Bioresource Technology 189 (2015) 341–348

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Salinity induced oxidative stress enhanced biofuel production potential of microalgae *Scenedesmus* sp. CCNM 1077

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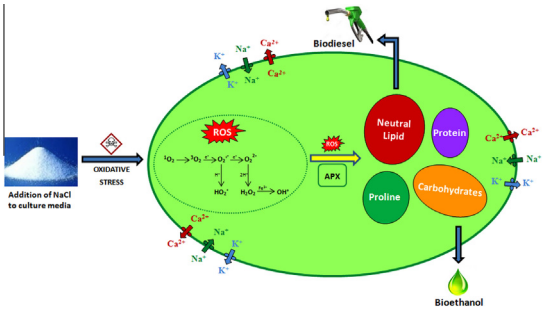
HIGHLIGHTS

- Salinity stress affects physiological and biochemical composition of microalgae.
- 33.13% lipid and 35.91% carbohydrate accumulated under 400 mM NaCl stress.
- Oxidative stress is important for neutral lipid accumulation.
- Two stage salinity stress is best approach for biomass and lipid production.
- *Scenedesmus* sp. is a potential feedstock for biofuels production.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 21 February 2015
Received in revised form 3 April 2015
Accepted 4 April 2015
Available online 16 April 2015

Keywords:
Microalgae
Salinity
Oxidative stress
Lipid
Carbohydrate

ABSTRACT

Microalgal biomass is considered as potential feedstock for biofuel production. Enhancement of biomass, lipid and carbohydrate contents in microalgae is important for the commercialization of microalgal biofuels. In the present study, salinity stress induced physiological and biochemical changes in microalgae *Scenedesmus* sp. CCNM 1077 were studied. During single stage cultivation, 33.13% lipid and 35.91% carbohydrate content was found in 400 mM NaCl grown culture. During two stage cultivation, salinity stress of 400 mM for 3 days resulted in 24.77% lipid (containing 74.87% neutral lipid) along with higher biomass compared to single stage, making it an efficient strategy to enhance biofuel production potential of *Scenedesmus* sp. CCNM 1077. Apart from biochemical content, stress biomarkers like hydrogen peroxide, lipid peroxidation, ascorbate peroxidase, proline and mineral contents were also studied to understand the role of reactive oxygen species (ROS) mediated lipid accumulation in microalgae *Scenedesmus* sp. CCNM 1077.

1. Introduction

Worldwide recent research is focused on finding the cost-effective, environmental friendly and renewable energy resources. Microalgae have gained much interest as a future promising renewable energy resources due to its fast generation time, high carbohydrate and lipid content, ability to grow in saline and various types of wastewater, higher photosynthetic ability compared to all other terrestrial energy crop plants (Chisti, 2007).

Culture conditions like nitrogen starvation (Pancha et al., 2014), light intensity (George et al., 2014) and mixotrophic growth condition (Pancha et al., 2015; Ghosh et al., 2013) significantly enhance the biomass, lipid and carbohydrate contents in various microalgal

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http://dx.doi.org/10.1016/j.biortech.2015.04.017
0960-8524/

species, which ultimately enhance the biofuel production potential of microalgae. Nutritional starvation like nitrate and phosphate limitations result in lower biomass production, which leads to lower biofuel yield; on the other hand, mixotrophic cultivation is costly due to high cost of organic carbon source. To overcome such problems, many researchers have proposed two stage cultivation strategy in which microalgae is first grown in nutrient sufficient condition and after producing sufficient biomass stress phase is applied to produce higher lipid and carbohydrate contents in microalgae (Sun et al., 2014).

Similar to nutritional starvation, salinity stress has also been reported to enhance lipid accumulation in various microalgae like *Chlamydomonas* sp. JSC4 (Ho et al., 2014), *Desmodesmus abundans* (Xia et al., 2014), *Nannochloropsis* sp. (Pal et al., 2011) etc. Effects of salinity on microalgae have been mainly studied in marine microalgae with focus on enhancement in lipid content. Very few reports are available on systematic study of salinity stress on freshwater microalgae for the production of lipid and carbohydrate which can be converted into biodiesel and bioethanol, respectively. Large scale cultivation of microalgae is mainly carried out in closed photobioreactors or open raceway ponds. Microalgal cultivation in closed photobioreactors is economically unsuitable for biofuel production due to its high operation cost. On other hand, open raceway ponds result in lower and poor quality of biomass due to problems like lack of control over various cultivation parameters like temperature, light intensity, pH etc. Salinity stress applied through addition of NaCl during two stage cultivation is practically easy compared to other two stage TAG accumulation methods in large scale open ponds (Yang et al., 2014).

Reactive oxygen species (ROS) are known to accumulate in microalgae under various environmental stress conditions like high temperature, salinity, heavy metal stress, high light intensity and various nutritional stress conditions (Chokshi et al., 2015). High level of oxidative stress results in higher accumulation of ROS in cells, which leads to degradation of cellular macromolecules. However, role of ROS in lipid accumulation is not well studied. Recent research shows that oxidative stress is a mediator for lipid accumulation in various microalgae like *Dunaliella salina* (Yilancioglu et al., 2014), *Chlorella pyrenoidosa* (Fan et al., 2014).

The present study was aimed to understand salinity induced physiological and biochemical changes in freshwater microalgae *Scenedesmus* sp. CCNM 1077 under single and two stage cultivation regimes. Biomass production, lipid, carbohydrate, protein, photosynthetic pigments, mineral contents and various stress biomarkers like membrane lipid peroxidation, hydrogen peroxide, proline, antioxidative enzyme ascorbate peroxidase (APX) were used to understand the physiological mechanism to overcome the salinity stress as well as to study its effects on lipid and carbohydrate contents in microalgae *Scenedesmus* sp. CCNM 1077.

2. Methods

2.1. Microalgae and experimental conditions

The microalgae used in this study, *Scenedesmus* sp. CCNM 1077, was cultured in BG-11 medium as described previously (Pancha et al., 2014). For single stage salinity stress experiment, cells were grown in BG-11 medium with different initial NaCl concentrations of 0, 50, 100, 150, 200 and 400 mM throughout 15 days of growth period. For second, two stage cultivation experiment, cells were first grown in BG-11 medium and 400 mM NaCl was added in the culture after 6, 9 and 12 days of cultivation to induce salinity stress in microalgae *Scenedesmus* sp. CCNM 1077.

All the experiments were carried out in triplicate in 1 L flasks containing 500 ml of respective culture medium and inoculated with 10% actively growing culture of *Scenedesmus* sp. CCNM 1077. All the experiments were performed in batch culture at 25 ± 2 °C with 12:12 h light dark period under 60 μmol m^2 s^{-1} light intensity. The flasks were manually shaken thrice a day.

2.2. Biomass and biochemical composition analysis

2.2.1. Determination of growth and biomass productivity

Microalgal growth was monitored every 3rd day by measuring optical density at 750 nm. The biomass productivity (mg/L/day) was calculated according to equation $P = (X_2 - X_1)/(t_2 - t_1)$, where, X_2 and X_1 are the dry cell weight (DCW) (mg/L) at time t_2 and t_1, respectively.

2.2.2. Determination of pigments

Pigments content was determined according to Lichtenthaler (1987). In detail, 2 ml culture was centrifuged at 10,000 rpm for 5 min, supernatant was discarded and pellet was mixed with 99.9% methanol and incubated in dark for 24 h at 45 °C. After incubation, pigment content was determined using following formulas:

$$\text{Chlorophyll a; Chl-a } (\mu g/ml) = 16.72\ A_{665.2} - 9.16\ A_{652.4}$$

$$\text{Chlorophyll b; Chl-b } (\mu g/ml) = 34.09\ A_{652.4} - 15.28\ A_{665.2}$$

$$\text{Carotenoids} = (1000\ A_{470} - 1.63\ \text{Chl-a} - 104.9\ \text{Chl-b})/221$$

Absorbencies at 470, 652.4 and 665.2 nm were corrected for turbidity by subtracting absorbance at 750 nm.

2.2.3. Determination of lipid, protein and carbohydrate

Lipid was extracted using chloroform:methanol (1:2 v/v) and determined gravimetrically (Bligh and Dyer, 1959). Total lipid was further fractionated into neutral lipid using chloroform/acetic acid (9:1, v/v), glycolipid using acetone/methanol (9:1, v/v) and phospholipid using methanol as described in Pancha et al. (2014).

Nitrogen content in microalgal biomass was determined through elemental analysis using CHNS analyzer (Perkin-Elmer Model 2 400, USA). Crude protein content of microalgae was calculated according to following equation:

Crude protein (%) = N (%) × 6.25 (Becker, 1994).

For carbohydrate estimation, 50 mg of lipid extracted biomass was first hydrolyzed with 500 μl of 72% (w/v) H_2SO_4 for 1 h. Concentration of H_2SO_4 was reduced to 4% by addition of distilled water and autoclaved at 121 °C for 1 h. After cooling down to room temperature content was made up to 50 ml with distilled water (Van Wychen and Laurens, 2013). The resulting solution was centrifuged at 10,000 rpm for 10 min and supernatant was used for sugar estimation by phenol sulfuric acid method (Dubois et al., 1956) using glucose as a standard.

2.2.4. Determination of stress biomarkers

Proline was extracted using 3% sulphosalicylic acid and estimated using L-proline as a standard as described in Bates et al. (1973).

For the determination of H_2O_2 content in microalgae, cells were centrifuged at 10,000 rpm for 10 min and pellet was homogenized in 0.1% w/v TCA solution. The homogenate was centrifuged at 10,000 rpm for 10 min. 0.5 ml supernatant was mixed with 0.5 ml of 10 mM phosphate buffer (pH 7.0) and 1 ml of 1 M KI. Absorbance was recorded at 390 nm. H_2O_2 content was expressed as μmol H_2O_2 g^{-1} fresh weight (FW) (Velikova et al., 2000).

For the determination of lipid peroxidation, microalgal cells were harvested by centrifugation at 10,000 rpm for 10 min. Cell pellet was homogenized in 2 ml of 80:20 (v/v) ethanol:water. The homogenate was centrifuged at 10,000 rpm for 10 min. 1 ml

supernatant was mixed with 1 ml of 0.65% TBA prepared in 20% TCA solution containing 0.01% BHT and heated at 95 °C for 25 min. After cooling at room temperature, content was centrifuged at 10,000 rpm for 10 min. Absorbances of supernatants were read at 450 nm, 532 nm, and 600 nm using Spectra max 190 spectrophotometer. MDA content was calculated using following equation (Chokshi et al., 2015):

$$\text{MDA } (\mu\text{molg}^{-1} \text{ fresh weight}) = [6.45 \times (OD_{532} - OD_{600})] - [0.56 \times OD_{450}]/\text{fresh weight (g)}$$

Activity of antioxidative enzyme APX (EC 1.11.1.11) was evaluated by the changes in absorbance at 290 nm due to ascorbate oxidation and calculated using an extinction coefficient of 2.8 mM^{-1} cm^{-1} (Nakano and Asada, 1981). One APX unit was defined as the enzyme amount that transforms 1 μmol of ascorbate per min.

2.2.5. Mineral composition analysis

For mineral content determination, 100 mg dried microalgal biomass was acid digested by 10 ml of conc. HNO_3 overnight (Santoso et al., 2006). Next day, 2.5 ml $HClO_4$ and 250 μl H_2SO_4 was added and heated over hot plate until white smoke eliminated. The content was dissolved in 100 ml of 2% HCl solution and filtered though 0.22-μm membrane filter. Mineralogical analysis was carried out using flame photometer (Systronics flame photometer 128).

2.3. Statistical analysis

All results are expressed as mean values ± standard deviation. The statistical differences between experimental groups were assessed by analysis of variance (ANOVA) using the Infostat software package (2012). The mean values were compared with LSD test ($p < 0.05$).

3. Results and discussion

3.1. Effect of salinity stress on dry cell weight (DCW) and biomass productivity of microalgae Scenedesmus sp. CCNM 1077

The growth and biomass production of microalgae *Scenedesmus* sp. CCNM 1077 was significantly affected by both single and two stage salinity stress. Addition of NaCl to the growth medium significantly ($p < 0.05$) decreased the DCW and biomass productivity of microalgae *Scenedesmus* sp. CCNM 1077 in dose dependent manner (Fig. 1). Highest amount of DCW (421.85 mg/L) and biomass productivity (22.72 mg/L/Day) was found in culture grown in BG-11 medium. Addition of 100 mM NaCl to the growth medium resulted in 30% reduction in total DCW production. In a similar study, *Tetraselmis subcordiformis* grown under 67.5 g/L NaCl had lower biomass productivity than 5.4 g/L NaCl grown culture (Yao et al., 2013). In order to evaluate the effect of two stage salinity stress, *Scenedesmus* sp. CCNM 1077 was first grown in BG-11 medium and stress was applied by addition of 400 mM of NaCl after 6, 9 and 12 days of cultivation. Fig. 2 shows effect of two stage salinity stress on DCW and biomass productivity of microalgae *Scenedesmus* sp. CCNM 1077. The results show that salinity stress for 3 days during two stage cultivation did not show much reduction in the DCW (404.18 mg/L) and biomass productivity (19.01 mg/L/day) compared to control culture (460.15 mg/L DCW and 22.74 mg/L/day). On the other hand, 6 and 9 days stressed cells showed significant reduction in DCW i.e. 377.99 and 273.52 mg/L, respectively. Similarly, reduction in biomass during two stage salinity stress has also been observed in *D. abundans* (Xia et al., 2014). Compared to single stage, two stage salinity stress did not show much retardation in cellular biomass production that suggests an efficient approach to produce higher lipid and carbohydrate contents without compromising the biomass production.

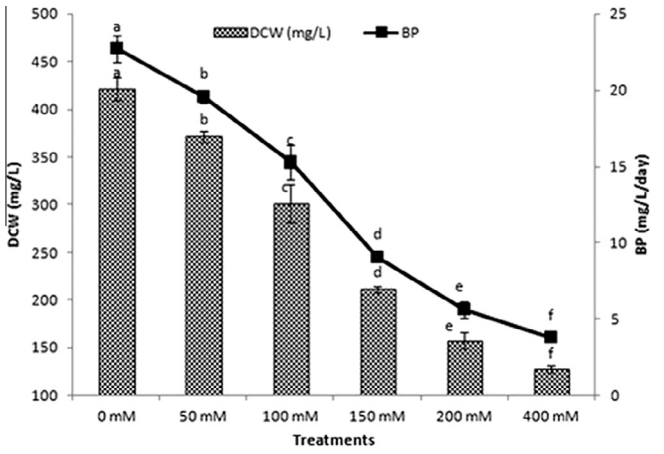

Fig. 1. Effect of different NaCl concentration on DCW and biomass productivity of *Scenedesmus* sp. CCNM 1077. [Different lower case letters above the bars indicate statistically significant difference between treatment means by a Fisher LSD test ($p < 0.05$).]

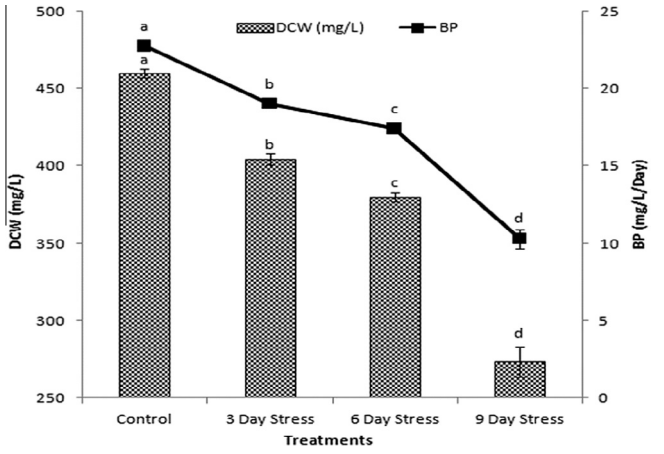

Fig. 2. Effect of two stage salinity stress on DCW and biomass productivity of *Scenedesmus* sp. CCNM 1077. [Different lower case letters above the bars indicate statistically significant difference between treatment means by a Fisher LSD test ($p < 0.05$).]

3.2. Effect of salinity stress on pigments composition of microalgae Scenedesmus sp. CCNM 1077

To assess the impact of salinity stress on photosynthetic process of microalgae *Scenedesmus* sp. CCNM 1077, its pigment composition was measured during single and two stage salinity stress conditions. Degradation or reduction in photosynthetic pigments like Chl-a is a common stress response in plants and microalgae. Under salinity induced oxidative stress higher amount of ROS is accumulated in cells which lower down photosynthetic efficiency of photosynthetic organisms by peroxidation of thylakoids lipid and degrade the PS II complex (Liu et al., 2012). Table 1 shows effect of different initial NaCl concentrations on pigment content of microalgae *Scenedesmus* sp. CCNM 1077. Increase in NaCl concentration in growth medium significantly ($p < 0.05$) reduced the Chl-a, Chl-b and total carotenoids content which was also evident from the color of the culture flasks as high NaCl grown cultures

Table 1
Effect of different NaCl concentration on pigments composition of *Scenedesmus* sp. CCNM 1077. (Different superscript letters within column indicates significant differences at $p < 0.05$.)

Treatments (mM)	Chl-a* (μg/ml)	Chl-b** (μg/ml)	Chl a + b (μg/ml)	Caro*** (μg/ml)	Chl a/b	Caro/Chl a + b
0	6.45 ± 0.42^{a}	4.08 ± 0.29^{a}	10.53 ± 0.71^{a}	1.36 ± 0.06^{a}	1.58 ± 0.02^{d}	0.13 ± 0.003^{e}
50	5.63 ± 0.12^{b}	3.55 ± 0.15^{b}	9.18 ± 0.26^{b}	1.24 ± 0.02^{b}	1.59 ± 0.04^{cd}	0.14 ± 0.004^{de}
100	4.12 ± 0.31^{c}	2.46 ± 0.17^{c}	6.58 ± 0.48^{c}	0.95 ± 0.07^{c}	1.68 ± 0.02^{b}	0.14 ± 0.001^{d}
150	3.21 ± 0.14^{d}	1.72 ± 0.10^{d}	4.92 ± 0.23^{d}	0.79 ± 0.03^{d}	1.87 ± 0.04^{a}	0.16 ± 0.006^{c}
200	2.56 ± 0.12^{e}	1.41 ± 0.07^{e}	3.97 ± 0.20^{e}	0.70 ± 0.01^{e}	1.82 ± 0.01^{a}	0.18 ± 0.005^{b}
400	1.14 ± 0.07^{f}	0.70 ± 0.04^{f}	1.84 ± 0.11^{f}	0.41 ± 0.01^{f}	1.64 ± 0.00^{bc}	0.22 ± 0.009^{a}

* Chl-a: chlorophyll-a.
** Chl-b: chlorophyll-b.
*** Caro: carotenoids.

were yellow in color compared to green color of the control flasks. Addition of 50 mM NaCl to the growth medium reduced the Chl-a content by 12% and total carotenoids by almost 9%. Further increase in the salinity of the growth medium to 400 mM resulted in only 1.14 μg/ml Chl-a and 0.41 μg/ml total carotenoids in microalgae *Scenedesmus* sp. CCNM 1077. However, ratio of Chl a/b or carotenoids/total Chl significantly increased with the increase in the salinity stress which is an indirect indication of salinity induced oxidative stress and reduced PS II activity. High ratios of Chl a/b or carotenoids/total Chl also suggest reduction in the antenna size that may be a protective function against photo oxidative damage. Similarly high Chl a/b or carotenoids/total Chl was also observed in microalgae *Nannochloropsis* sp. cultivated under high salinity stress (Pal et al., 2011). Similar to single stage salinity stress, two stage salinity stress also reduced the photosynthetic activity of microalgae *Scenedesmus* sp. CCNM 1077 and resulted in overall reduction in chlorophylls (Table 2). Almost 50% reduction in Chl-a and Chl-b was observed in 3, 6 and 9 days of salinity stressed cultures compared to BG-11 grown cells. Identical chlorophyll a and b in all treatments indicate that microalgae *Scenedesmus* sp. CCNM 1077 maintain almost similar photosynthetic efficiency and light-harvesting antenna size during salinity stress in two stage cultivation. Similar to present study, Park et al. (2014) also found almost similar photosynthetic pigments in microalgae *D. salina* in salinity range of 0.8–4.5 M. In the present study, no significant difference ($p < 0.05$) in total carotenoids content was found in two stage cultivation process which suggests the protective role of carotenoids under salinity induced photo-oxidative damage. Similar to single stage, two stage salinity stressed cultures had higher Chl a/b or carotenoids/total Chl ratio.

3.3. Effect of salinity stress on biochemical composition of microalgae *Scenedesmus* sp. CCNM 1077

Fig. 3 shows effects of various initial concentrations of NaCl on biochemical composition of microalgae *Scenedesmus* sp. CCNM 1077. Increase in NaCl concentration in growth medium significantly enhanced the total lipid content in microalgae *Scenedesmus* sp. CCNM 1077. Highest lipid content (33.13%) was found in cells cultivated at 400 mM of NaCl, which was about 1.8-folds higher than the control culture (18.98%). No significant difference ($p < 0.05$) in the lipid content was found in cells grown at 150 (24.50%) and 200 mM NaCl (24.57%). Similar to present study, Salama et al. (2014) also found increase in total lipid content with the increase in NaCl in the growth medium from 0 to 400 mM. Fig. 4 shows effect of two stage salinity stress on biochemical composition of microalgae *Scenedesmus* sp. CCNM 1077. Highest lipid content (31%) was found in 9 days stressed cells followed by 6 (29.37%) and 3 (24.77%) days stressed cultures, which were significantly ($p < 0.05$) higher than the control culture (18.23%).

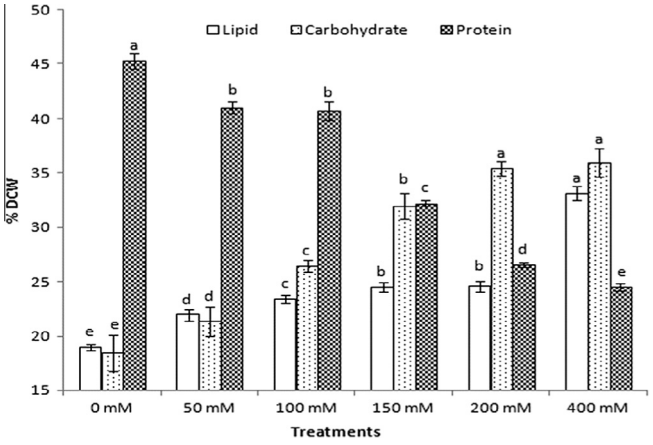

Fig. 3. Effect of different NaCl concentration on biochemical composition of *Scenedesmus* sp. CCNM 1077. [Different lower case letters above the bars indicate statistically significant difference between treatment means by a Fisher LSD test ($p < 0.05$).]

Microalgal total lipid was further fractionated into neutral, glyco and phospho lipids through column chromatography to understand lipid class composition under salinity stress. Fig. 5 shows that increase in salinity stress enhanced the neutral lipid (NLs) content in microalgae. Highest NL content (76% of total lipid) was found in cells cultivated in 400 mM NaCl supplemented growth medium, which was about 1.12-folds higher than the

Table 2
Effect of two stage salinity stress on pigments composition of *Scenedesmus* sp. CCNM 1077. (Different superscript letters within column indicates significant differences at $p < 0.05$.)

Treatments	Chl-a* (μg/ml)	Chl-b** (μg/ml)	Chl a + b (μg/ml)	Caro*** (μg/ml)	Chl a/b	Caro/Chl a + b
Control	6.85 ± 0.60^{a}	3.92 ± 0.39^{a}	10.77 ± 0.97^{a}	1.54 ± 0.10^{a}	1.75 ± 0.06^{b}	0.14 ± 0.01^{b}
3 Day stress	3.34 ± 0.18^{bc}	1.80 ± 0.08^{b}	5.14 ± 0.25^{bc}	0.87 ± 0.04^{b}	1.86 ± 0.05^{ab}	0.17 ± 0.00^{a}
6 Day stress	3.12 ± 0.13^{c}	1.67 ± 0.08^{b}	4.79 ± 0.21^{c}	0.82 ± 0.04^{b}	1.87 ± 0.01^{ab}	0.17 ± 0.00^{a}
9 Day stress	3.89 ± 0.07^{b}	2.0 ± 0.09^{b}	5.89 ± 0.11^{b}	0.91 ± 0.05^{b}	1.95 ± 0.10^{a}	0.15 ± 0.01^{b}

* Chl-a: chlorophyll-a.
** Chl-b: chlorophyll-b.
*** Caro: carotenoids.

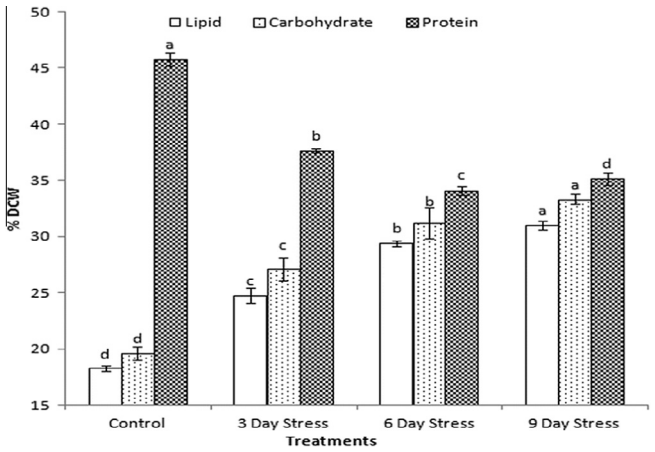

Fig. 4. Effect of two stage salinity stress on biochemical composition of *Scenedesmus* sp. CCNM 1077. [Different lower case letters above the bars indicate statistically significant difference between treatment means by a Fisher LSD test ($p < 0.05$).]

control culture (67% of total lipid). Addition of 100 and 150 mM NaCl to the BG-11 medium also enhanced the NL content i.e. 70% and 73% of total lipid, respectively. Increased NL content during salinity stress gives rigidity to the cell membrane thereby helping in maintenance of mineral ion regulation in microalgal cells (Lu et al., 2012). Similar to NL content, PL content was also found to increase with the increase in salinity stress. Highest PL content was found in 400 mM NaCl stress cultures. However, no difference in PL content was found in control and 50 mM NaCl (16%) grown cells. GL content decreased with the increase in salinity of growth medium. During two stage salinity stress highest amount of NL (77%) was found in 9 days stressed culture followed by 3 and 6 days stressed cultures (75% of total lipid). Similar to single stage cultivation, GL content also decreased with the increase in salinity stress during two stage cultivation. Enhancement in PL content in microalgae *Scenedesmus* sp. CCNM 1077 during salinity stress might be due to membrane remodeling that is important for compartmentalization to withstand the salinity induced osmotic stress. It has been shown by Yang et al. (2014) that microalgae *Monoraphidium dybowskii* LB50 synthesize more PL to reduce membrane fluidity and permeability, which helps microalgae to cope up osmotic balance under high salinity.

Carbohydrate content also increased with the increase in NaCl concentration in growth medium from 0 to 400 mM (Fig. 3). Highest amount of carbohydrate (35.91%) was found in cells cultivated in 400 mM salinity. However, there was no significant difference in carbohydrate accumulation by 200 mM (35.41%) and 400 mM (35.91%) salinity stressed cells. Similar amount of carbohydrate but higher lipid content in 400 mM grown culture might be because carbohydrate and lipid synthesis is parallel phenomenon in microalgae and reduced energy is first stored as a carbohydrate and excess is converted into lipids (Fan et al., 2012). Similar to single stage, two stage salinity stress also significantly ($p < 0.05$) enhanced the carbohydrate accumulation in microalgae *Scenedesmus* sp. CCNM 1077. Highest carbohydrate accumulation was found in 9 days stressed culture (33.33%) followed by 6 (31.24) and 3 (27.11%) days stressed cultures. During this study, carbohydrate content was estimated from lipid

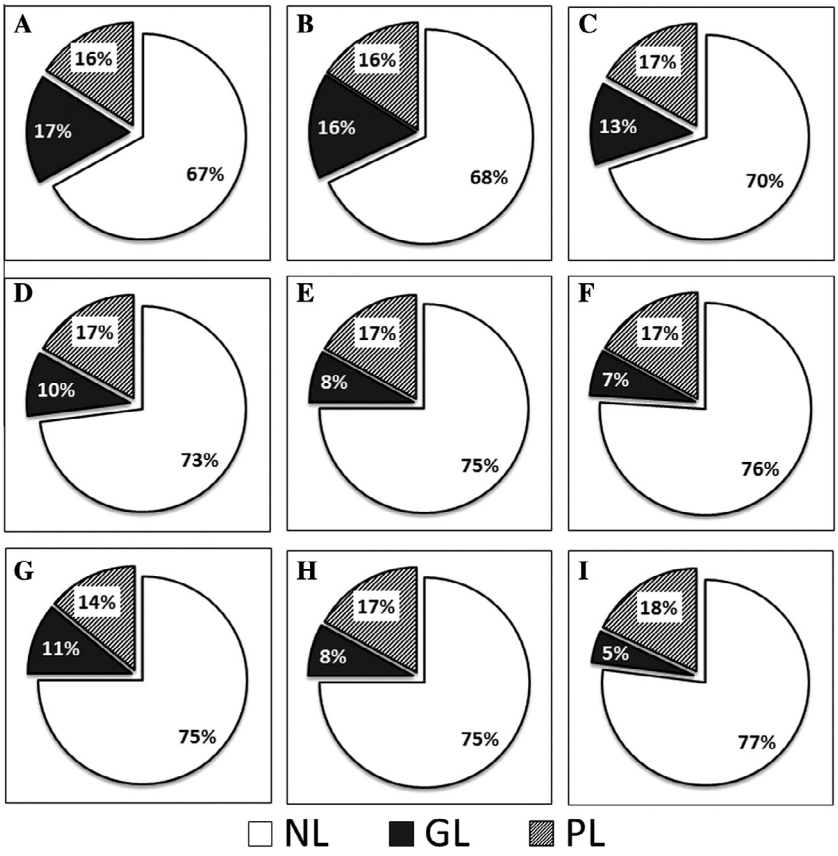

Fig. 5. Changes in neutral lipids (NL), glycolipids (GL) and phospholipids (PL) concentration of *Scenedesmus* sp. CCNM 1077 under single stage ((A) 0 mM NaCl; (B) 50 mM NaCl; (C) 100 mM NaCl; (D) 150 mM NaCl; (E) 200 mM NaCl; (F) 400 mM NaCl) and two stage ((G) 3 day stress of 400 mM NaCl; (H) 6 day stress of 400 mM NaCl; (I) 9 day stress of 400 mM NaCl) salinity stress.

extracted microalgal biomass, which helps in co-production of biodiesel and bio-ethanol simultaneously, which will ultimately reduce the overall cost of biofuel production process. Similar to this study, high glycogen accumulation was also observed when cyanobacteria *Synechococcus* sp. strain PCC 7002 grown in brackish or seawater as compared to fresh water (Aikawa et al., 2014). Compared to other nutritional starvations like nitrate starvation, comparatively lower lipid and carbohydrate accumulation during salinity stress was observed. One of the possible reasons behind this may be under salinity stress cells synthesize various low molecular weight organic osmolytes like proline, glycine betaine etc. to withstand high osmotic shock. Moreover, under high salinity, cell respiration activity also increases those results in the degradation of energy rich storage compounds like lipid and starch of microalgae (Yao et al., 2013).

Fig. 3 also shows effect of NaCl addition on protein content of microalgae *Scenedesmus* sp. CCNM 1077. Similar to pigments, salinity stress significantly ($p < 0.05$) reduced the cellular protein content in microalgae *Scenedesmus* sp. CCNM 1077. Lowest protein content was found in cells grown under 400 mM NaCl (24.53%). During two stage cultivation, protein content of control culture was 45.78%, while 3, 6 and 9 days salinity stressed cultures had marked reduction in protein content i.e. 37.66%, 34.14% and 35.15%, respectively (Fig. 4). Under salinity stress, down regulation of genes involved in primary metabolism and protein synthesis as well as activation of genes related to autophagy and protein degradation has been observed in higher plant and macroalgae (Dittami et al., 2011).

3.4. Effect of salinity stress on stress biomarkers of microalgae *Scenedesmus sp. CCNM 1077*

Under unfavorable environment conditions microalgae produce various reactive oxygen species (ROS) like H_2O_2, O_2^- OH^- etc. These ROS are highly toxic and damage protein, lipid, DNA and other cellular macromolecules thereby inhibiting the cell growth and ultimately leading to cell death. To protect cells from damage caused by ROS, plant and algae have several innate defense systems like antioxidative enzymes APX and catalase as well as non-enzymatic molecules like proline, ascorbic acid etc. (Chokshi et al., 2015). It has been known since long that under various nutritional starvation conditions microalgae accumulate large amount of TAGs that can be easily converted into biodiesel. However, no systematic study on salinity induced biofuel production potential as well as stress biomarkers is reported till today. In the present study, to understand the role of ROS in lipid accumulation, we studied H_2O_2 content, lipid peroxidation along with activity of antioxidative enzyme APX as well as proline accumulation under salinity stress conditions.

Table 3 shows effect of various NaCl concentrations on H_2O_2, MDA and APX content of microalgae *Scenedesmus* sp. CCNM 1077. From the data of growth and pigment content, it is clear that increase in the salinity of the growth medium significantly increases the stress in microalgae *Scenedesmus* sp. CCNM 1077. Similarly, higher accumulation of H_2O_2 content was also observed with the increase in NaCl in growth medium. Highest amount of H_2O_2 was observed in cells grown at 400 mM NaCl [36.82 μM g^{-1} fresh weight (FW)] followed by 200 mM (22.50 μM g^{-1} FW) and 150 mM (19.40 μM g^{-1} FW) NaCl grown cultures, which was significantly higher than the control culture (3.85 μM g^{-1} FW). During two stage cultivation, increase in stress duration significantly ($p < 0.05$) enhanced the H_2O_2 content in microalgae *Scenedesmus* sp. CCNM 1077. Highest H_2O_2 content was observed in 9 days (29.75 μM g^{-1} FW) followed by 6 days (15.09 μM g^{-1} FW) stressed cultures. However, 3 days stressed cultures had H_2O_2 content of 16.81 μM g^{-1} FW that was higher than the 6 days stressed culture. The sudden increase in H_2O_2 in 3 days stressed culture is mainly due to sudden change in the salinity of the culture medium.

Lipid peroxidation is another most commonly used stress biomarker measured in terms of malondialdehyde (MDA) content in the cells. The MDA level was significantly ($p < 0.05$) higher in 50 mM NaCl grown culture (0.052 μM g^{-1} FW) compared to control culture (0.042 μM g^{-1} FW). Its level further increased to 0.081 and 0.1 μM g^{-1} FW in 200 and 400 mM NaCl grown cultures, respectively. Similar to present study, biofuel producing microalgae *C. sorokiniana* C3 also accumulated high amount of MDA under 2 and 8 days of nitrogen stress (Zhang et al., 2013). The increased levels of MDA as well as H_2O_2 under salinity stress indicate generation of oxidative stress in the microalgae *Scenedesmus* sp. CCNM 1077. During two stage cultivation, cells with salinity stress of 400 mM NaCl had marginal increase in MDA level i.e. 0.065 and 0.056 μM g^{-1} FW in 6 and 3 days stressed cultures, respectively. However, 9 days stressed culture had significantly higher MDA (0.070 μM g^{-1} FW) compared to control (0.038 μM g^{-1} FW) culture. Culture stressed for 3 days at 400 mM NaCl had higher NL (75% of total lipid) with marginal loss in DCW and biomass productivity compared to control culture making it an efficient strategy to enhance biofuel production potential of microalgae *Scenedesmus* sp. CCNM 1077.

Highly accumulated ROS in the cells are neutralized by various antioxidative enzymes like APX, superoxide dismutase, catalase etc. (Chokshi et al., 2015). In order to evaluate the ability of cells to mitigate ROS generated through salinity stress, APX activity was measured during both single and two stage salinity stress conditions in microalgae *Scenedesmus* sp. CCNM 1077. From the results, it is clear that although cells were stressed in all tested salinity, increased activity of APX was observed with the increase in NaCl concentration in the growth medium. Highest APX activity (233.59% compared to control culture) was found in cells cultivated in 200 mM NaCl (Table 3). Similar to single stage, significantly higher activity of APX was also observed during two stage salinity stress i.e. 157.80%, 165.85% and 311.91% higher compared to control in 3, 6 and 9 days stressed cultures, respectively (Table 4). Recently, it has been reported that oxidative stress tolerant microalgae are efficient for biofuel production than non oxidative tolerant microalgae (Osundeko et al., 2013). Higher activities

Table 3
Effect of different NaCl concentration on H_2O_2, MDA, APX, proline and mineral ration of *Scenedesmus* sp. CCNM 1077. (Different superscript letters within column indicates significant differences at $p < 0.05$.)

Treatments (mM)	H_2O_2 (μM g^{-1} FW)	MDA (μM g^{-1} FW)	APX (% activity)	Proline (μM g^{-1} DW)	Na^+/K^+	Na^+/Ca^{2+}
0	3.85 ± 0.55^{c}	0.042 ± 0.012^{c}	100 ± 0.22^{f}	29.21 ± 0.57^{f}	0.35^{e}	0.69^{a}
50	11.81 ± 3.45^{bc}	0.052 ± 0.01^{c}	131.78 ± 0.26^{e}	56.33 ± 2.90^{e}	1.04^{d}	1.33^{d}
100	16.76 ± 0.77^{bc}	0.067 ± 0.009^{b}	133.03 ± 0.26^{d}	69.78 ± 3.15^{d}	1.23^{c}	1.38^{c}
150	19.40 ± 0.72^{b}	0.071 ± 0.009^{b}	178.73 ± 0.35^{b}	75.70 ± 2.23^{c}	1.41^{b}	1.44^{b}
200	22.50 ± 1.47^{ab}	0.081 ± 0.002^{b}	233.13 ± 0.46^{a}	109.27 ± 0.49^{b}	1.40^{b}	1.33^{d}
400	36.82 ± 19.58^{a}	0.1 ± 0.015^{a}	150.50 ± 0.3^{c}	131.51 ± 3.68^{a}	3.54^{a}	2.38^{a}

Table 4
Effect of two stage salinity stress on H_2O_2, MDA, APX, Proline and mineral ratio of *Scenedesmus* sp. CCNM 1077. (Different superscript letters within column indicates significant differences at $p < 0.05$.)

Treatments	H_2O_2 (μM g^{-1} FW)	MDA (μM g^{-1} FW)	APX (% activity)	Proline (μM g^{-1} DW)	Na^+/K^+	Na^+/Ca^{2+}
Control	3.26 ± 0.11^c	0.038 ± 0.002^b	100 ± 0.33^d	31.35 ± 2.4^c	0.37^d	0.71^c
3 Day stress	16.81 ± 4.75^b	0.056 ± 0.003^a	158.39 ± 0.59^c	56.77 ± 4.56^b	2.36^c	1.90^b
6 Day stress	15.09 ± 0.88^b	0.065 ± 0.013^a	165.94 ± 0.084^b	73.52 ± 4.22^a	7.15^a	2.86^a
9 Day stress	29.75 ± 2.34^a	0.070 ± 0.005^a	311.92 ± 0.039^a	69.64 ± 1.47^a	5.93^b	2.88^a

of APX under salinity stress shows ability of microalgae *Scenedesmus* sp. CCNM 1077 to tolerate high oxidative stress generated due to high salinity.

Apart from enzymatic antioxidant defense, microalgae also accumulate small organic non- enzymatic antioxidant compounds like proline, glycine betaine, ascorbic acid etc. under abiotic stress conditions. A dose dependent increase in proline content in microalgae *Scenedesmus* sp. CCNM 1077 under salinity stress indicate its adaptive response towards salinity induced oxidative stress (Table 3). Addition of 50 mM NaCl in the growth medium enhanced free proline content in microalgae *Scenedesmus* sp. CCNM 1077 by almost 1.9-folds compared to control.

Further increasing the salinity resulted in higher accumulation of proline in the cells i.e. 69.78, 109.27 and 131.51 μM g^{-1} DW in 100, 200 and 400 mM NaCl grown culture, respectively. Proline is known to accumulate under various adverse environmental conditions like nitrogen starvation (Pancha et al., 2014), temperature stress (Chokshi et al., 2015) etc. Proline helps in maintenance of cytosolic pH and stabilization of subcellular structures. It also works as a compatible solute and ROS scavenging agent. During two stage salinity stress, highest proline (73.52 μM g^{-1} DW) was accumulated in 6 days stressed culture while 3 and 9 days stressed cultures had 69.64 and 56.77 μM g^{-1} DW proline, respectively.

3.5. Effect of salinity stress on mineral content of microalgae *Scenedesmus sp. CCNM 1077*

The single and two stage salinity stress significantly affected the Na^+, K^+ and Ca^{2+} level in microalgae *Scenedesmus* sp. CCNM 1077. Increase in salinity in the growth medium significantly enhanced the Na^+/K^+ and Na^+/Ca^{2+} ratio in the cells (Tables 3 and 4). At higher salinity, cell growth rate and biomass productivity was reduced which might be due to electrolyte leakage which stimulates cellular protease and endonucleases and ultimately lead to cell death (Parida and Das, 2005). Highest Na^+/K^+ and Na^+/Ca^{2+} ratio (3.52 and 2.38, respectively) were found in cells cultivated under 400 mM salinity, which was significantly higher than those of the control culture (0.36 and 0.70, respectively). Maintenance of the level of these ions is mainly important for ions homeostasis and normal functioning of the cells. At high salinity, ion homeostasis is generally maintained by various enzymes like Na^+-ATPase, H^+-ATPase. Salinity tolerance of microalgae is mainly due to removal of toxic Na^+ ions by controlling their influx and efflux activated by salinity induced Na^+/H^+ antiporter (Strizh et al., 2004). Addition of 50 mM NaCl in the growth medium enhanced the Na^+/K^+ and Na^+/Ca^{2+} ratios by 2.97 and 1.92-folds, respectively, compared to control culture. However, cells grown with 150 and 200 mM salinity stress did not show much alteration in Na^+/K^+ and Na^+/Ca^{2+} ratio. Recent study shows importance of Ca^{2+} signal transduction in higher accumulation of neutral lipid by microalgae *Chlorella* sp. C2 (Chen et al., 2014). In two stage salinity stress, ratios of Na^+/K^+ and Na^+/Ca^{2+} increased with the increase in duration of stress. Highest Na^+/K^+ ratio (7.15) was found in cells cultivated with 6 day salinity stress , whereas no significant difference was found in Na^+/Ca^{2+} ratio in cells cultivated in 6 (2.86) and 9 (2.88) days of salinity stress.

4. Conclusion

In summary, results of the present study show that *Scenedesmus* sp. CCNM 1077 grown with 400 mM salinity stress under single stage cultivation accumulated higher carbohydrate and lipid contents but resulted in lower biomass. During two stage cultivation, 3 days of stress of 400 mM NaCl enhanced the lipid and carbohydrate contents with negligible biomass reduction. Higher H_2O_2, MDA, APX and proline contents show link between ROS and lipid accumulation. Findings of the present study gives an idea about physiological mechanism to withstand salinity induced oxidative stress and biofuel production potential of freshwater microalgae *Scenedesmus* sp. CCNM 1077.

Acknowledgements

CSIR-CSMCRI Registration Number: 024/2015. I.P. and R.M. would like acknowledge CSIR for SRF fellowship. K.C., K.T. and S.K.P. would like to acknowledge CSC 0203 and CSC 0105 for their financial support. The continuous support from Dr. Arvind Kumar, DC, SMC and the entire staff of the division is gratefully acknowledged. The authors would like to thank Dr. Parimal Paul, DC, ADCIF, CSIR-CSMCRI, Bhavnagar for their help during the analysis. I.P., K.C., R.M. and K.T. also acknowledge AcSIR for their Ph.D. enrollment. We would also like acknowledge all lab colleagues for their help during the study.

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