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Effect of salinity on some physiological and biochemical responses in the cyanobacterium *Synechococcus elongatus*

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ABSTRACT

In this study, some physiological and biochemical responses of *Synechococcus elongatus* to salt stress were investigated. The cyanobactrium was grown in BG-11 medium under different concentrations of NaCl (0, 0.5, 1 M). The results indicated that the growth of *S. elongatus* was significantly inhibited under salt stress on days 5, 9 and 12 after treatment. Protein content increased in *S. elongatus* on day 12 in presence of salt. Salinity induced proline accumulation at 1 M NaCl on day 12 and caused a significant increase in hydrogen peroxide content on day 5. Catalase (CAT) activity continuously increased on day 5. An increasing trend in polyphenol oxidase (PPO) activity was indicated on days 5 and 9. Superoxide dismutase (SOD) activity gradually induced with increasing NaCl concentrations on day 5. Salt stress decreased chlorophyll content compared to that of control in three stages of growth, and carotenoid content declined on days 9 and 12. The contents of phycobiliprotein (PBP), phycoerythrin (PE) and phycocyanin (PC) enhanced significantly under different NaCl concentrations on days 5 and 9. These results show that *S. elongatus* has limited adaptive potential to salinity, and the optimum medium for its culture should not bear NaCl even at a moderate level, if production of carotenoids is aimed.

Keywords: Antioxidant enzymes; Carotenoids; Phycobiliprotein; Cyanobacterial culture; Adaptation; Salt stress.

Introduction

High concentration of salt causes crucial changes in ion and water homeostasis and thus lead to ionic and osmotic stress as a primary effect and oxidative stress as a secondary effect. Osmotic stress is rather of a physical stress and occurs when the concentration of molecules in solution outside of the cell is different than that inside the cell. Salinity affects water potential inside cells and causing loss of water and elevated ion concentration (Na^+ and Cl^-) (1, 2). Cyanobacteria are photosynthetic prokaryotic organisms that might tolerate wide range of salt concentration. Environmental factors such as nutrient availability, light, temperature and salinity affect biochemical composition of cyanobacteria (3).

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Cyanobacteria based on their tolerance to salinity are classified as halophilic (requiring salt for optimum growth) and halotolerant (able to survive at high salt concentrations but do not require these conditions for growth). Cyanobacteria produce usually some metabolites to protect from salt stress and to balance their osmotic conditions (4). Cyanobacteria have developed multi-protection mechanisms in order to adapt to various conditions. These microorganisms are able to tolerate a certain degree of drought and salinity by accumulation of extracellular substances, activating anti-oxidative system and adjustment of other metabolic mechanisms (5).

It is known that physiological stress leads to formation of Reactive Oxygen Species (ROS) that might damage most cellular components. Furthermore, acclimation to altered osmotic conditions particularly to salinity cause change in physiological processes including antioxidant enzymes [superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidease (APX) and glutathione reductase (GR)] and non-enzymatic antioxidants (ascorbate, glutathione and carotenoids). All these processes function to alleviate the cellular hypo/hyper osmolarity, ion disequilibrium and detoxification of ROS which cause oxidative destruction to cells (6).

In earlier investigations, the type of compatible solutes synthesized or accumulated in cyanobacteria was used to differentiate the degree of tolerance to external salinity. For example, strains with low salt tolerance (max 0.7 M) have been shown to synthesize sucrose or trehalose, strains with moderate salt tolerance (max 1.8 M) produce glucosylglycerol and strains with high salt tolerance amalgamate glycine betaine (7). Proline is accumulated more than any other amino acid under unfavorable environmental conditions in cyanobacteria. It could be involved in stress resistance mechanisms by acting as an osmoprotectant. Cyanobacterial pigments, i.e., chlorophyll a, carotenoids and phycocyanin are also affected under stress conditions. Change in chlorophyll content depends on the nature, habitat, and morphology of cyanobacteria. Salt stress decreased chlorophyll content in *Anabaena doliolum*, but increased it in *Spirulina platensis* (8).

Cyanobacterium *S. elongatus* is photoautotrophic organism, which converts light energy into useful chemical energy using a complex biological process. It grows best in BG-11 nutrient composition. In addition to physiological features, the genetic code of S. *elongatus* has been established and makes it an effective host for molecular genetics techniques (9, 10). The main aim of this study was to determine the physiological and biochemical responses against salinity conditions in *Synechococcus elongatus*.

Materials and Methods

Algal strain and cultivation

Synechococcus elongatus isolated during a screening program from Ramsar hot springs (36°54′11″N and 50°39′30″E), located in north of Iran (11, 12). The sequence of 16S rDNA had been already generated and submitted in the GenBank under accession number JQ771323.1. The cyanobacterium was grown and maintained in sterile BG-11 media (13) agar slants and subcultured freshly before use in later experiments. For short storage, the cyanobacteria were maintained at 4°C on BG-11. They were transferred to fresh medium every 2 months. The cyanobacterium strain was then individually inoculated in 100 ml of BG-11 medium in a 500 ml Erlenmeyer flask and incubated at 25°C under continuous illumination of 60 μmol photons m^{-2} s^{-1}. The initial pH of the medium was adjusted at 7.2 using Tris-HCl (40 mM) prior to sterilization by autoclave. To study the effect of salt stress on growth and physiological and biochemical parameters, *S. elongatus* was cultured under various concentrations (e.g., 0, 0.5, and 1 M) of NaCl and the cultures sampled during three harvesting times (5, 9 and 13 days after treatment).

Determination of growth and protein content

Growth rate of the cyanobacterium was measured using a spectrophotometer (UV-Visible160, Shimadzu, Japan) with wavelength 684 nm. Protein content was determined according to Bradford (14) using bovine serum albumin (BSA) as a standard.

Proline and hydrogen peroxide assay

Proline content was determined as suggested by Bates et al. (15). Cyanobactrium material, 1 g, was homogenized in 4 ml 3% aqueous sulphosalicylic acid. Two

ml of the extract was treated with 2 ml acid ninhydrin and 2 ml of concentrated glacial acetic acid, shaken vigorously, and boiled for 1 h at 100°C. The reaction mixture was extracted with 4 ml toluene, and the absorbance was measured at 520 nm. The content of proline was determined using a standard curve.

Hydrogen peroxide (H_2O_2) content was determined according to Velikova et al. (16). Cyanobactrium material, 1 g, was homogenized in an ice bath with 1 ml 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 12,000 rpm for 15 min, and 0.5 ml of the supernatant was added to 0.5 ml of 10 mM potassium phosphate buffer (pH 7.0) and 1 ml of 1 M KI. The absorbance of the supernatant was measured at 390 nm. The H_2O_2 content was then determined using a standard curve.

Antioxidant enzymes assay

Catalase (CAT) activity was assayed by measuring the initial rate of disappearance of H_2O_2 according to the method suggested by Aebi (17). The reaction mixture contained 50 mM phosphate buffer (pH 7.0), H_2O_2 (3%) and 10 µl enzyme extract. The decrease in absorption was followed for 180s and CAT activity was expressed as units per milligram of protein. Polyphenol oxidase (PPO) activity was estimated following the method of Raymond et al. (18) at 40°C. The reaction mixture contained 2.5 ml of 200 mM potassium phosphate buffer (pH 7), 200 µl pyrogallol 20 mM, and 20 µl enzyme extract. The increase in absorbance was recorded at 430 nm. The PPO activity was defined as Unit mg^{-1} protein. Superoxide dismutase (SOD) activity was determined by monitoring the inhibition of photochemical reduction of nitro blue tetrazolium (NBT) at 560 nm as described by Giannopolitis and Ries (19) in a reaction mixture containing 50 mM sodium phosphate buffer (pH 7.5), 13 mM methionine, 75 µM NBT, 75 µM riboflavin, 0.1 mM EDTA, and 0.1 ml of enzyme extract. The reaction mixture was irradiated for 10 min, and absorbance was read at 560 nm and the results were expressed as U mg^{-1} protein. SOD isoforms were examined in 10% acrylamide gel using the procedures of Laemmli (20). Gels were incubated in 0.2 M Tris-HCl (pH 8.0) containing 4% riboflavin, 4% EDTA, and 20% NBT for 40 min in the dark at room temperature and then exposed to white light until white bands appeared in violet background.

Determination of chlorophyll, carotenoid, and phycobiliprotein content

Chlorophyll content (Chl) was measured according to Marker method (21), performing overnight extractions using 90% aqueous methanol. Centrifuged extracts were measured at 665 nm and calculated using the extinction coefficient of Marker. Carotenoid (Car) content was determined according to the method of Chamovitz et al. (22) using, and 80% acetone as the solvent. Absorbance of acetone extract was taken at 461 and 665 nm by Spectrophotometer.

Phycobiliproteins were extracted by the osmotic shock method (23). One ml of cyanobacterial cell suspension was centrifuged and the pellets homogenized in 60–150 µl of glycerol and incubated in the dark at 4ºC for 24 h. Water was then added to osmotically lysed cells. The cell lysates were centrifuged and phycobiliproteins (PBPs) were determined in the supernatant according to Bennett and Bogorad (24). As a control, absorption spectra of the supernatants were measured (400–750 nm) to confirm the absence of chlorophyll contamination, because the chlorophyll absorbance at 665 nm could interfere with the absorbance of allophycocyanin (APC) at 650 nm. Finally, the absorbance was determined spectrophotometrically at 652, 615, 562 and 750 nm.

Statistical analysis

Each experiment was repeated three times and the data were analyzed using either one- or two-way analysis of variance (ANOVA) in SPSS (ver. 21) and means were compared by Duncan's test at the 0.05 level of confidence. PCA **(**Principal Component Analysis**)** and HCA (Hierarchical Cluster Analysis) were used for evaluating correlation between each pair of variables and performed using XLSTAT (2016) and online CIMminner software, respectively.

Results

Salinity effects on growth and protein content

We observed a decreasing trend in growth under salt stress during various stages (Fig. 1). According to correlation analyses using Pearson's coefficient, the

growth exhibited positive correlations with protein, proline, APC, phycocyanin (PC), phycoerythrin (PE), PBP, Car and Chl (Fig. 7) in *S. elongatus*. Protein content declined significantly in *S. elongatus* under salt stress on days 5 and 9, but it enhanced from 2.7 to 5.02 (mg g^{-1} FW) on day 12 (Fig. 2a).

Table 1. Results of two-way analysis of variance (ANOVA) in *Synechococcus elongatus*

Dependent variable	Independent variable		
	Salinity	Time	Salinity × Time
Growth	33543.459***	6336.459***	2003.073***
Protein content	20696.944***	13558.807***	279090.310***
Proline content	2055.869***	31408.746***	47001.943***
H_2O_2 content	1451.237***	10529.381***	2816.888***
CAT activities	.303^{ns}	3.076^{ns}	.939^{ns}
PPO activities	23922.756***	8876.408***	46236.118***
SOD activities	121124.257***	15755760.200***	5296664.429***
Chl content	7986882.029***	2155599.457***	1575688.100***
Car content	.985^{ns}	.998^{ns}	.997^{ns}
AP content	463971.247***	4757834.589***	312765.267***
PC content	197069.706***	2704549.328***	1207125.054***
PE content	1533129.627***	1099960.855***	700089.084***
PBP content	356342.650***	2903122.673***	894562.551***

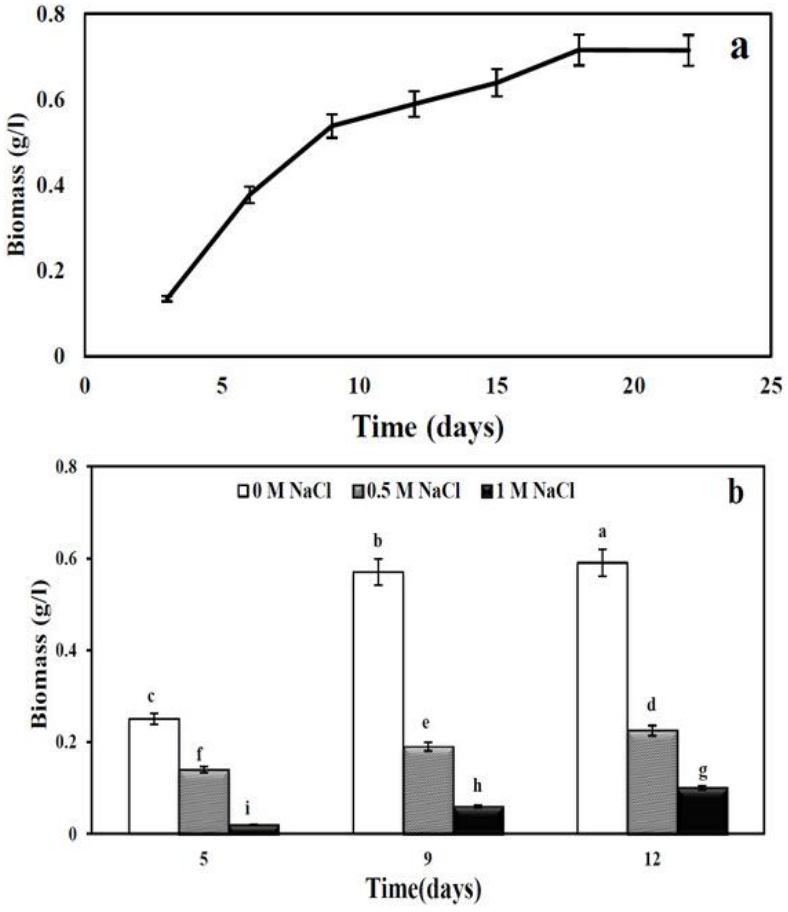

Figure 1 Effect of different concentrations NaCl on the growth in *Synechococcus elongatus.* Values marked with different letters are significantly different according to Duncan test at $P < 0.05$.

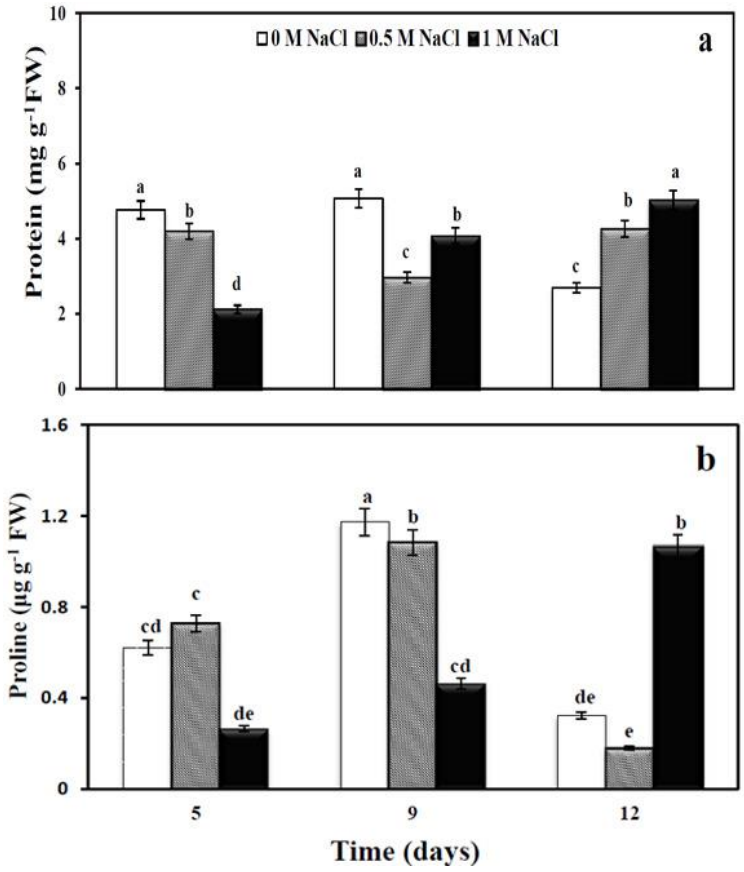

Figure 2 Effect of different concentrations of NaCl on protein and proline contents in *Synechococcus elongatus.* Values marked with different letters are significantly different according to Duncan test at $P < 0.05$.

Proline and hydrogen peroxide contents

Salinity induced proline accumulation at 1 M NaCl after 12 days, whereas proline content decreased from 1.1 to 0.4 (µg g^{-1} FW) after 9 days (Fig. 2b). There was an obvious trend in proline content under salt stress in *S. elongatus*. In terms of proline content, the effects of salinity, time and combination of salinity and time treatments were significant according to a two-way ANOVA (Table 1). Salt stress increased H_2O_2 content on day 5, but salinity had no significant effect on H_2O_2 content after 9 and 12 days (Fig. 3a). According to correlation analyses using Pearson's coefficient, H_2O_2 showed negative correlations with protein, proline, CAT, PPO and Car in *S. elongatus* (Fig. 7).

Antioxidant enzymes

CAT activity enhanced from 8.7×10^{-2} to 20.7×10^{-2} (U mg^{-1} Protein) with increasing NaCl concentration on day 5 (Fig. 3b). Salinity considerably induced the PPO activity on day 9. There was an increasing trend in SOD activity on day 5. Staining analysis of SOD activity by PAGE revealed two SOD isoforms with altering intensities under salt stress (Fig. 4). According to correlation analyses, positive correlations were observed between CAT, SOD and PPO under salt stress (Fig. 7).

Chlorophyll, carotenoid and phycobiliprotein contents

Salt stress decreased chlorophyll content in three stages of growth. Similar trends were observed in carotenoid content on days 9 and 12 (Fig. 5). The contents of PBP, PE, PC, and APC enhanced significantly under different NaCl concentrations after 5 and 9 days, whereas the contents of PBP, PE, and PC gradually decreased with increasing NaCl concentrations after 12 days (Fig. 6). AP, PC, PE and PBP contents showed positive correlation among each other in *S. elongatus* (Fig. 7).

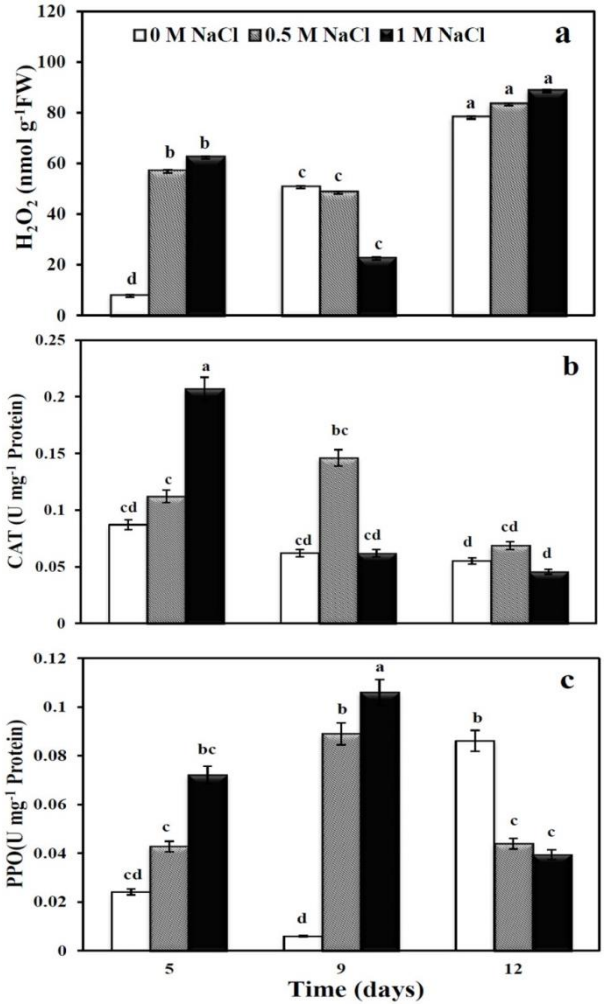

Figure 3. H_2O_2 content and activity of CAT and PPO [U mg^{-1} (protein)] in *Synechococcus elongatus* under salt stress. Values marked with different letters are significantly different according to Duncan test at $P < 0.05$.

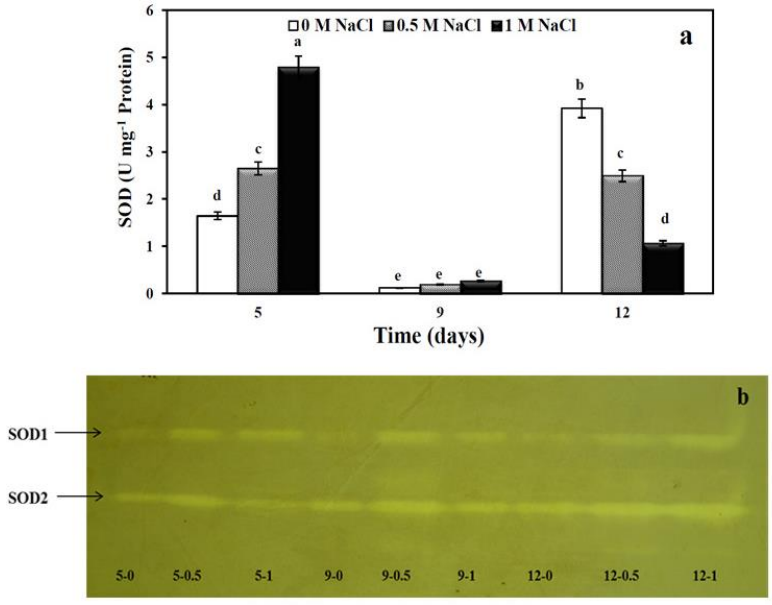

Figure 4. Changes in the activity of SOD (a) and the isoform patterns of SOD enzyme in *Synechococcus elongatus* subjected to differnt salt concentrations during three harvesting times (5, 9 and 12 days).

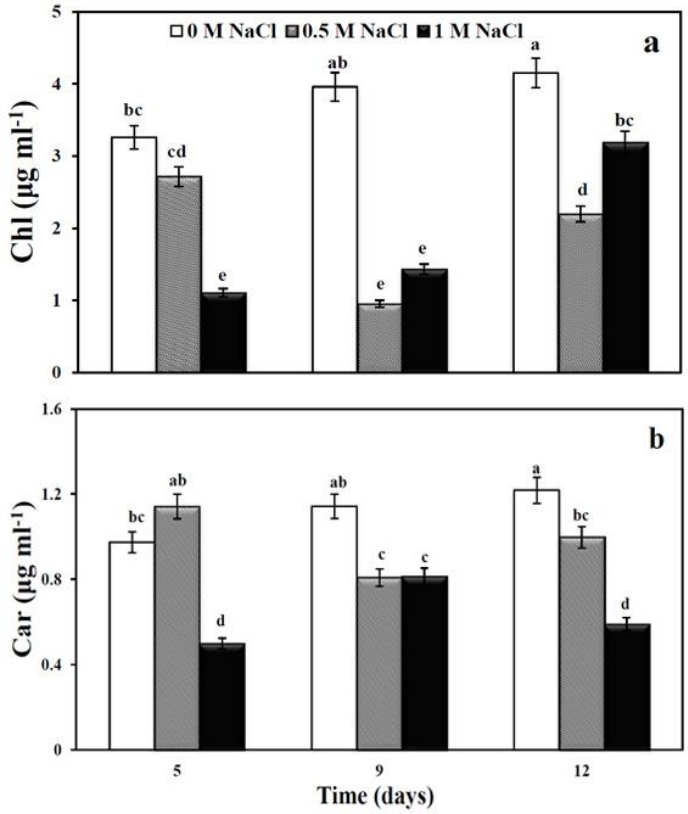

Figure 5. Chlorophyll and carotenoid contents in *Synechococcus elongatus* subjected to various salt concentrations. Values marked with different letters are significantly different according to Duncan at $P < 0.05$.

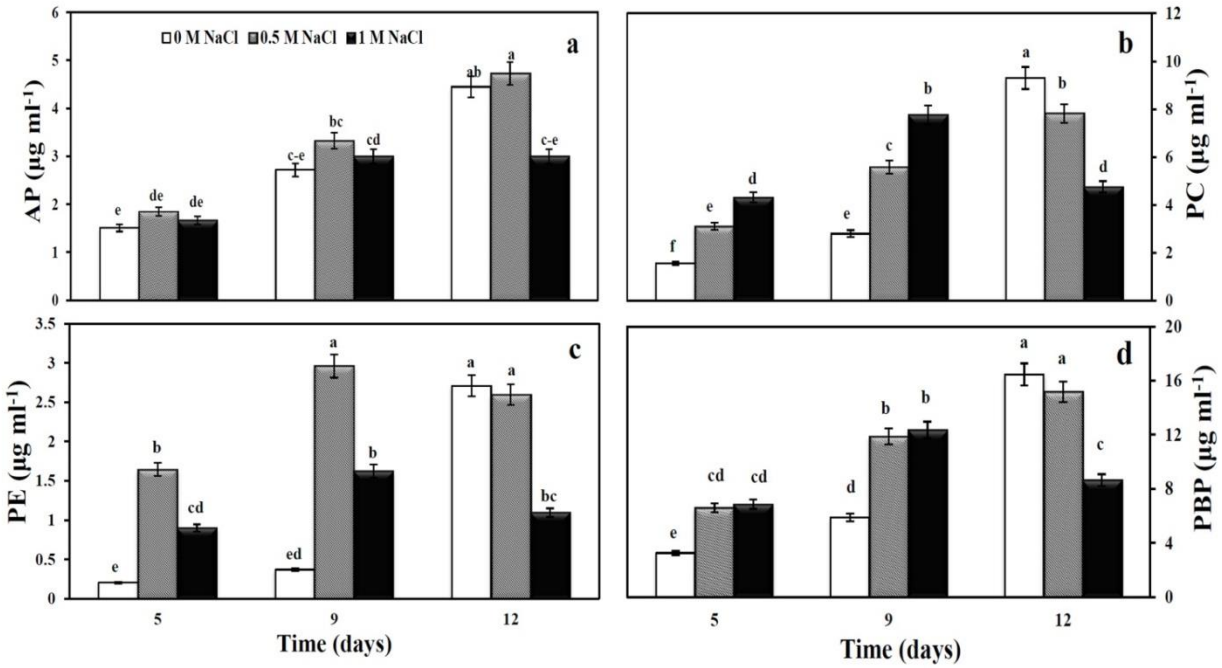

Figure 6. AP, PC, PE and PBP contents in *Synechococcus elongatus* subjected to different salt concentrations during three harvesting times (5, 9 and 13 days). Data are mean ± SE of three replicates. Bars for each harvesting time followed by different letters show significant difference at P≤0.05 according to Duncan's multiple test.

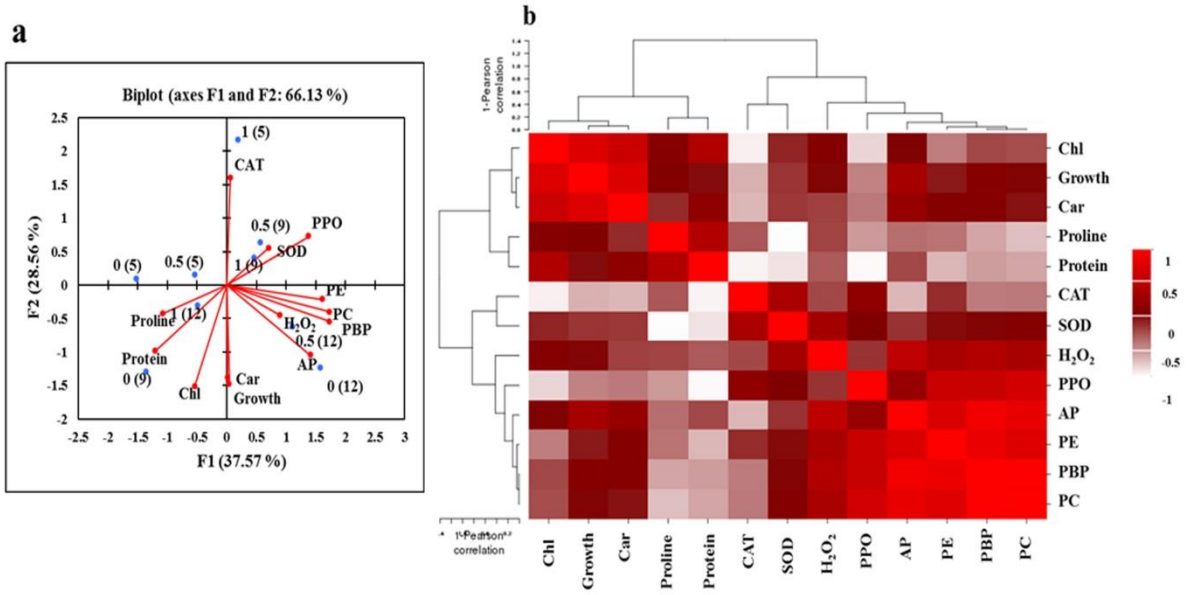

Figure 7. Loading plots of principle components 1 and 2 of the PCA (a), and Heatmap (b) of physiological and biochemical changes in *Synechococcus elongatus*.

Discussion

Salinity is one of the most important abiotic stresses which affects the growth and survival in cyanobacteria. The effect of salinity on growth are associated with, reduces the availability of water to plants (water stress), nutritional imbalance, salt stress (effect on specific ions) and combination of all these three factors (25). High salinity causes inhibition of photosynthetic and respiratory system and reduces the specific growth rate (26). Similar findings on decreased growth in salinity condition were reported in *Spirulina fusiformis* and *Scenedesmus quadricauda* (26, 27). All salinity-induced changes in physiological and biochemical processes are associated with alteration in protein profile. Proteins are one of the most important targets of salinity, and the NaCl-induced oxidative damage is the foremost reason in misfolding of proteins. The oxidation of cysteine SH to cysteine S-S, methionine to methionine sulfoxide, and formation of 3,4-dihydroxyphenylalanine (DOPA) and various hydroxyleucines from tyrosine and leucine, respectively, are some of the examples of protein damage by reactive oxygen species (ROS). The physiological basis of cellular responses to salt stress includes the alteration in protein profile, thus the final line of salt tolerance is change in proteins (28).

The accumulation of organic compounds also known as osmoprotectant or compatible solutes is one of the foremost tolerance mechanisms adopted by cyanobacteria. Compatible solutes have low molecular masses and usually lack a net charge that accumulated in the cytoplasm without interfering with the cellular metabolism. The main function of compatible solutes is to increase the internal osmolality, prevent the water loss and plasmolysis and therefore maintain the osmoticum of the cell. Proline contributes in cytoplasmic osmotic adjustment, stabilizing membranes and protein and scavenging free radicals under stress conditions (29).

The photosynthetic electron transport system is the major source of ROS in plant tissues. The over-reduction of electron transport chain in chloroplasts and electron leakage to oxygen by photosystem I (PSI) and photosystem II (PSII) components resulted in formation of ROS in the light (30). Salt stress lead to increase of ROS and oxidative stress. ROS are highly reactive and might cause oxidative damage to lipids, proteins and nucleic acids (25). Our result is consistent with the findings in *Ulva fasciata* (31) and *Scytonema javanicum* (25).

Salt stress, like other abiotic stresses, lead to oxidative damage through the increase of ROS such as

superoxide, hydrogen peroxide, and hydroxyl radicals. These ROS are highly reactive and can alter normal cellular metabolism through oxidative burst to lipids, proteins, and nucleic acids. To mitigate the oxidative burst caused by ROS, cyanobacteria have developed a complex antioxidative system, including enzymatic antioxidative consists of SOD and CAT. SOD is a major O_2^- scavenger and produces H_2O_2 and O_2, then CAT scavenges the produced H_2O_2 (32). PPO is the major enzyme responsible for oxidation of phenolic compounds (33). Similar findings on antioxidant enzymes under stress condition in *Ulva fasciata* (31) confirm the results performed here. Marine cyanobacterium *Synechococcus* sp. has both Fe-SOD and Cu/ZnSOD (34), whereas *Synechocystis* sp. contains only the Fe-SOD (35).

Decreasing in Chl content could be a symptom of oxidative stress, that might be due to increase in pigment degradation or decrease in its synthesis. Reductions in Chl may also be explained by decrease in Rubisco activity under stress (36). Cyanobacteria are valuable resources for natural non-enzymatic antioxidants such as PBPs and Cars. Consequently, investigations were carried out aiming az assessment and production of these antioxidants under salinity stress. Salt stress causes peroxidation of lipids in the thylakoids and confers damage to PSII complex and change electron transport chain of PSII. PBPs serve as the primary light-harvesting antenna for PSII and changed their composition under stress conditions (32). Our results are in agreement with the findings of Rafiqul et al. (26), Kumar et al. (37), and Kirrolia et al. (27).

In conclusion, salt stress decreased the growth in *S. elongatus*. This cyanobacterium has a number of defense mechanisms to reduce the damaging effects of salinity. These mechanisms include scavenging of ROS by enzymatic antioxidant, proline accumulation and inducing of PBPs.

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