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ORIGINAL PAPER

Physiological characterization and stress-induced metabolic responses of *Dunaliella salina* isolated from salt pan

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Abstract A *Dunaliella* strain was isolated from salt crystals obtained from experimental salt farm of the institute (latitude 21.46 N, longitude 72.11°E). The comparative homology study of amplified molecular signature 18S rRNA, proves the isolated strain as *D. salina*. The growth pattern and metabolic responses such as proline, glycine betaine, glycerol, total protein and total sugar content to different salinity (from 0.5 to 5.5 M NaCl) were studied. The optimum growth was observed at 1.0 M NaCl and thereafter it started to decline. Maximum growth was obtained on 17th day of inoculation in all salt concentrations except 0.5 M NaCl, whereas maximum growth was observed on 13th day. There were no significant differences ($P < 0.01$) in chlorophyll *a*/*b* contents (1.0–1.16 ± 0.05 μg chl. *a* and 0.2–0.29 ± 0.01 μg chl. *b* per 10^6 cells) up to 2.0 M NaCl, however at 3.0 M NaCl a significant increase (2.5 ± 0.12 μg chl. *a* and 0.84 ± 0.4 μg chl. *b* per 10^6 cells) was observed which declined again at 5.5 M NaCl concentration (2.0 ± 0.1 μg chl. *a* and 0.52 ± 0.03 μg chl. *b* per 10^6 cells). Stress metabolites such as proline, glycine betaine, glycerol and total sugar content increased concomitantly with salt concentration. Maximum increase in proline (1.4 ± 0.07 μg), glycine betaine (5.7 ± 0.28 μg), glycerol (3.7 ± 0.18 ml) and total sugar (250 ± 12.5 μg) per 10^5 cells was observed in 5.5 M NaCl. A decrease in total protein with reference to 0.5 M NaCl was observed up to 3.0 M NaCl, however, a significant increase ($P < 0.01$) was observed at 5.5 M NaCl (0.19 ± 0.01 μg per 10^5 cells). Inductive coupled plasma (ICP) analysis shows that intracellular Na^+ remained unchanged up to 2.0 M NaCl concentration and thereafter a significant increase was observed. No relevant increase in the intracellular level of K^+ and Mg^{++} was observed with increasing salt concentration. Evaluation of physiological and metabolic attributes of *Dunaliella salina* can be used to explore its biotechnological and industrial potential.

Keywords *Dunaliella* · Proline · Glycine betaine · Glycerol · Metabolites · Salinity

Introduction

Life exists over wide range of salt concentrations encountered in natural habitats: from freshwater to hypersaline environment saturated with respect to sodium chloride. The diversity in the characteristics of saline and hyper-saline habitats on Earth is reflected by great diversity within the microbial communities adapted to life under extreme conditions. Halophiles are salt-loving organisms that inhabit hyper-saline environment. They include prokaryotic and eukaryotic microorganisms with the capacity to balance osmotic pressure of the environment and resist the denaturing effects of salt.

Dunaliella is recognized as the only eukaryotic and photosynthetic organism, which grows in wide range of salt concentration, ranging from 0.05 M to saturation (5.0 M) [20]. This biflagellate unicellular alga is responsible for most of the primary production in hyper-saline environment

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worldwide [46]. Michael Felix Dunal was the first person to report *Dunaliella* occurring in salt evaporation pond in southern France in 1838. *Dunaliella* is named as such in respect to Dunal by Teodoresco in 1905. The cell of *Dunaliella* is enclosed in a thin elastic plasma membrane surrounded by mucus "surface coat" [6] with cell volume of 50–100 μm^{-3}. It has typical cellular organelles like other green alga but lacks rigid polysaccharide cell wall. Lack of cell wall makes *Dunaliella* a model organism to study the transporters [11, 40, 51] and understand the biochemical process involved in cell osmoregulation [9].

The ability of cells to survive and flourish in saline environment under the influence of osmotic stress has received considerable attention. Cells develop many adaptive strategies in response to different abiotic stresses such as high salinity, dehydration, cold, heat and excessive osmotic pressure. Against these stresses, cells adapt themselves by undergoing different mechanisms including change in morphological and developmental pattern as well as physiological and bio-chemical processes [7]. Stress adaptation is associated with metabolic adjustments that lead to the accumulation of several organic solutes and osmolytes. The extracellular osmotic pressure is combated in *Dunaliella* by production of intracellular glycerol [4]. Glycerol is metabolized by two inter-related but distinct reaction sequences and intracellular concentration of glycerol is proportional to the extracellular NaCl concentration [35]. Intracellular glycerol accumulation has been studied in response to temperature, metal toxicity, nutrient depletion and salinity (ranging 0.5–3.0 M NaCl) [9, 16, 49, 50, 60].

Salinity stress leads to a series of changes in basic biosynthetic functions, including photosynthesis, photorespiration and amino acid synthesis [37, 48, 55]. Compatible osmolytes such as proline, glycine betaine, sugars, polyols and amino acids are synthesized in response to salt stress. In higher plants, these are well studied [30, 32] however, in *Dunaliella*, proline and glycine betaine response to salinity have not been reported yet, while other metabolites, such as glycerol, was studied only up to 3.0 M NaCl stress [16, 50]. One of the main biological constraints on algal biotechnology is limited availability of a wide variety of algal species or strains which respond favorably to varying saline conditions. The present work was aimed to isolate and characterize microalgae *Dunaliella*, surviving on salt crystals obtained from experimental salt farm and to study its metabolic responses in varying salt concentration. *Dunaliella* isolated from salt crystals may serve as an ideal model for further study of hyper salt responsive gene(s) as well as physiological and metabolic attributes of *Dunaliella* will play an important role in its biotechnological and industrial potential.

Material and methods

Salt crystals were collected from the salt farm of Central Salt and Marine Chemicals Research Institute (CSMCRI), Bhavnagar (latitude 21.46 N, longitude 72.11°E), Gujarat, India.

Unialgal culture and growth pattern

Some salt crystals, containing *Dunaliella* were dissolved in 250 ml of De Walne's media (5 μM $FeCl_3 \cdot 6H_2O$, 1.82 μM $MnCl_2 \cdot 4H_2O$, 7.28nM $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.54 mM H_3BO_3, 0.12 mM Na_2EDTA, 0.13 mM $NaH_2PO_4 \cdot 2H_2O$, 1.18 mM $NaNO_3$, 0.10 μM $ZnCl_2 \cdot 4H_2O$, 0.08 μM $CoCl_2 \cdot 6H_2O$, 0.08 μM $CuSO_4 \cdot 5H_2O$; pH-8.0) [47] and incubated for 15 days. Culture was microscopically analyzed to find out the presence of *Dunaliella* as per available morphological description [8, 46]. The alga was isolated and axenized [28]. The contaminant removal procedure started with a series of centrifuge washes. After washing, cell pellet was inoculated into fresh enriched media to obtain algal culture of *Dunaliella.* After 1 week of growth, the culture was again observed under microscope and a loopful of this culture was streaked onto solid agar medium (De Walne's media with 1% agar), aseptically to get single isolated colonies. Selection of the clone was done and unialgal culture of *Dunaliella* was prepared [56]. The step of streaking and selection of single isolated colony was repeated five times. Bacterial contamination was removed by adding streptomycin, ampicillin, kanamycin and tetracycline antibiotics (50 μg/ml) in culture media during screening, as *Dunaliella* has shown high degree of resistance to these antibiotics [21]. The purity of algal cultures from bacterial contamination was verified first by spreading culture on TYG agar and sub-culturing in TYG broth (0.5% tryptone [Difco], 0.25% yeast extract [Difco] and 0.1% glucose) and incubating for several weeks at 26–37 °C [19, 61]. The final unialgal culture was again carefully examined under microscope and for the final proof of purity, 16S rRNA gene amplification was performed. Genomic DNA of algal culture was isolated using SDS method [12] and 16S rRNA gene(s) were amplified by means of universal primers (fD1-5′-AGA GTT TGA TCC TGG CTC AG-3′ and rP2-5′-ACG GCT ACC TTG TTA CGA CTT-3′) [62] in 25 μl reaction mix containing 1 μg template DNA, 1.0 unit of *Taq* DNA polymerase, 1 × assay buffer, 200 μM of each dNTPs and 2 μM of each primer to cross-check bacterial contamination. PCR was carried out in thermal cycler (Bio-rad, USA) with initial denaturation temperature of 95 °C for 5 min, subsequent 35 cycles of 95 °C denaturation for 2 min, 42 °C annealing for 30 s, 4 min extension at 72 °C and a final extension step at 72 °C for 15 min [62]. PCR amplifications were analyzed

by 1% agarose gel electrophoresis. The axenized algal culture was established under controlled laboratory conditions at 25 ± 2 °C under 12:12 h (light/dark cycle) with white fluorescent lamp of 38μmol photons m^{-2} s^{-1} light intensity.

Ten milliliter (5×10^6 cells) unialgal culture was inoculated in triplicate into 250 ml of De Walne's culture media containing different salt concentrations; 0.5, 1.0, 2.0, 3.0 and 5.5 M (super saturated containing salt crystals) and were grown under controlled laboratory conditions to study the growth pattern. Growth was measured in terms of cell numbers using Neubaur haemocytometer.

Chlorophyll content

Ten milliliter culture (17 days old) was centrifuged for 5 min at 3,000 rpm, supernatant was aspirated out and a pinch of $MgCO_3$ was added followed by the addition of 1 ml 90% acetone and cells were homogenized. Completely homogenized cells were subjected to quick centrifugation and supernatant was transferred to fresh tube. The centrifugation and transfer of the supernatant was continuously done until clear solution is obtained. The absorbance of supernatant was taken at 664 and 647 nm in UV–Vis spectrophotometer (Varian). Chlorophyll is light sensitive pigment hence light was avoided and all steps were done in dark. Chlorophyll content was calculated using following formula [33]:-

$$\text{chl.}a\,(\mu g/ml) = 11.93A_{664} - 1.93A_{647}$$

$$\text{chl.}b\,(\mu g/ml) = 20.36A_{647} - 5.50A_{664}$$

$$\mu\text{mol of chl. in extract} = \frac{\mu\text{ g chl. in extract}}{\text{mol. wt. of chl.}}$$

$$\text{Molecular wt. of chl.}a = 894$$

$$\text{chl.}\,b = 908$$

$$\mu\text{mol of chl. per cell} = \frac{\mu\text{M of chl.in extract}}{\text{No. of cells in sample}}$$

Molecular identification

Genomic DNA of *Dunaliella* was isolated using SDS method [12]. A master mix (25 μl) was prepared, containing 50 ng of template DNA, 0.4 μM of each primer (MA1-5′-CGG GAT CCG TAG TCA TAT GCT TGT CTC-3′ and MA2-5′-CGG AAT TCC TTC TGC AGG TTC ACC-3′) [45], 200 μM of each dNTPs, 1 × assay buffer and 1.5 unit of *Taq* DNA polymerase, in a 0.2 ml of sterile, thin walled PCR tubes (Axygen) to amplify 18S rRNA gene. PCR was carried out in thermal cycler (Bio-Rad, USA) with initial denaturation temperature of 95 °C for 5 min, subsequent 40 cycles of 95 °C denaturation for 1 min, 55 °C annealing for 1 min and 2 min extension at 72 °C. Final extension was done at 72 °C for 5 min. PCR amplification was analyzed by 1% agarose gel electrophoresis. Amplified 18S rRNA was purified using MinElute PCR Purification kit (Qiagen, Germany), according to manufacturer's instructions and cloned in pGEM *T*-easy vector (Promega, USA). Positive clone was sequenced using T-7 and SP-6 primers and sequence was analyzed using bioinformatics tools and submitted to NCBI (http://www.ncbi.nlm.nih.gov) gene data bank.

Salt stress metabolites

In this study, proline, glycerol, total protein content, total sugar content and uptake/accumulation of ions in the presence of different salinity were studied using 17th day old (log phase) cultures.

Proline content

Proline content was measured as described by Bates et al. [5]. A measure of 20 ml culture was centrifuged at 5,000 rpm for 5 min and pellet was homogenized in 5 ml of 3% aqueous sulfosalicylic acid. Homogenized cells were centrifuged for 10 min at 15,000 rpm and 2 ml upper aqueous solution was transferred in a fresh test tube. Ninhydrin (2 ml) and 2 ml of glacial acetic acid was added and reaction mixture was incubated at 100 °C for 1 h. Reaction was stopped by placing the reaction mixture on ice and proline was extracted with 4 ml toluene. The chromophore containing toluene was warmed to room temperature and its optical density was measured at 520 nm.

Glycerol content

Twenty milliliter culture was centrifuged at 5,000 rpm for 5 min and pellet was homogenized in 500 μl alcoholic KOH (4%). Homogenized cells were kept in water bath at 60 °C for 30 min. Content was allowed to cool at room temperature and total glycerol was extracted with 700 μl chloroform and 250 μl glacial acetic acid. Lambert and Neish [39] method was used for the quantitation of total glycerol content in *Dunaliella*, grown in different molar concentrations of salt.

Glycine betaine content

The amount of Glycine betaine was estimated following the method of Grieve and Grattan [27]. A measure of 20 ml culture was centrifuged at 5,000 rpm for 5 min and pellet was homogenized in 200 μl of deionized water. Homogenized samples were placed in ice bath and equal volume of 2 N H_2SO_4 was added to the content and left for 2 h. Equal volume of cold KI–I_2 reagent (1.75 g I_2 and 2 g KI in 10 ml deionized water) was added and content was thoroughly

mixed. Test tubes were kept over-night at 4 °C. Next day, contents were centrifuged and supernatant was carefully removed, betaine periodic complexes were formed and seen on side or bottom of the tubes. Betaine residue complex was resuspended in 1–2 dichloro-ethane. Contents were kept for 2 h in the dark after that absorbance was recorded at 365 nm. A standard curve was made simultaneously using known amount of glycine as standard following the same method. Reading of samples was compared to the standard to find out total glycine betaine per 10^5 cells.

Total sugar content

Total sugar content was estimated by the procedure of Dubois et al. [13]. Ten milliliter culture was centrifuged at 5,000 rpm for 5 min and pellet was resuspended in 2 ml distilled water. A measure of 1 ml phenol solution (5%) was added and test tube was placed in ice bath, followed by the addition of 5 ml sulphuric acid and test tube was left for some time for cooling of the content. Test tube was kept at room temperature for 30 min. Upper color phase was taken out for the absorbance reading at 485 nm. Reading was compared to find out total sugar content of samples with a standard curve, which was drawn by same method, using known amount of glucose as standard.

Total protein content

Ten milliliter culture was centrifuged as above and pellet was homogenized vigorously in 1.5 ml distilled water in ice bath. A measure of 1 ml aqueous phase was taken for the quantitation of total protein by Lowery method [42] using Folin–phenol reagent and BSA as standard.

Ion content determination by ICP

Twenty ml culture was centrifuged at 5,000 rpm for 5 min and after extensive washing, pellet was transferred to porcelain crucible for the digestion in HNO_3:HCl (3:1) at 150 °C for 4 h. Digested samples were kept at 450 °C for ashing. Dry ash was dissolved in 5% HNO_3 and filtered through 0.45 μm filter. The filtrate was used for the detection of Na^+, K^+ and Mg^{++} by inductive coupled plasma (ICP).

Statistical analysis

Analysis of variance (ANOVA) was used to analyze data and determine differences [57]. Data were expressed as means ± SE. A Tukey HSD multiple comparisons of means test was used when significant differences were found and $P < 0.01$ was considered as significant.

Results and discussion

Dunaliella strain, isolated from salt crystal was biflagellated, devoid of cell wall and as per available morphological descriptions [8, 46]. The alga was axenized [28] and bacterial contamination was eliminated by using antibiotics [21]. The purity of algal culture from bacterial contamination was verified first by the absence of bacterial growth on TYG agar and in TYG broth [19, 61] at 26–37 °C (both in light and dark) incubation for the period of 30 days. The unialgal culture was carefully examined under microscope and finally, purity of culture was again verified by non-amplification of 16S rRNA [62]. *Dunaliella* strain was subjected to different salt concentration and all parameters were studied on 17th day (log phase) of inoculation.

Growth curve

An exponential growth pattern was observed in each salt concentration (Fig. 1). *Dunaliella*, grown in De Walne's media with 0.5 M NaCl, gave maximum number of cells on 13th day of inoculation but at higher salt concentration (1.0–5.5 M NaCl) maximum growth was observed on 17th day. Maximum number of cells and optimum growth was observed in 1.0 M NaCl concentration. A reduced growth was observed in 5.5 M NaCl containing medium. Cell number was increased on increasing NaCl concentration from 0.5 to 1.0 M, which may be due to halophilic character of *Dunaliella* there-after cell number gets decreased on further increase in salt concentration. Takagi et al. [59] also observed same result as an increase in *Dunaliella* concentration up to 1.0 M salt thereafter growth was inhibited by salt concentration. Optimum growth rate of *Dunaliella salina* was observed at ionic strength of 2.0 M NaCl by Muñoz et al. [44] and García et al. [20]. Different growth patterns, exhibited by

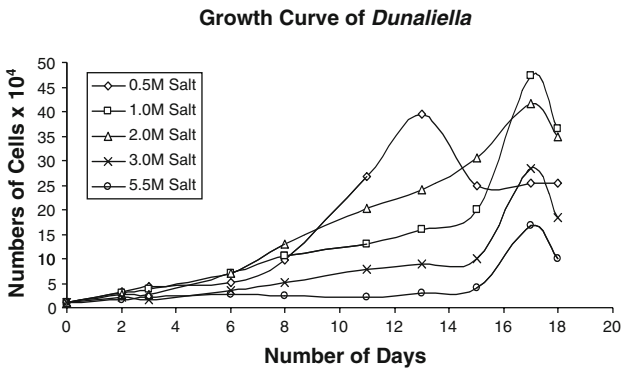

Fig. 1 Growth kinetics of *Dunaliella salina* grown in De Walne's media with different salt concentrations

geographically distinct strains, confirm the hypothesis that these algae do not adapt to a specific saline condition, but can tolerate a wide range of salinities [20]. Growth of isolated *Dunaliella* was observed in the range of 0.5–5.5 M NaCl concentration.

Chlorophyll content

Dunaliella cells contain almost same chlorophyll *a/b* content (1.0 to 1.16 ± 0.05 μg chl. *a* and 0.2 to 0.29 ± 0.01 μg chl. *b* per 10^6 cells) grown in 0.5–2.0 M salt concentration. A significant increase ($P < 0.01$) (2.5 ± 0.12 μg chl. *a* and 0.84 ± 0.4 μg chl. *b* per 10^6 cells) was observed in 3.0 M NaCl which declined again at 5.5 M NaCl concentration (2.0 ± 0.1 μg chl. *a* and 0.52 ± 0.03 μg chl. *b* per 10^6 cells) (Fig. 2a). In previous studies, maximum chlorophyll content was observed at 1.0 M salinity in *D. salina* and thereafter it decreased with increasing salt concentration [10] while chlorophyll a was observed significantly higher at 3.0 M NaCl in *D. tertiolecta* [10, 18]. In higher plants, it has been observed that salt treatment (0.3 M NaCl) stimulates chlorophyll production per unit leaf area, such as in tomato [1]. *Dunaliella* responds to high salinity by enhancing photosynthetic CO_2 assimilation, by diversion of carbon and energy resources for the synthesis of glycerol and the osmotic element [41]. In this study, significant increase in chlorophyll content at 3.0 M NaCl was observed which is remarkable because, in cyanobacteria and most plants, salt stress inhibits chlorophyll production.

Molecular identification

Identification of *Dunaliella* from natural samples has been made easy by using modern research methodology [26, 45, 63] and primers MA1 and MA2 allowed the amplification of the full length of 18S rDNAs in *Dunaliella* species [53]. The primer pair was designed to identify *D. salina* as it strictly amplifies the chromosomal DNA of *D. salina*. The amplified 1.8 kb PCR product of *Dunaliella* (Fig. 3), used in this study was similar in size to the previously amplified PCR product of *D. salina* [45, 53], and it confirms that the isolated microalga is *D. salina*. The amplified 18S rRNA of size 1,800 bp was cloned in pGEM-*T* easy vector (Promega, USA), sequenced by T7 and SP6 universal primers and submitted to NCBI gene bank (Gene bank accession no. EF195157). Sequences were subjected to NCBI blast for identification and approximately 99% sequence homology was observed with existing *D. salina* 18S rRNA nucleotide sequences (AF506698), further confirming that the isolated microalga is in fact, *D. salina*.

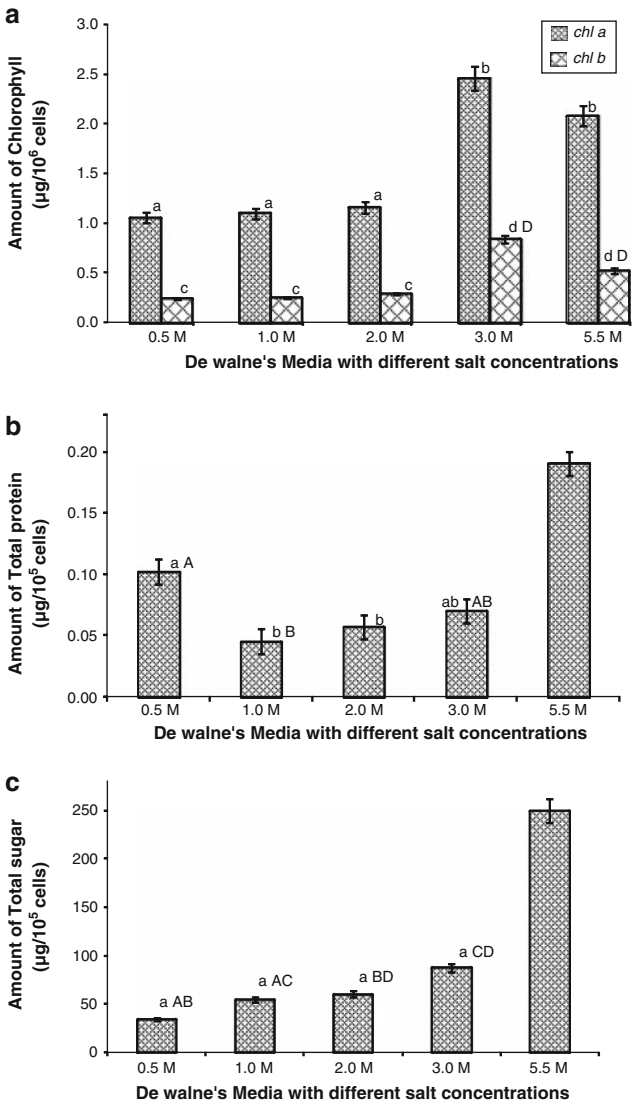

Fig. 2 Biochemical composition of *Dunaliella salina* grown over a salinity gradient. **a** Chlorophyll content, **b** total protein contents and **c** total sugar contents. *Error bars* indicate ±SE and similar *lower case letters* indicate no significant difference ($P < 0.01$), while similar *capital letter* indicate significant differences at only $P < 0.05$

Salt stress metabolites

Cells resort to many adaptive strategies in response to different abiotic stresses such as high salinity, dehydration, cold, heat and excessive osmotic pressure which ultimately affects growth and productivity [17, 65]. Against these stresses, cells adapt themselves by different mechanisms including change in morphological and developmental pattern as well as physiological and bio-chemical processes [7]. Adaptation to stress is associated with metabolic adjustments which lead to the accumulation of several organic solutes and osmolytes. These osmotic adjustments protect sub-cellular structures and reduce oxidative damage

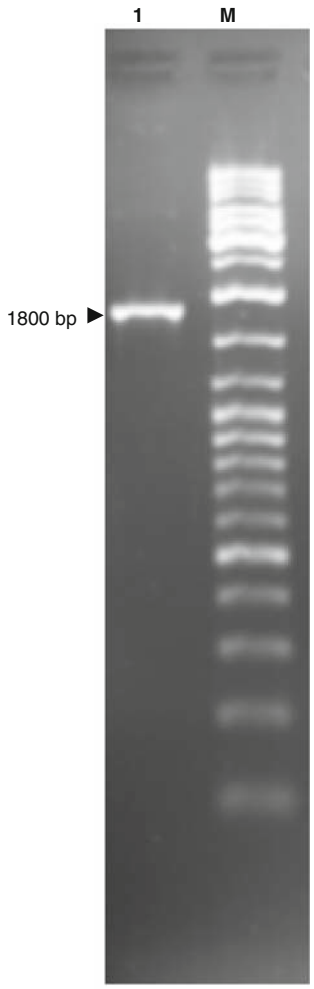

Fig. 3 PCR amplification of 18S rRNA of *D. salina* isolated from CSMCRI Salt Farm, Gujarat, India. *Lane-1* amplified 18S rRNA of *D. salina*. *Lane-M* molecular weight marker 10 kb DNA ladder

caused by free radicals, produced in response to high salinity [29, 31].

Total protein content

Total protein was observed decreasing upto 3.0 M NaCl with reference to 0.5 M NaCl ($P < 0.05$), however a significant increase ($P < 0.01$) in total protein was observed at 5.5 M (0.19 ± 0.01 μg per 10^5 cells) (Fig. 2b). At 5.5 M salt concentration, total protein was found double to that in 0.5 M NaCl (0.10 ± 0.01 μg per 10^5 cells) and it may be concluded that some proteins were hyper salt responsive and expressed in saturated salt concentration. Previously, all up regulated proteins were identified and studied from 3.0 M salt concentration [41], the present view opens new area of proteomics, isolation and characterization of hypersaline responsive protein(s) and/or gene(s).

Total sugar content

Total sugar increased concomitantly with salt concentration (0.5 M, 34.5 ± 1.73 μg; 1.0 M, 54.87 ± 2.74 μg; 2.0 M, 60.4 ± 3.02 μg and 3.0 M, 87.67 ± 4.38 μg per 10^5 cells) however, a significant increase ($P < 0.01$) of approximately 3.0-fold (250 ± 12.5 μg per 10^5 cells of *Dunaliella*) was observed in high salt stress 5.5 M NaCl (Fig. 2c). Among different solutes accumulating in response of stress, sugars play a key role to maintain the osmotic regulation of cells. There are earlier reports on carbohydrate accumulation in response to various abiotic stress during reproductive development [3, 43]. Accumulation of sugars is enhanced in response to the variety of environmental stresses [22, 52].

Proline content

In this study, proline content was found almost the same in range of 0.53–0.67 ± 0.03 μg per 10^5 cells, up to 3.0 M salt stress thereafter a significant increase ($P < 0.01$) of approximately 2.0-fold (1.4 ± 0.07 μg per 10^5 cells) was observed at 5.5 M salt concentration (Fig. 4a). In *Dunaliella*, proline response to salinity has not been reported yet. In higher plants, proline is considered to play an important role in defense mechanism of stressed cells providing carbon, nitrogen and energy source after stress by degradation [58]. Proline improves tolerance of cells to partial dehydration as well as exogenous proline improves growth of salt stressed cell cultures. The improvement was attributed to the role of proline as an osmoprotectant for enzymes and membranes against salt inhibition [38].

Glycerol content

The accumulation of glycerol was increasing from 0.13 ± 0.01 ml (grown in 0.5 M NaCl) to 3.7 ± 0.18 ml (grown in 5.5 M NaCl) per 10^5 *Dunaliella* cells on 17th day of inoculation (Fig. 4b). Previously, it was observed that addition of NaCl in culture medium leads to an increase in glycerol [2]. It shows a linear relationship between glycerol accumulation and a wide range of salt concentration. Accumulation of glycerol in high concentration is required to adjust intracellular osmotic potential against high salt stress. The ability of the halotolerant green alga *Dunaliella* to survive in wide range of salt concentrations is attributed to its ability to adjust osmotic potential by changing intracellular glycerol concentration [34]. Such adjustments in the intracellular glycerol content of *Dunaliella* cells occur rapidly as a consequence of variation in salt concentration. Glycerol production is one of the important features of *Dunaliella*.

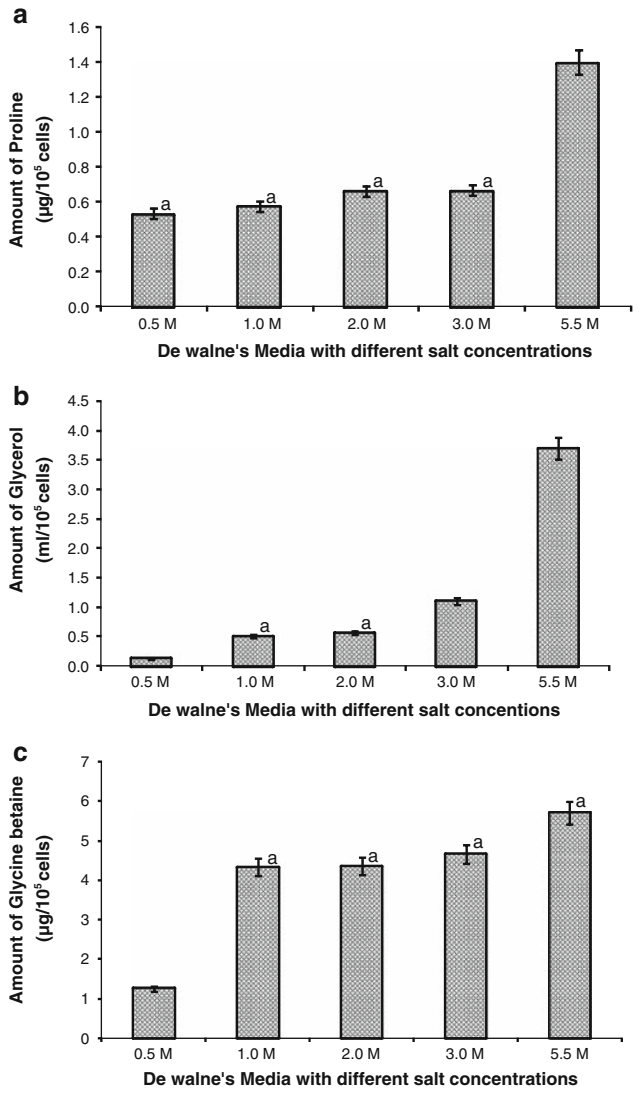

Fig. 4 Stress metabolites of *Dunaliella salina* grown over a salinity gradient. **a** Proline, **b** Glycerol and **c** Glycine betaine. *Error bars* indicate ±SE and similar *lower case letters* indicate no significant difference ($P < 0.01$)

Glycine betaine content

Glycine betaine content significantly increased nearly 3.5-fold (4.32 ± 0.22–4.66 ± 0.23 µg per 10^5 cells) with reference to 0.5 M NaCl (1.25 ± 0.06 µg per 10^5 cells), in response to salt concentration and the increased amount of glycine betaine was maintained up to 5.5 M salt concentrations (5.7 ± 0.28 µg per 10^5 cells) (Fig. 4c). Several species of marine algae have been reported to contain glycine betaine as a stabilizing osmolyte. As far as previous literature is concerned, variation in glycine betaine content of *Dunaliella* in response to salt concentration has not been reported yet. In this study, contribution of glycine betaine was significant and it played a major role as an osmoprotectant in the osmoregulation of *Dunaliella* cells. Glycine betaine carries no net charge at physiological pH and is nontoxic even at higher concentrations [64].

Ion content determination by ICP

Intracellular Na^+ concentration was observed almost unchanged up to 2.0 M salt concentration but significant increase was seen beyond 2.0 M salt concentration (Fig. 5). Non-significant increase was observed in K^+ and Mg^{++} (Fig. 5). Previously it was found that in *D. parva*, Na^+ ions were involved in osmoregulation whereas K^+ ions were not [23, 24]. In another species, *D. marina,* it was suggested that both Na^+ and K^+ ions play a part in volume regulation [54]. Ehrenfeld and Cousin [14] have shown that ions in *D. tertiolecta* were compartmentalized; they postulated that a large compartment regulates its ion concentration, maintaining low Na^+ and $C1^-$ and high K^+ concentrations, while a second compartment is in equilibrium with the external medium. They described a Na^+/K^+ exchange mechanism which helps to regulate the ionic composition of cells over wide range of salinity. Ehrenfeld and Cousin [15] observed that in initial hypertonic shock, *Dunaliella* cells suddenly increased Na^+ content as a consequence to increase in Na^+ influx through the cell membranes. *Dunaliella* can grow in media containing wide range of salt concentrations, but the intracellular Na^+ concentration under all these conditions is low. It was therefore suggested that the antiporter in *Dunaliella* may play a major role in the regulation of intracellular Na^+ concentration [36].

Increase in intracellular Na^+ concentration indicates that *Dunaliella salina* cell is accumulating Na^+. Na^+ uptake may have role in osmoregulation of cells, in the initial stages of high salt concentration and there is compartmentalization in *Dunaliella salina* for the accumulation of salt. Na^+/K^+ ratio was found to remain the same in all

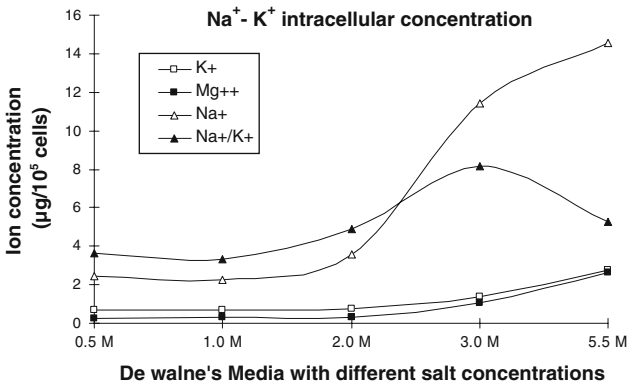

Fig. 5 Intracellular Na^+, K^+ and Mg^{++} ion concentration in *Dunaliella salina* grown in De walne's media with different salt concentrations

molar concentrations, whereas a sudden increase in Na^+/K^+ ratio was observed (Fig. 5) at 3 M concentration which later on intend to remain constant. It may be explained as there is a rapid adjustment by the cells to maintain their osmoregulation during transition stages of salt concentration from high to hyper-saline condition.

Conclusion

Intraspecific physiological variability has been reported in *D. salina* [18, 20, 25], which lead to erroneous assumptions about the industrial potential of the microalga. *Dunaliella* isolated from salt crystals may serve as an ideal model for further study of hyper salt responsive gene(s) and its physiological and metabolic attributes may play an important role in its biotechnological and industrial potential. In this study, we isolated *Dunaliella* from salt crystals and identified as *D. salina* using morphological descriptions and molecular signature, 18S rRNA gene sequences. Physiological attributes of *Dunaliella* (growth pattern and chlorophyll content) and metabolic responses (proline, total sugar and protein contents, glycine betaine and glycerol) to different salinity (0.5–5.5 M NaCl) were studied. Extracellular salt stress affects the accumulation of osmolytes concomitantly. Biosynthesis of proline and glycine betaine was observed as an osmoprotectant in *Dunaliella salina* and it may be the first report on it. Significant advancement in glycerol production was observed at higher salinity (5.5 M NaCl). These beneficial properties of *D. salina* clearly indicate that the alga possesses inherent abilities including the incredible metabolic and physiological versatility which allows it to inhabit the hostile saline environment and make *D. salina* as a promising candidate for the commercial exploitation.

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