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Salt stress enhancing the production of Phytochemicals in *Chlorella vulgaris* and *Chlamydomonas reinhardtii*

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Abstract

Increase of salinity in freshwater reservoirs due to the let outs of industrial effluents has threaten the aquatic organisms against toxicity, stress and acclimatization to the changing environment. In the present investigation, abiotic stress response of *Chlorella vulgaris* and *Chlamydomonas reinhardtii* was studied by subjecting to 50, 150, 250 and 350 mM of sodium chloride. Physiological, bio-chemical and phytochemical variations were assessed. Steep and steady growth was observed at low concentration of 50 mM in both the cultures. The increase in concentration of salt decreased the growth density and lowered the cell division rate. Total chlorophyll, carotenoid and astaxanthin content was enhanced at 50 mM and 150 mM concentration of NaCl among treated cells. In *C. vulgaris*, the total chlorophyll and carotenoid contents were estimated to be 36.58 mg/L and 6.08 mg/L whereas in *C. reinhardtii*, 19.85 mg/L and 3.90 mg/L respectively. Protein levels elevated at all concentrations of NaCl in both of the treated cultures when compared to control cells. Elevated levels of protein in treated cultures of *C. vulgaris* and *C. reinhardtii* were 14 µg/mL and 17 µg/mL on 20th d; 7-8 times higher than that of control cells (2 µg/mL). Carbohydrates and lipids showed no significant elevation in the levels up to 5th d, this was followed by declination in the follow up days of both the cultures. Among secondary metabolites, *C. vulgaris* alone showed increase in phenol (33 µg/mL) at low salinity of 50 mM NaCl whereas increase in flavonoid was observed in both of the microalgae at high salinity of 350 mM NaCl.

1. Introduction

Algal cells are generally able to live within a certain range of enhanced salt concentrations or changing salinities, since most probably all life originated in the oceans, i.e. a highly saline environment. However, during evolution, the degree of salt resistance and salt tolerance became very divergent among the present-day aquatic organisms (Bohnert, 1996). Algae and cyanobacteria have attracted considerable attention in this respect, since they are inhabitants of biotopes characterized by changing salinities and can serve as model organisms for a better understanding of salt acclimation in the more complex physiological processes of higher plants (Bohnert, 1998; Fogg, 2001). Abiotic stress such as salt stress leads to the over production of highly reactive and toxic reactive oxygen species (ROS) in plants which causes damages to proteins, lipids, carbohydrates and DNA in turn resulting in oxidative stress. Salt stress also causes an imbalance of the cellular ions resulting in ion toxicity and osmotic stress; leading to retardation of growth either directly by salt or indirectly by oxidative stress induced by ROS (Emad *et al* 2010).

The ROS comprises both free radical ($O_2^{\bullet -}$, superoxide radicals; OH, hydroxyl radical; H_2O; perhydroxy radical and $RO^{\bullet}$, alkoxy radicals) and non-radical (molecular) forms (H_2O_2, hydrogen peroxide and $^1O_2$, singlet

oxygen) (Gill and Tuteja, 2010). The antioxidant defense machinery protects algae against oxidative stress damages. Similar to plants, algae possess very efficient enzymatic (superoxide, SOD; catalase, CAT; ascorbate peroxidase, APX; glutathione reductase, GR; monodehydro ascorbate reductase, MDHAR; dehydroascorbate reductase, DHAR; glutathione peroxidase, GPX; guaicol peroxidase, GOPX and glutathione-S-transferase, GST) and non-enzymatic (ascorbic acid, ASH; glutathione, GSH; phenolic compounds, carotenoids, alkaloids, non-protein amino acids and α-tocopherols) antioxidant defense systems that work in concert to control the cascades of uncontrolled oxidation, protecting plant cells from oxidative damage by scavenging ROS. Thus, stress may influence the expression of number of genes that in turn controls many processes like growth, cell cycle, programmed cell death (PCD), abiotic stress responses, pathogen defense, systematic signaling and development.

In addition to antioxidant defense mechanism, adaptability to salinity in algae is also associated with metabolic adjustments such as accumulation of several organic solutes and osmolytes (Hasegawa *et al* 2000; Hoque *et al* 2007). Osmotic adjustments protect sub-cellular structures and reduces oxidative damage caused by free radicals (Hare *et al* 1998; Hong *et al* 1992). Microalgae are considered as promising alternative source for antioxidants such as carotenoids, flavonoids and phenols (Li *et al.* 2007; Natrah *et al.,*2007; Hajimahmoodi *et al.,* 2010; Rodriguez-Garcia and Guil-guerrero 2008; Chacón-Lee and González-Mariño 2010; Lee *et al*., 2010) besides being rich sources of proteins, carbohydrates and fatty acids. Therefore in the present study, salt stress response of *Chlorella vulgaris* and *Chlamydomonas reinhardtii* against sodium chloride was assessed by observing the variation in growth, pigment (chlorophyll a, chlorophyll b, astaxanthin, carotenoid), biochemical (carbohydrate, protein, lipid) and phytochemical (phenol, flavonoid) production.

2. Materials and methods

2.1 Test organism

The fresh water algal strains of *Chlorella vulgaris* and *Chlamydomonas reinhardtii* were collected from Algal Culture Collection, Center for Advanced Studies in Botany, University of Madras, Chennai, India.

2.2 Culturing condition

Microalgae, *Chlorella vulgaris* and *Chlamydomonas reinhardtii* were inoculated into an inorganic Bold Basal medium and were cultured at 24 ± 1 ºC in a thermostatically controlled room and were illuminated with cool inflorescence lamps (Phillips 40 W, cool dlight 6500 K) at an intensity of 2000 lux in a 12:12 h light dark regime.

2.3 Treatment to salt stress

To study the impact of salt stress, different concentrations of NaCl such as 50 mM, 150 mM, 250 mM and 350 mM were taken in 250 mL conical flasks containing 100 mL of Bold's basal medium. Then the medium was autoclaved and inoculated with exponentially growing algal suspension and kept under observation for 25 days. The samples were drawn periodically (0^{th}, 5^{th}, 10^{th}, 15^{th}, 20^{th} and 25^{th} d) during growth from control and different concentrations of NaCl containing medium and were subjected to the analysis of growth, pigment, biochemical and phytochemical parameters.

2.4 Growth measurement

Growth of algal cultures were monitored at the regular intervals of 5 days. Measurement of growth and synthesis of primary and secondary metabolites included the determination of optical density at 678 nm (Robert, 1979), chlorophylls, carotenoids, astaxanthin and total pigment according to Lichtenhaler (1987), total carbohydrates (Dubois et al., 1951), proteins (Bradford, 1976), lipid (Folch et al., 1956), phenols (McDonald et al., 2001) and flavonoids (Chang et al., 2002).

2.5 Statistical analysis

Results obtained were statistically analyzed by using the analysis of variance (ANOVA) and Fisher's Least Significance (LSD) at $p<0.05$. Experiments were repeated thrice individually.

3. Result and discussion

In both of the algal cultures, growth was steady and steep at low concentration of 50 mM NaCl whereas further increase in concentration of salt decreased algal growth. Among both of the cultures though the tolerance to salt were similar i.e., the growth density was observed up to 350 mM, the response and rate of growth varied (Fig. 1 a, b). This indicated that both of the algae exhibit variable response to high salinity and conforms to the observations of Munns et al. (1983) who reported that the effect of salt on growth of microalgae varies dramatically between species. Enhanced salt concentrations may change the growth conditions in a manner unfavourable for most

organisms. The increase in external concentrations of inorganic ions impairs the osmotic balance between the cells and their surrounding medium and forces water efflux (exosmosis) from the cells, leading to the loss of turgor pressure. In parallel, the increased exogenous ion concentration tightens the influx of these ions into the cells according to their electrochemical gradients (Fisher et al., 1997, Kinraide, 1999; Oren, 1999; Serrano, 1999).

The salinity induced growth reduction may be attributed to the accumulation of reactive oxygen species (Menezes-Benavente et al., 2004). The optical density of all the cultures treated to different concentrations of salt showed profuse increase to meager increase depending upon the stress. The maximum optical density was observed among untreated cells and were recorded to be 1.360 with *C. vulgaris* and 1.372 with *C.reinhardtii*. Among the stress treated ones low salinity, 50mM NaCl showed steady growth in both *C.vulgaris* and *C. reinhardtii*.

Observation of pigment level variation for 25 days suggested that total chlorophyll and carotenoid level increased among the 50mM and 150mM treated cells of both *C. vulgaris* and *C. reinhardtii* when compared to untreated cells. Similar type of observation was made by Shaila and Pratima (2010) in microalgae, *Chlorella vulgaris*. Increased concentrations of 250 mM and 350 mM NaCl showed no increase in pigment levels. Maximum level of total chlorophyll and carotenoid in *C. vulgaris* was 36.58 mg/L and 6.08 mg/L (Fig. 2 a, c) and in *C. reinhardtii* was 19.85 mg/L and 3.90 mg/L (Fig 2 b, d). Astaxanthin content remained to be high among untreated cells alone, 0.36 mg/L in *C. vulgaris* and 0.07 mg/L in *C. reinhardtii* (Fig. 2 e, f). *C. vulgaris* showed more stability and increased levels of pigment at all concentrations of salt when compared with *C. reinhardtii.* Salt treatment significantly increased the accumulation of the total secondary carotenoids (Dan Pelah *et al.,* 2004). Carotene is an important source of provitamin A and was found to increase at all the concentrations of NaCl. Similar observations were made by Reddy *et al.* (2003) in cyanobacterial isolates.

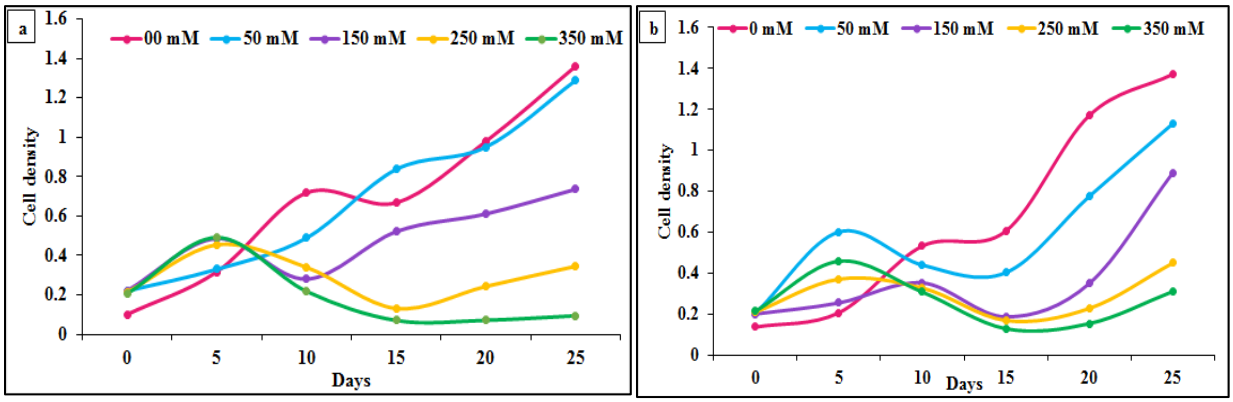
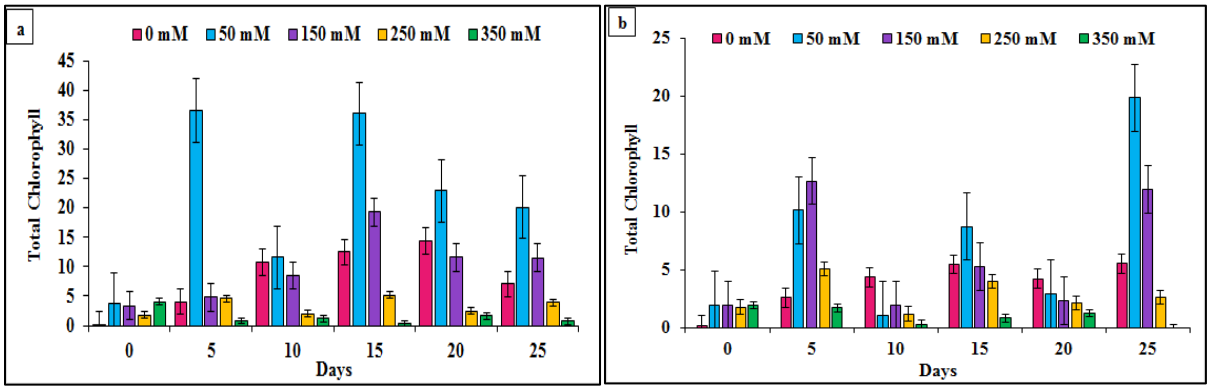


Fig. 1 (a) and (b) Variation in cell density of *Chlorella vulgaris* and *Chlamydomonas reinhardtii* treated to salt stress



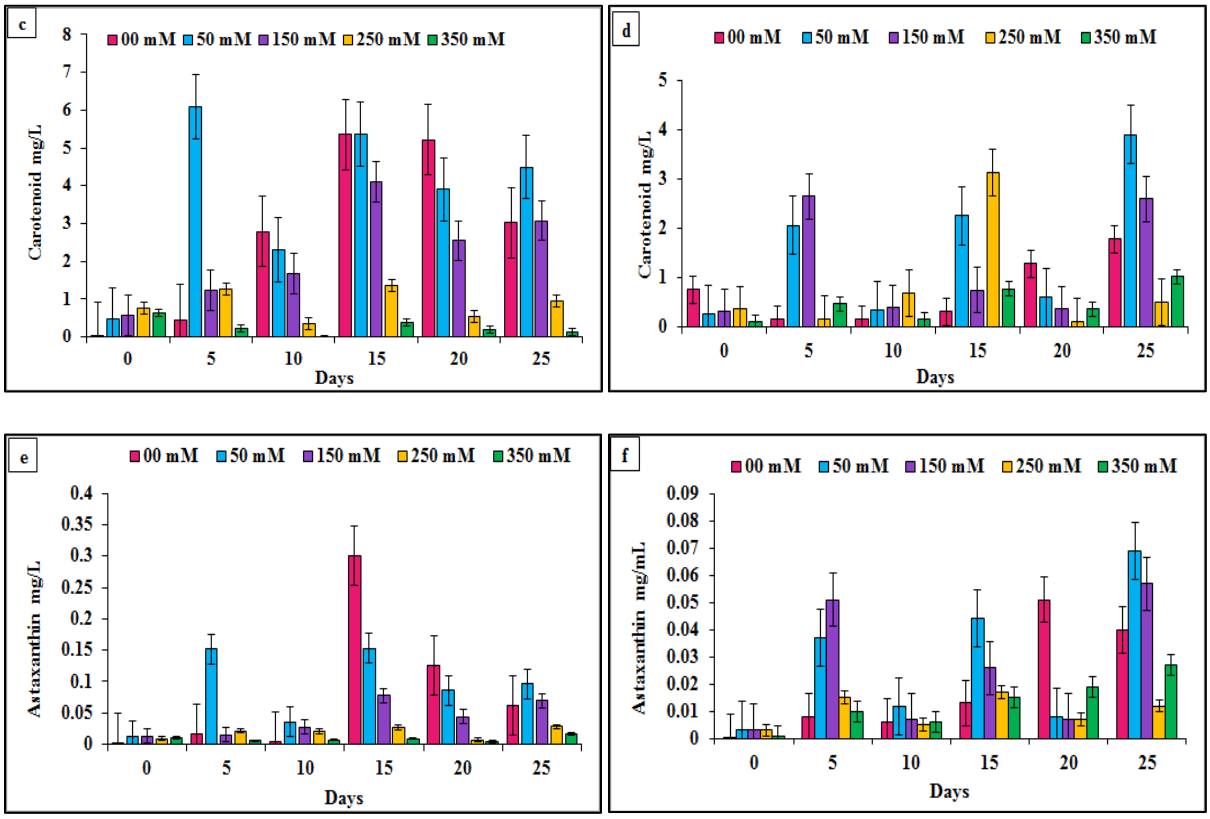


Fig. 2. Effects of NaCl on primary and accessory pigments (a) and (b)- variation in total chlorophyll content of *C. vulgaris* and *C. reinhardtii* (c) and (d)- variation in carotenoid content of *C. vulgaris* and *C. reinhardtii* (e) and (f)- variation in astaxanthin content of *C. vulgaris* and *C. reinhardtii*

According to Moradi and Ismail (2007), reduced chlorophyll contents at higher salinities are due to decrease in photosynthetic rate because of salt osmotic and toxic ionic stress. Many other previous studies report that the cultivation with higher saline concentrations had lower chlorophyll and protein contents along (Vonshak *et al.* 1996) with net assimilation rate and reduced growth (Rai 1990; Rai and Abraham 1993).

Among different solutes accumulating in response to stress, sugar may play a key role to maintain the osmotic regulation of cells. Increase in the carbohydrate content may be an adaptive measure under saline conditions in our study. Increase in the level of carbohydrate content was observed at all concentrations of salt up to 5th d but when compared to control due to toxicity and intolerance, the deprivation might have occurred in other follow up days. Similar to this report, many studies also report increased synthesis of carbohydrates stimulated by stress conditions (Shaila and Pratima 2010; Gill *et al.*, 2002; Tomaselli *et al.,* 1987; Warr *et al.,* 1985). Thus carbohydrates play an important role in the osmotic regulation of cells and increases in content up to certain level of tolerance. Protein synthesis was also triggered under salt stress as 14 µg/ mL and 17 µg/mL of protein content was observed among treated cells of *C. vulgaris* and *C. reinhardtii* which was 7-8 times greater to the amount among untreated cells (2 µg/mL). Salt treated cells of *C. vulgaris* and *C. reinhardtii* did not show any significant increase in lipid content when compared to control cells except 50 mM of NaCl concentration increased lipid content to the maximum of 63. 21 µg/mL in *C. vulgaris.* This suggests that low salinity improves lipid production in *C. vulgaris* but not in *C. reinhardtii* (Table 1).

Phenol content increased at the low salinity of 50mM NaCl rather than 150, 250 and 350 mM in *C. vulgaris* up to 33±0.7 µg/mL whereas gradual decrease in phenol content was observed at all concentrations of NaCl in *C. reinhardtii*. Flavonoid content in both *C. reinhardtii* and *C. vulgaris* content remarkably increased higher among treated cells than in control cells. Maximum level of flavonoid in *C. vulgaris* was reported to be 232 µg/mL on 15th of 350 mM NaCl treated cells while in *C. reinhardtii* maximum level of flavonoid was reported to be 197 µg/mL on 15th d

of 50 mM NaCl treated cells (Table 1). In both the cultures, flavonoid content was enhanced up to 15th d of growth curve followed by diminishment while in control cells there was a gradual increase in flavonoid from 0th d to 25th d.

4. Conclusion

Microalgae *C.vulgaris* and *C.reinhardtii* can acclimate to high salt concentration; under these condition the rate of cell division slowed with the gradual increase in salt concentration. Low and moderate salinity influenced the synthesis of total chlorophyll, carotenoids and astaxanthin in both the cultures. Salt tolerance protein might have also been produced since remarkable increase in protein synthesis was observed with the increase of salinity. Secondary metabolites, phenol and flavonoid synthesis varied among *C.vulgaris* and *C.reinhardtii* in response to salt concentration. Higher salt tolerance was expressed mainly with increase in protein level and elevation in carbohydrate and lipid levels on the initial days of acclimatization; suggesting salt stress protein and solutes has an efficient protective mechanism against physiological drought caused by the hypertonic environment and against the toxicity induced by excessive amounts of the sodium ion.

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References

Bohnert, H.J., Jensen, R.G. 1996. Metabolic engineering for increased salt tolerance - the next step. Aust. J. Plant Physiol. 23, pp. 661-667.

Bohnert, H.J., Nelson, D.E., Jensen, R.G. 1995. Adaptations to environmental stress. Plant Cell. 7, pp. 1099-1111.

Bohnert, H.J., Sheveleva, E. 1998. Plant stress adaptations-making metabolism move. Curr. Opin. Plant Biol. **1:** 267-274.

Bradford, M.M. 1976. Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:** 248-254.

Chacón-Lee, T.L., González-Mariño, G.E. 2010. Microalgae for “healthy” foods-possibilities and challenges. *CRFSFS*. **9(6):** 655-675.

Chang, C., Yang, M., Wen, H., Chern, J. 2002. Estimation of total flavonoid content in propolis by two complementary colorimetric methods. J. Food Drug Analaysi. **10:** 178-182.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F. 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. **28:** 350-356.

Fathi, A.A., Zaki, F.T., Ibraheim, H.A. 2005. Response of tolerant and wild type strains of *Chlorella vulgaris* to copper with special references to copper uptake system. Protistology. **4:**73-78.

Fisher, M., Gokhman, I., Pick, U., Zamir, A. 1997. A structurally novel transferrin-like protein accumulates in the plasma membrane of the unicellular green alga *Dunaliella salina* grown in high salinities. J. Biol. Chem. **272 (3):** 1565-l570.

Fogg, G.E. 2001. Algal adaptation to environmental stresses- some general remarks. New York: Springer- Berlin; p. 1-19.

Folch, J., Lees, M. 1951. Proteolipides, a new type of tissue lipoproteins: their isolation from brain. J. Biol. Chem. **191:** 807-817.

Gill, P.K., Sharma, A.D., Singh, P., Bhullar, S.S. 2002. Osmotic stress induced changes in germination, growth and soluble sugar contents of *Sorghum bicolor* (L.) Moench seeds under various abiotic stresses. Plant Physiol. **128:** 12-25.

Gill, S.S., Tuteja, N. 2010. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiol. Biochem. **48:** 909-930.

Hagemann, M.L., Wolfel Kruger, B. 1990. Alterations of protein synthesis in *Syncechocytes* Sp. PCC 6803 after salt shock. J. Gen. Microbiol. **136:** 1390-1399.

Hajimahmoodi, M., Faramarzi, M.A., Mohammadi, N., Soltani, N., Oveisi, M.R., Nafissi-Varcheh, N. 2010. Evaluation of antioxidant proper- ties and total phenolic contents of some strains of microalgae. J. Appl. Phycol. **22:** 43-50.

Hare, P.D., Cress, W.A., Van-Staden, J. 1998. Dissecting the roles of osmolyte accumulation during stress. Plant Cell Environ. **21:** 535-554.

Hasegawa, P.M., Bressan, R.A., Zhu, J.K., Bohnert, H.J. 2000. Plant cellular and molecular esponse to high salinity. Annu. Rev. Plant Physiol. Plant Mol. Biol. **51:** 463- 499.

Hong, B., Barg, R., Ho, T.H. 1992. Development organ specific expression of an ABA- and stress induced protein in Barley. Plant Mol. Biol. **18:** 663-874.

Hoque, M., Okuma, E., Banu, M.N.A, Nakamura, Y., Shimoishi, Y., Murata, Y. 2007. Exogenous proline mitigates the detrimental effects of salt stress more than the betaine by increasing antioxidant enzyme activitie. J. Plant Physiol. **164:** 553-561.

Kinraide, T.B. 1999. Interactions among Ca^{2+}, Na^{+} and K^{+} in salinity toxicity: Quantitative resolution of multiple toxic and ameliorative effects. J. Exp. Bot. **50:** 1495- 1505.

Lee, S.H., Lee, J.B., Lee, K.W., Jeon, Y.J. 2010. Antioxidant properties of tidal pool microalgae, *Halochlorococcum porphyrae* and *Oltamannsiellopsis unicellularis* from Jeju Island, Korea. Algae. **25:** 45- 56.

Levy, J.L., Stauberand, J.L., Jolley, D.F. 2007. Sensitivity of marine microalgae to Copper: The effect of biotic factors on Copper adsorption and toxicity. Sci. Total Environ. **387:** 141- 154.

Li, H.B., Cheng, K.W., Wong, C.C., Fan, K.W., Chen, F., Jiang, Y. 2007. Evaluation of antioxidant capacity and total phenolic content of different fractions of selected microalgae. Food Chem. **102:** 771-776.

Lichtenthaler, H.K. 1987. Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. Methods nzymol. **148:** 350-382.

McDonald, S., Prenzler, P.D., Autolovich, M., Robards, K. 2001. Phenolic content and antioxidant activity of olive extracts. Food Chem. **73:** 73-84.

Menezes-Benavente, L., Teixeira, F.K., Kamei, C.L.A., Margis-Pinheiro, M. 2004. Salt stress induces altered expression of gene encoding antioxidant enzymes in seedlings of a Brazilian indica rice (*Oryza sativa* L.). Plant Sci. **2:** 323-331.

Moradi, M., Ismail, A.M. 2007. Responses of Photosynthesis, chlorophyll fluorescence and ROS- scavenging systems to salt stress during seedling and reproductive stages of rice. Ann. Bot. **99:** 1161-1173.

Munns, R., Greenway, H., Krist, G.O. 1983. *Halotolerant Eukaryotes*. New York: Springer-Verlag; p. **12:** 59-136.

Natrah, F.M.I., Yusoff, F.M., Shariff, M., Abas, F., Mariana, N.S. 2007. Screening of Malaysian indigenous microalgae for antioxidant properties and nutritional value. J. Appl. Phycol. **19:** 711-718.

Oren, A. 1999. Bioenergetic aspects of halophilism. Microbiol. Mol. Biol. Rev. **63:** 334-337.

Pelah, D., Sintov, A., Cohen, E. 2004. The effect of salt stress on the production of canthaxanthin and astaxanthin by *Chlorella zofingiensis* grown under limited light intensity. World J. Microbiol. Biotechnol*.* **20:** 483-486.

Rai, A.K., Abraham, G. 1993. Salinity tolerance and growth analysis of the cyanobacterium *Anabaena doliolum*. B. Environ. Contam. Tox. **51:** 724-731.

Rai, A.K. 1990. Biochemical characteristics of photosynthetic response to various external salinities in halotolerant and fresh-water cyanobacteria. FEMS. Microbiol. Lett. **69:** 177-180.

Reddy, O.V., Wattal-Dhar, D., Prasanna, R., Singh, P.K. 2003. The influence of sodium stress on selected cyanobacterial isolates. Indian J. Plant Physiol. **8:** 332-338.

Robert, R.L.G. 1979. Growth measurements. Division rate. Cambridge University Press- Cambridge; p. 275.

Rodriguez-Garcia, I., Guil-Guerrero, J. 2008. Evaluation of the antioxidant activity of three microalgal species for use as dietary supplements and in the preservation of foods. Food Chem. **108:** 1023-1026.

Serrano, R., Mulet, J.M., Rios, G., Marquez, J.A., de Larrinoa, I.F., Leube, M.P., Mendizabal, I., Pascual, A.A., Proft, M., Ros, R., Montesinos, C. 1999. A glimpse of the mechanisms of ion homeostasis during salt stress. J. Exp. Bot. **50:** 1023-1036.

Shaila, H., Pratima, M. 2010. Impact of Salinity on the Physiological and Biochemical Traits of *Chlorella vulgaris* Beijerinck. J. Algal Biomass Utln. **1(2):** 51-59.

Shalaby, E.A., Shanab, S.M.M., Singh, V. 2010. Salt Stress Enhancement of Antioxidant and Antiviral Efficiency of *Spirulina platensis.* J. Med. Plants Res. **4(24):** 2622-2632.

Sheikh, T.A., Baba, Z.A., Parvez, S. 2006. Effect of NaCl on growth and physiological traits of *Anabaena cylindrica* L. Pak. J. Biol. Sci. **9(13):** 2528-2530.

Tomaselli, L., Torzillo, G., Giovanetti, L., Bocci, F., Tredici, M.R., Pusharaj, B., Pupuazzo, T., Balloni, W., Meterassi, R. 1987. Recent research of Spirulina in Itali Hydrobiol. **151:** 79-82.

Vonshak, A., Kancha ra ksa B. Bunnag, Tanticha roen, M. 1996. Role of Right and Photosynthesis on the acclimation process of the cyanobacteria *Spirulina platensis* to salinity stress. J Appl Phycol. **8:** 119-124.

Warr, S.R.C, Reed, R.H., Chudek, J.A., Foster, R., Stewart, W.D.P. 1985. Osmotic adjustment in *Spirulina platensis*. Planta. **163:** 424-429.

Table. 1. Variation in bio-chemical and phyto-chemical parameters Data are the mean ± SD (n = 3). *P < 0.05, **P < 0.01, ***P<0.001 compared with control. Note: C- Control, T_1- NaCl 50 mM, T_2- NaCl 150 mM, T_3- NaCl 250 mM, T_4- NaCl 350 mM. C_1, C_2- Carbohydrate; P_1, P_2- Protein; L_1, L_2- Lipid; Ph_1, Ph_2-Phenol; F_1, F_2- Flavonoid

Salt and its conc.	Days	*Chlorella vulgaris*					*Chlamydomonas reinhardtii*				
		Primary metabolites			Secondary metabolites		Primary metabolites			Secondary metabolites	
		C_1 µg/mL	P_1 µg/mL	L_1 µg/mL	Ph_1 µg/mL	F_l µg/mL	C_2 µg/mL	P_2 µg/mL	L_2 µg/mL	Ph_2 µg/mL	F_2 µg/mL
C	0	0	1±0.02*	5.4±0.1**	4±0.09*	0.017	0	0	8.8±0.20*	4±0.09**	0.014
	5	42±0.9*	3±0.07*	7.2±0.16*	5±0.11*	0.022	19±3.6*	2±0.04*	6.4±0.14*	0	0.004
	10	115±2.6*	1±0.02*	47.1±1.0*	5.5±0.1*	0.142	62±1.4**	0	9.8±0.22*	21±0.49*	0.062
	15	251±5.8**	1±0.02*	15.6±0.3*	11±0.2*	0.134	109±2.5*	0	13.6±0.3*	6±0.14**	0.08
	20	124±2.8*	2±0.04*	44.1±1.0*	21±0.4*	0.139	124±2.8	2±0.04*	45.9±1.0*	11±0.25*	0.101
	25	254±5.9**	1±0.02*	31.7±0.7*	20±0.4*	0.145	203±4.7*	1±0.02*	12.6±0.2*	20±0.4*	0.152
T_1	0	24±0.5*	1±0.02*	11.2±0.2*	2±0.04*	0.050	47±1.0*	1±0.02*	8.5±0.19*	2±0.04*	0.035
	5	76±1.7***	4±0.09*	16.6±0.3*	33±0.7*	0.069	32±0.7**	2±0.04*	23±0.53*	0	0.023
	10	21±0.4*	5±0.1**	63±1.4**	20±0.4*	0.082	22±0.5**	2±0.04*	11.7±0.2*	2.5±0.05*	0.041
	15	58±1.3**	2±0.04	9.6±0.2*	16±0.3*	0.016	12±0.28*	8±0.1**	4.8±0.11*	5.5±0.12*	0.197
	20	37±0.8***	11±0.25	23.9±0.5*	10±0.2*	0.176	7±0.16**	17±0.3*	18.6±0.4*	2±0.04*	0.059
	25	120±2.8*	1±0.02*	18±0.42*	29±0.6*	0.198	4±0.09**	1±0.02*	8.4±0.19*	10±0.23*	0.084
T_2	0	27±0.6***	5±0.1**	12±0.28*	0	0.028	56±1.3*	1±0.02*	9.6±0.22*	0	0.018
	5	86±2.0*	1±0.02*	4.3±0.1*	4±0.09*	0.033	43±1.0**	3±0.07*	15.6±0.3*	0	0.014
	10	38±0.8**	2±0.04*	16.5±0.3*	8±0.1**	0.063	19±0.4*	3±0.07*	9.6±0.22*	0	0.021
	15	30±0.7**	8±0.18*	5.9±0.13*	1±0.02*	0.005	101±2.3*	13±0.3*	8.8±0.20*	4±0.09**	0.157
	20	47±1.0**	12±0.2*	12.8±0.2*	5±0.1**	0.183	22±0.51*	15±0.3*	10.6±0.2*	0	0.042
	25	112±2.6	1±0.02*	8.5±0.19*	12±0.2*	0.038	7±0.16**	1±0.02*	6±0.14**	5±0.1***	0.039
T_3	0	25±0.5**	2±0.04*	8.5±0.1**	0	0.028	30±0.7**	1±0.02*	9.2±0.21*	0	0.017
	5	59±1.3**	1±0.02*	10±0.23*	8±0.18*	0.030	45±1.0**	1±0.02*	8.6±0.20*	2±0.04**	0.019
	10	21±0.4*	0.5±0.1*	17.7±0.4*	14±0.3*	0.024	19±0.44*	1±0.02*	9.6±0.22*	0	0.009
	15	40±0.9**	8±0.18*	4.1±0.09*	8±0.01*	0.194	7±0.16**	12±0.2*	10.8±0.2*	2±0.04**	0.178
	20	13±0.3*	14±0.3*	12.8±0.2*	2±0.04*	0.059	19±0.44*	17±0.3*	9.4±0.21*	0	0.039
	25	68±0.3*	1±0.02*	6.6±0.15*	11±0.2*	0.033	35±0.81*	1±0.02*	5.2±0.1**	2.5±0.05*	0.051
T_4	0	30±0.7*	4±0.09*	2.5±0.05*	0	0.025	39±0.91*	1±0.02*	11±0.26*	0	0.019
	5	32±0.7*	1±0.02*	4.5±0.1*	4.5±0.1*	0.059	31±0.7**	1±0.02*	8.4±0.19*	2±0.04**	0.045
	10	4±0.09**	1±0.02*	10±0.23*	2±0.04*	0.002	40±0.93*	3±0.07*	15±0.35*	5±0.1***	0.008
	15	4±0.09**	12±0.28	7.8±0.18*	4±0.09*	0.232	26±0.60*	13±0.3*	16.4±0.3*	2±0.04**	0.147
	20	5±0.1***	1±0.02*	6.8±0.15*	0	0.042	10±0.23*	16±0.3*	5.6±0.13*	0	0.031
	25	19±0.4*	0.5±0.1*	5.2±0.1**	2±0.04*	0.006	26±0.60*	1±0.02*	6.6±0.1**	2±0.04**	0.017