, 4.9 mM Tris Buffer, 8.5 µM EDTA di-Na salt, 0.58 µM $ZnCl_2$, 19.0 µM H_3BO_3, 0.12 µM $CoCl_2$, 0.46 µM $CuCl_2$, 6.3 µM $MnCl_2$, 9.2 µM $FeCl_3$ and 1.4 mM KH_2PO_4. It was grown in 250 mL Erlenmeyer flasks containing 100 mL medium at 26±1°C temperature and a 16:8 h light:dark cycle with photoperiods of 50 µmol $m^{-2}s^{-1}$ illumination provided by cool white fluorescent lamps (Philips, India). Bacterial contaminations were eliminated by adding the mixture of ampicillin, streptomycin, tetracycline (total 200 µg/mL; 2:1:1 ratio). Genomic DNA was isolated using TEN buffer (Tris 10 mM pH 8.0, EDTA 10 mM, NaCl 150 mM) method [21] to check other algal species.

2.2 Estimation of cell number and chlorophyll content

Pure culture of *Dunaliella* was inoculated at seven different concentrations of NaCl (0.5 M, 1.0 M, 1.7 M, 2.0 M, 2.5 M, 3.5 M, and 4.5 M). Samples were taken 11 times during the 20 days incubation period. Cell number was counted using a Neubauer haemocytometer. Chlorophyll-a was extracted from the algal pellet with 80% acetone (v/v) and assayed according to Lichtenthaler and Wellburn method [22].

2.3 Stress parameters for expression analysis

Two different salt conditions were chosen to study the expression of photosynthesis genes. First is hypo saline shock treatment. The *D. salina* taken from Sambhar Lake was transferred to the algal growth medium containing 1.7 M NaCl. This was a shift from high salinity to low salinity (hypo-saline shock) and under this condition mRNA samples were collected on the 1st, 3rd, 5th, and 7th day. The algal cultures were acclimatized and grown in medium containing 1.7 M NaCl since the standard cultures grew best in this concentration. The mRNA isolated at 1.7M NaCl on 8th day after inoculation was taken as control for comparative analysis of photosynthesis associated genes expression of *Dunaliella* cultures.

The other treatments were to grow algal cells taken from standard cultures to the medium containing 1.0 M, 1.7 M, 2.5 M, and 3.0 M NaCl, and mRNA samples were collected on the 8th day after inoculation. The medium containing 1.7 M NaCl was taken as a control. All the experiments were performed in triplicate.

2.4 Primer Construction

Sequences of photosynthesis-related genes i.e. *rbcL*, *rbcS*, *lhcIIb*, *ca*, and *atpA* were taken from National Center for Biotechnology Information (NCBI). Primers were constructed using NCBI primer designing tool (Table 1).

Table 1: Nucleotide sequences of primer pair used during real-time PCR.

Genes*	Primer Sequences	Amplicon Size
rbcL	Set 1: CCG GGC ACT GGT TCA AGG TCG GTG TGG TGC AGC GGT TGC TG	120 bp
	Set 2: AGC GTG CAT CGC ACG GTG AA AGT GTG GTG CAG CGG TTG CT	743 bp
rbcS	GTC GGC AAC GGC TGG ATC CC GGG GTC GGT GCA GCC AAA CA	159 bp
lhcIIb	ATC AAG AAC GGC CGC CTG GC CCG GGG GTG AAC TTG GTG GC	152 bp
ca	GCC ACC TAG CTG TGC TGG GC GGC AGC AGC ATG ACG GGG TT	143 bp
atpA	AGG TCG TGG TCA ACG TGA GCT TCA CGA CCT GGT GGA CGA CGT	375 bp

* = Gene sequence identifier in NCBI of photosynthesis associated gene- *Dunaliella salina* strain CCAP 19/18 chloroplast, complete genome; 246880718:168236-169663 (*rbcL*); *Dunaliella salina* ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit (rbcs) mRNA, complete cds; nuclear gene for chloroplast product; 53793875:99-671 (*rbcS*); *Dunaliella salina* major light-harvesting chl a/b protein 2.1 mRNA, complete cds; 90191868:36-797 (*lhcIIb*); *Dunaliella salina* carbonic anhydrase gene, complete cds; 29569134:696-756, 1189-1214, 1631-1669, 2030-2290, 2712-3015, 3488-3894, 4167-4369, 4513-4708 (*ca*); *Dunaliella salina* strain CCAP 19/18 chloroplast, complete genome; 246880718:83203-83694, 85401-86423 (*atpA*)

2.5 RNA Isolation and Real-time PCR

RNA was isolated from aliquots of ~10^6 cells using the RNA purification kit (Fisher Bioreagents). Quality of total RNA was checked spectrophotometrically (A_{260}/A_{280}) and by agarose gel electrophoresis. One-step real-time PCR (LightCycler 2.0, Roche, Germany) was performed using Verso SYBR Green 1-step QRT-PCR Kit (Thermo Fisher Scientific Inc). Real-time PCR reaction was performed in a total volume of 25 µl, containing 1.0 ng of

RNA and 1.75 nM of each primer. All reactions were performed in triplicate. The following thermal profile was used for reactions: 50°C for 15 min (cDNA synthesis), 95°C for 15 min (Thermo start activation), 40 cycles at 94°C for 15 s, 60°C for 30 s, and 72°C for 30 s. Specificity of the PCR reaction was confirmed by melt curve program.

Agarose gel electrophoresis of real-time PCR product was performed to check unique amplicons of the expected size. The comparative C_T method (also referred to as $2^{-\Delta\Delta C}{}_T$ method) [23] was used to analyze relative expression of genes. The 5′-TTG GGT AGT CGG GCT GGT C-3′ (sense) and 5′-CGC TGC GTT CTT CAT CGT T-3′ (antisense) primer for 18S rRNA were used as the internal control for normalization [16].

3. RESULTS

The water sample collected from Sambhar Lake was analysed under microscope and inoculated in Petri dishes (solid medium) for one day to check any bacterial contamination. Genomic DNA was isolated and resolved in 0.8% agarose gel and a single band of DNA indicated that this sample was unialgal (this unialgal culture was transferred to 1.7 M NaCl concentrated medium in hyposaline shock treatment). Daily observations under microscope were also continued.

3.1 Cell number and Chlorophyll-a content

Maximum cell number was observed at a NaCl concentration of 1.7 M *i.e.* 19.62 x10^5 cells/ml on the 8th day of incubation (Fig 1a). Log phase of culture was observed up to 8-12 days after which the cell number decreased. No growth of *Dunaliella* culture was observed at a NaCl concentration of 4.5 M. Highest chlorophyll-a (chl a) content (8.2 µg/ml on 8th day) was observed at 1.7 M NaCl (Fig 1b).

3.2 RuBisCo large and small subunits

The effect of hypo-saline shock (transfer from Sambhar Lake to 1.7 M NaCl medium) on the expression of the *rbcL* gene was observed as ~1700.8, 14.6, 2.6, and 2.3 fold decrease on 1st, 3rd, 5th, and 7th day, respectively (Fig 2a), whereas expression of *rbcS* decreased to ~ 60,000.0, 700.0, 73.0, and 3.3 fold on the 1st, 3rd, 5th, and 7th day, respectively (Fig 2b). This decrease was in comparison to gene expression in the standard cultures maintained on 1.7 M NaCl-containing medium. The effect of NaCl on the expression of *rbcL* was observed as ~11.6, 12.2, and 25.7 fold decrease on 1.0 M, 2.5 M, and 3.0 M NaCl, respectively (Fig 3a) whereas expression of *rbcS* showed ~1.3, 42.9, and 42.9 fold decrease on 1.0 M, 2.5 M, and 3.0 M NaCl, respectively (Fig 3b). This decrease was in comparison to gene expression in the standard cultures. The expected real-time PCR products of 120 bp (Fig 4a), 743 bp (Fig 4b), and 159 bp (Fig 4c) were obtained from *rbcL* and *rbcS* genes, respectively. Two sets of primers were used for *rbcL* gene which showed similar results (Fig 4a and 4b).

3.3 Major light-harvesting chlorophyll a/b protein of photosystem II

The expression of the *lhcIIb* gene in hypo-saline shock treatment was observed as ~14388.4, 184.1, 20.8, and 1.1 fold decrease on the 1st, 3rd, 5th, and 7th day, respectively (Fig 2c). The effect of NaCl was observed as ~272.9, 676.2, and 1477.7 fold decrease on 1.0 M, 2.5 M, and 3.0 M NaCl, respectively (Fig 3c). The expected real-time PCR product of 152 bp was obtained (Fig 4d).

3.4 Carbonic anhydrase

The effect of hypo-saline shock on expression of *ca* gene was observed as ~41.7, 11.8, 3.3 fold decrease on the 1st, 3rd, 5th day, respectively and 1.2 fold increase on 7th day (Fig 2d). The effect of NaCl was observed as ~1.6, 1.8, and 4.6 fold decrease on 1.0 M, 2.5 M, and 3.0 M NaCl, respectively (Fig 3d). A 143 bp expected real-time PCR product was obtained (Fig 4e).

3.5 ATP Synthase alpha subunit

The expression of *atpA* gene was observed as ~922.3, 15.1, 2.4, and 1.4 fold decrease on the 1st, 3rd, 5th, and 7th day, respectively (Fig 2e) due to hypo-saline shock. The effect of NaCl was observed as ~3.8, 406.3, and 714.9 fold decrease on 1.0 M, 2.5 M, and 3.0 M NaCl, respectively (Fig 3e). The expected real-time PCR product of 375 bp was obtained (Fig 4f).

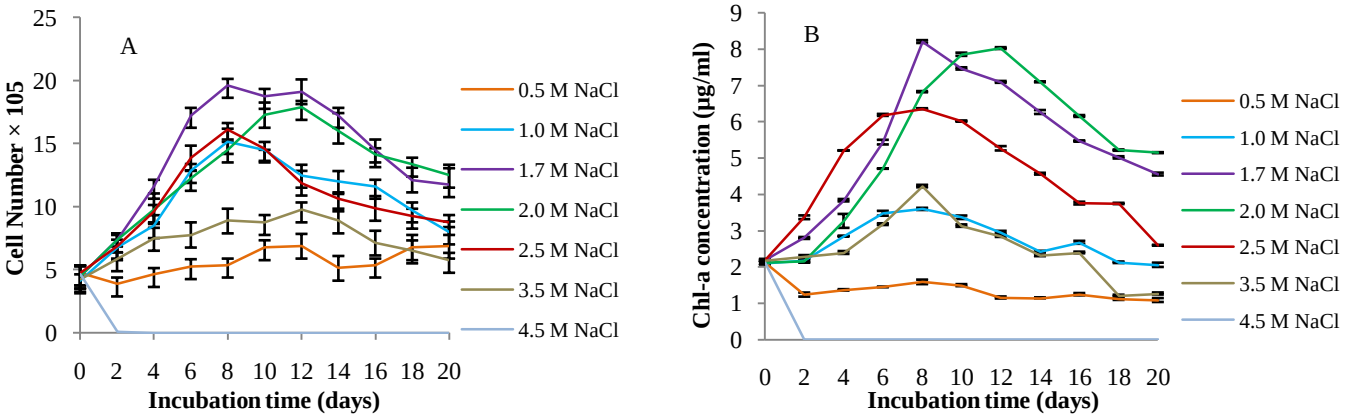

Fig. 1: Effect of NaCl on (a) Cell number, (b) Chl a content.

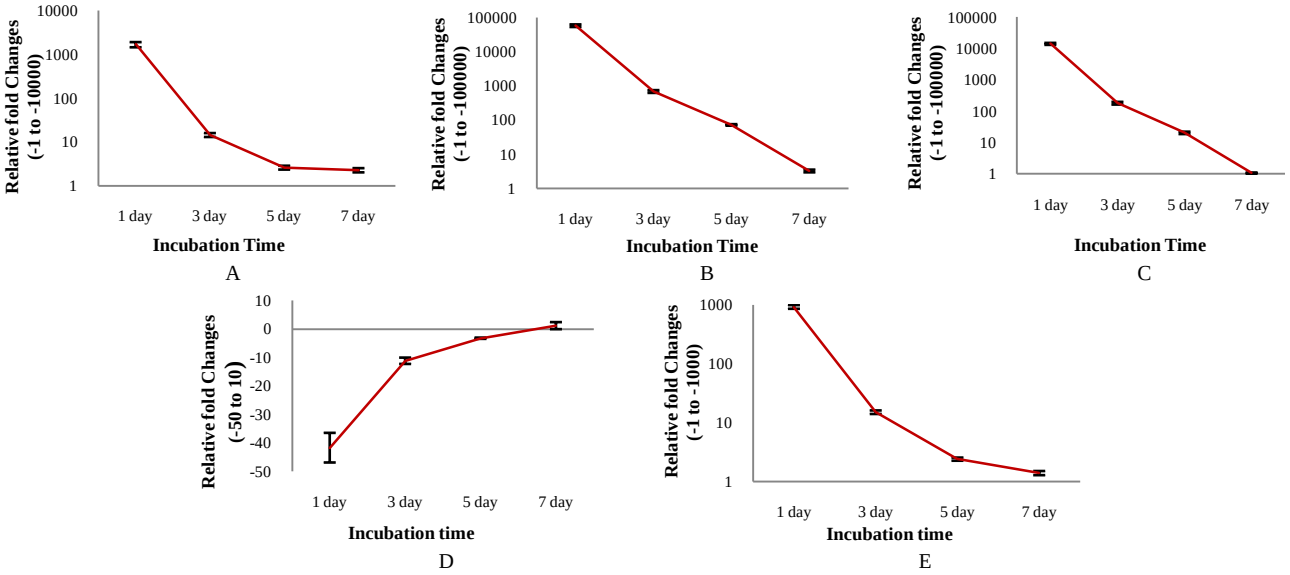

Fig. 2: The effect of hypo-saline shock treatment (from >5.0 M to 1.7 M NaCl) on relative expression of photosynthesis genes (a) *rbcL*, (b) *rbcS*, (c) *lhcIIb*, (d) *ca*, and (e) *atpA* [Y axis= Logarithmic scale (figure "d" is not in logarithmic scale)] on 1st, 3rd, 5th and 7th day of inoculation, 1.7 M NaCl on 8th day of inoculation used as a control.

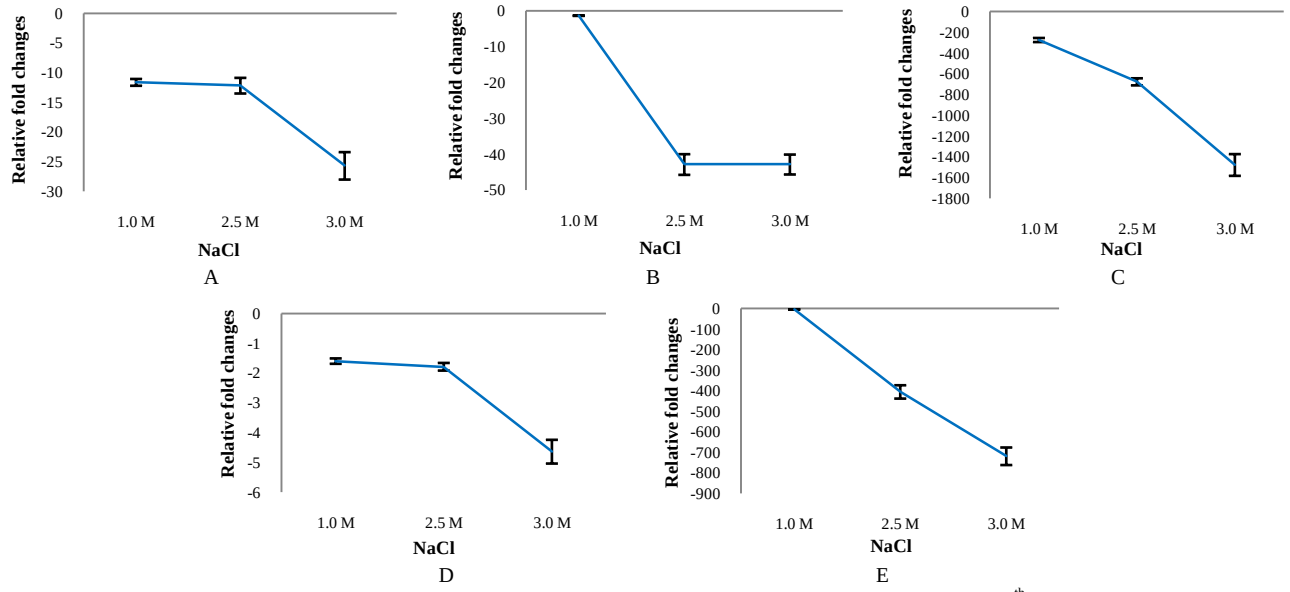

Fig. 3: The relative expression level of photosynthesis genes at different NaCl concentrations (culture were collected on 8th day after inoculation) (a) *rbcL*, (b) *rbcS*, (c) *lhcIIb*, (d) *ca*, and (e) *atpA*, 1.7 M NaCl on 8th day of inoculation used as a control.

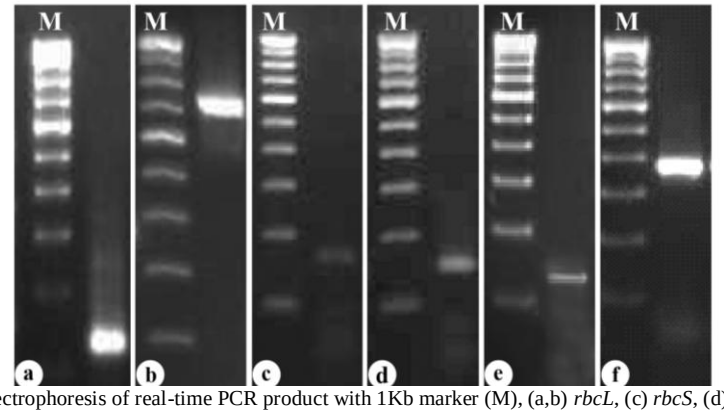

Fig. 4: The Agarose gel electrophoresis of real-time PCR product with 1Kb marker (M), (a,b) *rbcL*, (c) *rbcS*, (d) *lhcIIb*, (e) *ca* and (f) *atpA*.

4. DISCUSSION

The optimum salt concentration for growth and chl a synthesis in *Dunaliella salina* was found to be 1.7 M NaCl. In previous studies, optimum growth was observed at 1.0 M [8], 1.7 M [24] and 2.0 M NaCl [10]. These results indicate that *Dunaliella* has the ability to tolerate different salinity concentrations. It is a common observation that *Dunaliella* cultures taken from their natural habitat (high salinity) respond differently under laboratory growth condition as shown in the present study. Growth rate was reduced under salinity extremes [25] and similar result was observed in *D. tertiolecta* [1]. High growth rate and high chl-a content have been reported to be related with photosynthesis in the marine alga *Fucus vesiculosus* [26]. Ben-Amotz and Avron [27] found that the high salt concentration required for *in vivo* photosynthesis is not manifested by *in vitro* photosynthesis and other mechanisms.

Liska et al. [6] reported that 61 proteins are up-regulated in *Dunaliella* species at 3.0 M NaCl. In the present study, it was observed that the genes related to photosynthesis showed optimum response at high salinity (1.7 M NaCl). Salt concentration in Sambhar Lake varies from 7% (w/v) to more than 30% (w/v) as reported by Upasani and Desai [28]. Therefore, for the present study, the samples were collected from the nearly NaCl saturated Lake area. The mRNA extraction from *Dunaliella* sample (from Sambhar Lake) revealed that this sample was not suitable for expression analysis because of the low concentration of 18S rRNA gene at saturated NaCl concentration. The low concentration of 18S rRNA was not similar with concentration of 18S rRNA gene of standard. Therefore, in the hypo-saline shock experiment, mRNA was isolated from 1st day of inoculation (18S rRNA was used as the internal control for normalization, so concentration of 18S rRNA in test and standard sample should be similar).

There is a long history of relationship between salinity and photosynthesis in algae and plants. In salt-susceptible plants like potato (*Solanum* sp.) [29], pea (*Pisum sativum*) [30], and curcas bean (*Jatropha curcas*) [31], Chl content is reported to decrease with increasing salt concentrations. Chl content increases in salt tolerant plants like mustard (*Brassica nigra*) [32]. Carotenoid content is reported to increase under salt stress in rice plants (*Oryza sativa*) [33] and halo-tolerant algae like *Dunaliella* sps. Chl a content remained unaffected in *Spirulina platensis* grown under 0.8 M NaCl [34]. In the present study, Chl a content in *D. salina* increased at 0.5-1.7 M NaCl concentrations and decreased in salt extremes. In the blue green alga *Scytonema javanicum*, physiological and biochemical responses were studied under salt stress by Tang *et al.* [35] who found that 50 mM NaCl inhibited the growth of the alga (100 mM NaCl concentrations, proved lethal) and there was a decline in photosynthesis efficiency. In *Synechocystis* sp. PCC 6803, carotenoids content increased at high salinity (1026 mM NaCl) [36]. Johnson *et al.* [37] found that various enzymes in *Dunaliella viridis* were inhibited at high salt level, the response of the whole cell was different with salts and showed optimal growth between 0.8- 2.0 M NaCl.

Salt stress causes decrease in growth and productivity of plants by disrupting various physiological processes such as membrane instability, increased ion toxicity, and decreased efficiency of photosynthesis. The effect of salinity on photosynthetic activity is primarily exerted as osmotic stress modifying the permeability of the chloroplast envelope and affecting the activity of stroma enzymes or proteins like RuBisCo, thus resulting in the decrease of overall CO_2 fixation capacity [38]. In the present study, RuBisCo (subunit) gene expression was negligible on the 1st day and very low until the 5th day of inoculation with hypo saline shock treatment but thereafter increased rapidly. The expression of RuBisCo in *D. salina* at saturating level of NaCl (*in vivo* condition) was very low and expression increased at optimum salt concentration. The gene expression of RuBisCo (subunit) at 1.0 M NaCl was higher compared to gene expression at 2.5 M and 3.0 M NaCl. In the present study it was observed that *rbcL* gene expression was higher than *rbcS* gene expression with hypo saline shock treatment. A possible explanation of difference in *rbcL* and *rbcS* gene expression is that the RuBisCo small subunit protein is synthesized as precursors in the cytoplasm and then it is processed during transport into the chloroplast [39]. The effect of high salinities on RuBisCo content in the halotolerant cyanobacterium, *Aphanothece halophytica* was studied by Takabe *et al.* [40] and it was found that RuBisCO levels increased with increasing salinity. Park *et al.* [41] found that expression of RuBisCo enzyme increased with increasing salinity in *D. tertiolecta* but not in *D. salina*.

Several studies demonstrated that thylakoid membrane proteins are affected by salt stress. Photosystem-I (PSI), photosystem-II (PSII) and ATP synthase complex are involved in the electron transport process in the thylakoid membrane. Salt stress interrupts the energy transfer in PSII reaction centre. Photosystem II is damaged by both osmotic and ionic effects at high external salinity [42, 43]. In the present study, the expression of the *lhcIIb* gene at 1.0 M NaCl was higher than it was at 2.5 M and 3.0 M NaCl. The expression of the *lhcIIb* gene was very low untill the 5th day but it increased rapidly on the 7th day with hypo-saline shock treatment. Chl a and Chl b molecules are present in association with PSII, so *lhcIIb* is related with photosynthesis as well as growth. The PSII inhibition under salt stress was characterized by Misra *et al.* [44]. Allakhverdiev *et al.* [43] showed that salt stress changes $[K^+]/[Na^+]$ ratio which inactivated both PSI and PSII in *Synechococcus* cells. The salt stress damaged the PSII electron transport chain in *Spirulina platensis* [45]. According to Satoh *et al.* [46], the high salinity reduced the amount of light energy that reached the reaction centers of PSII in the intertidal red alga *Porphyra perforata*. Kim *et al.* [47] showed that under high or low salinity conditions *lhcIIb* and *atpA* genes were down- and up-regulated, respectively. In the present study, these genes were down-regulated at 1.0 M, 2.5 M, and 3.0 M NaCl-concentrating medium. Carbonic anhydrase and ATP synthase alpha subunit gene expression in hypo-saline conditions was low from the 1st to the 5th day and then it increased rapidly on

the 7th day of inoculation. Both the genes were down-regulated at high (3.0 M) NaCl concentrations. Increased salinities and alkaline conditions induced the activity of external carbonic anhydrase in *Dunaliella*. Fisher *et al.* [48] showed maximal carbonic anhydrase activity at 1.0 M NaCl and retained considerable activity at higher salt concentrations. It provides CO_2 to RuBisCo enzyme under high salt stress conditions. The present study also showed that the expression of carbonic anhydrase gene was higher than the expression of other photosynthesis-associated genes of *Dunaliella* under high salinity conditions.

5. CONCLUSIONS

In conclusion, the optimum salt concentration for growth of *D. salina* was 1.7 M NaCl. The expression of photosynthesis-associated genes was affected by different NaCl concentrations. These genes were low expressed in low (1.0 M) or high concentration (more than 2.5 M) of NaCl containing medium and optimum expressed in 1.7 M NaCl concentrated medium. The hypo-saline shock showed that Sambhar Lake is suitable for carotene production thus photosynthesis-associated genes were negligible expressed. This study is very beneficial to understand the interplay between abiotic factors and photosynthesis. It provides a good platform to understand the expression of photosynthesis genes in natural habitat. This study will help in understanding the salinity tolerance and carbon concentration mechanism in *D. salina*.

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