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A N I N T E R N A T I O N A L J O U R N A L

Changes of the Total Lipid and Omega-3 Fatty Acid Contents in two Microalgae *Dunaliella Salina* and *Chlorella Vulgaris* Under Salt Stress

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ABSTRACT

Effect of salt stress on biomass, cell number, contents of total lipid, omega-3 fatty acids, including ALA (Alpha Linolenic Acid), EPA (Eicosapentaenoic Acid) and DHA (Docosahexaenoic Acid) and their biosynthetic pathway intermediates (palmitic acid, stearic acid, oleic acid and linoleic acid) of two microalgae Dunaliella salina and Chlorella vulgaris were investigated. Dilution stress from 1.5 to 0.5 M NaCl and salt stress from 1.5 to 3 M NaCl were incorporated into the D. salina medium. Salt stress of 200 mM NaCl was also applied to C. vulgaris culture. Results indicated that increasing salt concentration resulted in the reduced growth rate of C. vulgaris and substantial increase of the total lipid content in both species. Proper growth rate of D. salina observed at 1.5 M of NaCl, but higher and lower concentrations led to the decreased growth rate of D. salina. In addition, considerable increase in the degree of fatty acid unsaturation and thereby the total omega 3 fatty acid content of D. salina was observed under salt stress. Salt stress had little positive effect on the amount of total omega-3 fatty acid of C. vulgaris due to the slight increase of the EPA content. Results showed that salt stress is an effective way for enhancing the total lipid and omega-3 fatty acid production in D. salina.

Key words: *Dunaliella*; *Chlorella*; Salt stress; Lipid; Omega-3

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INTRODUCTION

One of the most important components of cells is lipids. They are essential for storing energy [1]. Microalgae such as *Dunaliella*, *Chlorella* and *Spirulina* are rich sources of lipid and PUFAs (Poly Unsaturated Fatty Acids) such as ALA (Alpha Linolenic Acid) (C18:3 ω3), EPA (Eicosapentaenoic Acid) (C20:5 ω3), DHA (Docosahexaenoic Acid) (C22:6 ω3), Arachidonic acid (C20:4 ω6) and Gamma linolenic acid (C18:3 ω6) [2, 3]. These algae are capable of rapid growth, high biomass production and have simple nutritional requirements. In recent years, many studies were undertaken to evaluate the effect of various treatments on microalgal lipid quantity and quality in particular, omega-3 fatty acids including ALA, EPA, and DHA [4-6]. As, EPA and DHA are important for reducing blood cholesterol, prevention of cardiovascular disease, obesity, depression and other disorders [7-9], these compounds could be used for improving human health. Recently, high amount of lipid extracted from microalgae has been considered for converting into biodiesel [10]. There are some reports that conditions like salt stress, nutrient limitation, high irradiance and temperature affect the lipid contents and fatty acid compositions in microalga [11-13]. Microalgae suggested as a new source of omega-3 fatty acids. Fish is the current source of omega-3 which is an inadequate source for increasing omega-3 fatty acid demands [14]. On the other hand, microalgal omega-3 fatty acids have no contaminations by heavy metals and other poisons [15]. It is worth noting that omega-3 fatty acids of fish oil fundamentally derived from microalgae which eaten by fish [16]. In this viewpoint, microalgae are more suitable source than fish for EPA and DHA provision. *Dunaliella* is a halotolerant, unicellular green alga, which does not have cell wall [17]. Therefore, its lipid could be extracted easily. Moreover, it can grow to high biomass concentration and survive under environmental stresses such as high salt concentration of sea water [18]. *Chlorella* is a unicellular, fresh water alga, which has a cell wall and is able to produce high lipid amounts [19]. *Chlorella* has simple growth conditions and limited nutritional demands. Furthermore, it can produce high amounts of biomass in a short time [2]. Salt stress is one of the most important stresses that microalgae subjected to it [21]. The present study was performed to test the effect of different concentrations of NaCl on the growth rate and amounts of total lipid, omega-3 fatty acids and their biosynthetic intermediates in *Dunaliella salina* and *Chlorella vulgaris*.

MATERIAL AND METHODS

Algae source

Microalgae *Dunaliella salina* UTEX 200 and *Chlorella vulgaris* UTEX 265 were obtained from the algae collection held by the University of Texas.

Algal culture conditions

The modified Johnson's medium [22] was used for the cultivation of *D. salina*. *C. vulgaris* was grown in the modified Basal medium [23]. The cultures prepared in separated 3000 ml cotton plugged Erlenmeyer flasks containing 1000 ml of sterilized medium supplemented with the various NaCl concentrations. The pH of media was adjusted to 7-7.5. *D. salina* cells cultured in the concentration of 1.5 M NaCl as control. The dilution stress from 1.5 to 0.5 M NaCl was applied by adding the required amount of Johnson's medium without NaCl to *D. salina* cells grown in 1.5 M of medium as control. In contrast, the salt stress from 1.5 to 3M NaCl, was applied by adding appropriate amount of 4.5 M NaCl of Johnson's medium to the control cultures of *D. salina* to obtain final concentration of 3 M NaCl. As *D. salina*

is a halotolerant microalga but *C. vulgaris* is not, the NaCl concentrations used for salt stress in *D. salina* cultures are lethal for *C. vulgaris*. Accordingly, the most suitable concentration of NaCl for salt stress in *C. vulgaris* determined by applying concentrations ranging from 50 to 600 mM of NaCl. As the severe growth reduction of *C. vulgaris* cells occurred in 300 and 400 mM and the death of them observed in 600 and 700 mM, therefore, 200 mM concentration preferred for NaCl treatment of *C. vulgaris*. All the cell inoculations and stresses were applied in the aseptic conditions into the each Erlenmeyer flask to nearly reach the initial cell number of 3.1×10^6 per ml in *D. salina* and 1.8×10^6 per ml in *C. vulgaris* cultures. The cultures were illuminated using fluorescent lamps with 100 µmol photon m^{-2} s^{-1} intensity in the light cycle of 16 h light/ 8 h dark. The algae cultures were maintained in the controlled condition with orbital shaking at 100 RPM and 25±1 ºC for the 15 days period. The algae samples were taken on the days of 0, 5, 10 and 15 to estimate the variation of cell number, dry weight and total lipid, omega-3 fatty acids and their biosynthetic intermediates contents of both species under salt stress. All the experiments were performed in three replicates.

Algae growth assessment

The cell number was calculated [24] with the hemocytometer using the light microscope.

Biomass dry weight assay

100 ml of the culture was sampled and centrifuged at 1000 g for 15 minutes, which was led to the formation of two phases. The upper phase as supernatant was removed and the lower phase as the sedimented cells was dried at the room temperature. For *C. vulgaris* cells treated with NaCl, the pellet was washed gently by Basal medium containing no NaCl. The suspension was centrifuged again and the pellet was dried and then weighted. As *D. salina* has no cell wall, washing the cells with distilled water or medium containing no NaCl led to swelling cell and membrane disruption. Therefore, the weight of the NaCl residue in the pellet was calculated and then subtracted from the total weight for measuring the exact biomass dry weight.

Lipid extraction

The biomass was obtained through the centrifugation of 100 ml of the culture at 1000 g for 15 minutes. The lipids extracted according to the Bligh and Dyer method [25]. Chloroform and methanol added to the biomass in two steps and mixed well. The cell disruption was performed using a homogenizer (Heidolph DIAX 900, Germany). Afterward, 1% NaCl added to obtain the ratio of 2 chloroform: 1 methanol: 1 NaCl, and then, was centrifuged at 1000 g for 10 minutes. The lower phase containing all the lipids and chloroform was separated and desiccated under the nitrogen gas. The remained lipids were weighed and maintained at -20°C. All the materials were provided by Merck Corporation.

Fatty acid analysis

The extracted lipids were transmethylated through the Ortega *et al*. method [26]. The obtained methyl esters of fatty acids analyzed with the gas chromatograph (Agilent 19091J-413, USA) equipped with the FID detector and the column (HP-5) with a 30 m long, 0.32 mm inside diameter and 0.25 µm stationary phase thickness. Helium was utilized as the carrier gas. The initial column temperature was 60ºC. The detector and injector temperatures were 250ºC. The transmethylated fatty acids were injected into the gas chromatograph in the volume of 1 µL and the 1:10 ratio in the

split manner. Types and amounts of fatty acids consist of ALA, EPA, DHA, palmitic acid, stearic acid, oleic acid and linoleic acid were identified using the chromatogram of transmethylated samples compared with that of fatty acid standard (Sigma Chemical Co).

Statistical analysis

In all experiments, statistical comparisons performed using ANOVA with Duncan test at $P<0.05$.

RESULTS

Effect of salt stress on the cell number and biomass dry weight

As observed in Fig. 1A and C, the cell number and biomass dry weight of *D. salina* was decreased by the changing NaCl concentration in the medium from 1.5 (control) to 0.5 M and 1.5 to 3 M. The optimum cell division and biomass dry weight obtained at 1.5 M, which is respectively equivalent to 83×10^5 cell.ml^{-1} and 12×10^2 mg.L^{-1} on the 15th day. The cell number and biomass dry weight values of *C. vulgaris* significantly decreased to 11×10^5 cell.ml^{-1} and 14×10^2 mg.L^{-1} respectively at 200 mM of NaCl in the medium as compared to control (Fig. 1B and D). In general, cell division and biomass production in *C. vulgaris* is higher than *D. salina*.

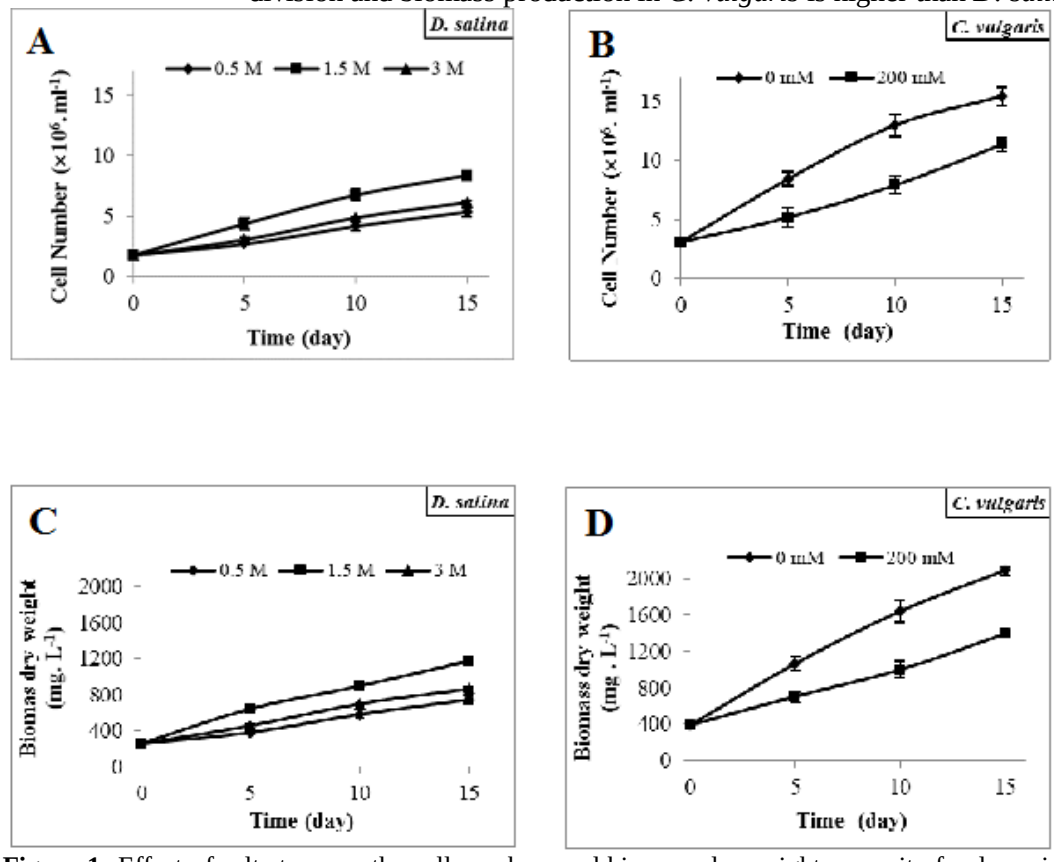

Figure 1- Effect of salt stress on the cell numbers and biomass dry weight per unit of volume in *D. salina* (A, C) and *C. vulgaris* (B, D). The data are expressed as mean ± standard deviation of three replicates.

Effect of salt stress on the total lipid content

According to Fig. 2A, the highest amount of total lipid was obtained at 3M NaCl in *D. salina* cells. This was nearly equivalent to 143 mg. g dw^{-1} on 15th day, which was about 14% more than the start of the experiment and 43% higher than the control. There was no significant difference between the total lipid content of 3M NaCl on

days 5, 10 and 15. When the NaCl concentration was 1.5 M (control), a 21% decrease was observed until the 15th day, whereas dilution stress from 1.5 to 0.5 M was led to 16% decrease as compared to the control and 33% reduction compared with the commencement of the experiment (Fig. 2A). While, 200 mM NaCl in *C. vulgaris* media, resulted in a 82% enhancement in the total lipid production (154 mg. g dw-1) compared with no NaCl in the medium (control) on the 15th day (Fig. 2B).

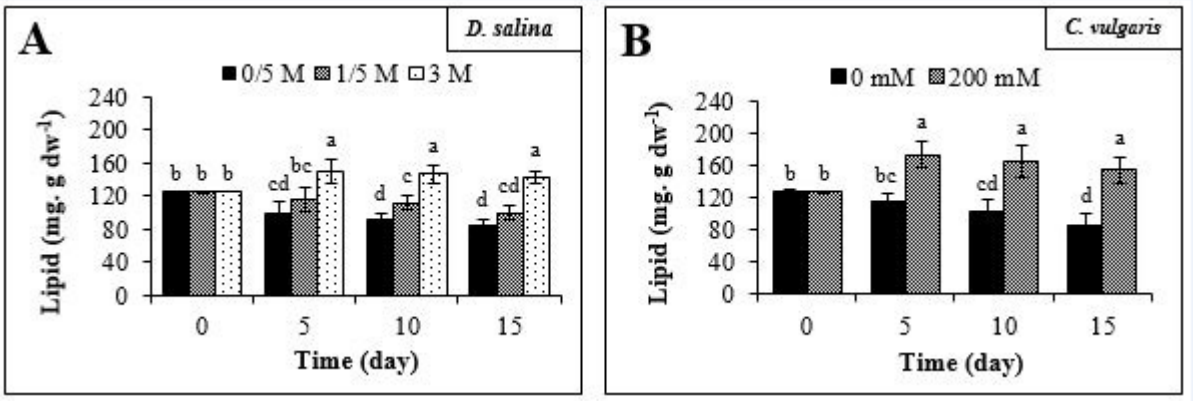

Figure 2- Effect of salt stress on the total lipid content per unit of biomass dry weight in *D. salina* (A) and *C. vulgaris* (B). The data are expressed as mean ± standard deviation of three replicates. Different letters indicate significant differences between various concentrations in each species at $p<0.05$ according to Duncan test.

Effect of salt stress on the ALA content

The *D. salina* cells grown at the NaCl concentration higher than 1.5 M produced the highest yield of ALA, which increased 70% after 15 days and also showed 66% as compared to the control (Fig. 3A). While, the minimum yield of ALA occurred in the culture with 0.5 M of NaCl, which indicated about 44% decrease in comparison with the beginning of the experiment and decreased 46% as compared to the control (1.5 M) on day 15. As presented in Fig. 3B, in *C. vulgaris* cultures, the higher ALA content corresponded to 200 mM of NaCl, which increased about 154% as compared with the control and 75% after 15 days.

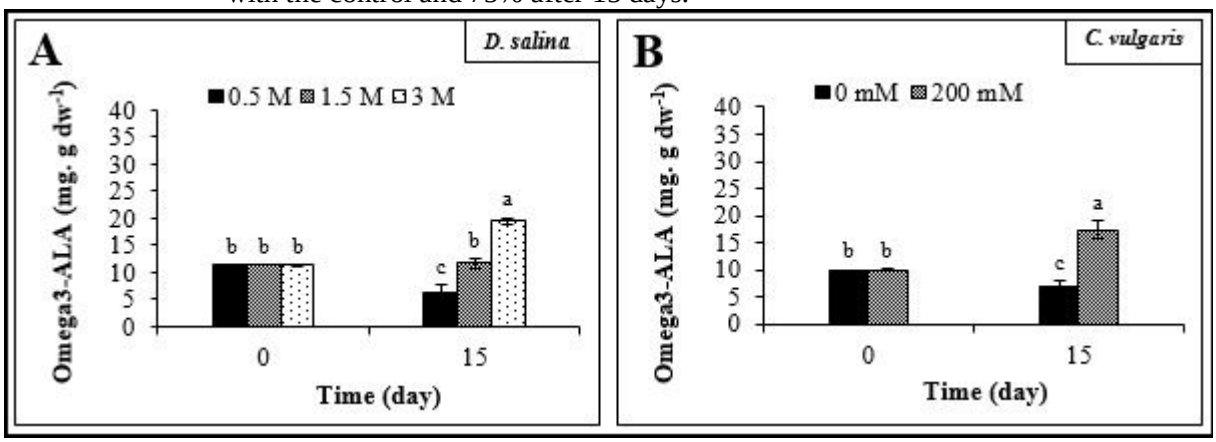

Figure 3- Effect of salt stress on the ALA content per unit of biomass dry weight in *D. salina* (A) and *C. vulgaris* (B). The data are expressed as mean ± standard deviation of three replicates. Different letters indicate significant differences between various concentrations in each species at $p<0.05$ according to Duncan test.

Effect of salt stress on the EPA content

As shown in Fig. 4A, hyper osmotic shock in *D. salina* culture brought about the 92% increase in EPA value in comparison with the control and enhanced 60% as compared with day 0. Additionally, hypo osmotic shock was led to the lowest EPA value on day 15. As well as, the higher EPA production occurred at 200 mM of NaCl

in *C. vulgaris*, which enhanced the EPA content about 76% compared to day 0 and 136% as compared with the control 15 days after commencement of the experiment (Fig. 4B).

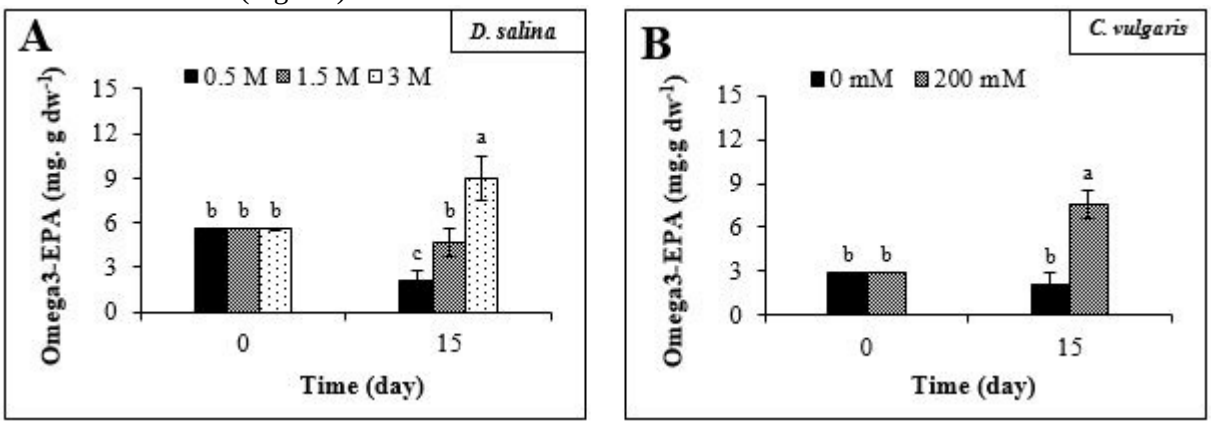

Figure 4 - Effect of salt stress on the EPA content per unit of biomass dry weight in *D. salina* (A) and *C. vulgaris* (B). The data are expressed as mean ± standard deviation of three replicates. Different letters indicate significant differences between various concentrations in each species at $p<0.05$ according to Duncan test.

Effect of salt stress on the DHA content

As shown in Fig. 5A, by adding NaCl to the control medium from 1.5 M to 3 M, significantly enhanced the DHA content of *D. salina*, which is equivalent to about 22% in comparison with day 0 and 309% compared with the control on the 15th day. Conversely, by diluting the control medium (1.5 M) to 0.5 M, considerably reduced the DHA content insofar as not detected on day 15. The DHA value of *C. vulgaris* was not influenced by 200 mM concentration of NaCl (Fig. 5B).

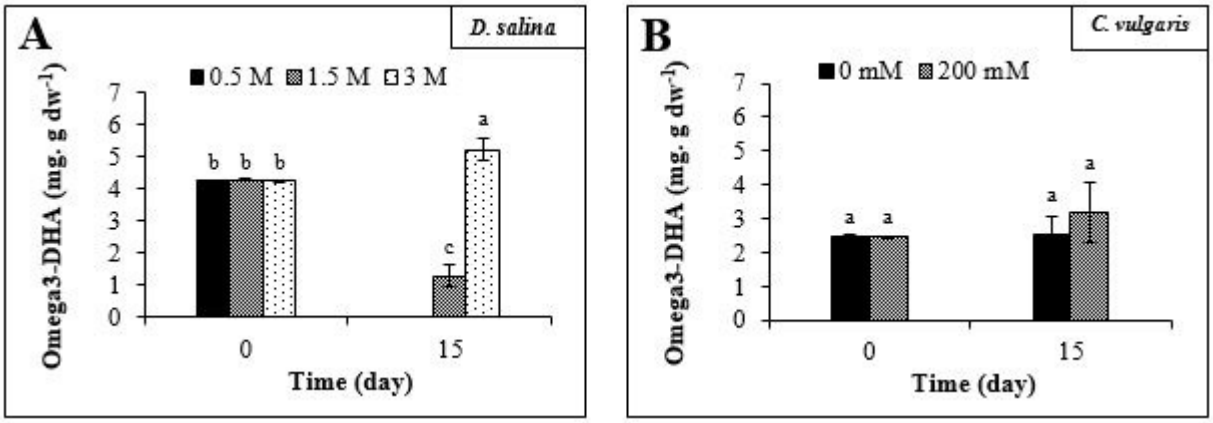

Figure 5 - Effect of salt stress on the DHA content per unit of biomass dry weight in *D. salina* (A) and *C. vulgaris* (B). The data are expressed as mean ± standard deviation of three replicates. Different letters indicate significant differences between various concentrations in each species at $p<0.05$ according to Duncan test.

Effect of salt stress on the total omega-3 content

As presented in Fig. 6A, the total omega-3 fatty acid content in *D. salina* was enhanced to 33.675 mg. g dw^{-1} with the increase of salinity to 3 M which is generally about 90%. The 52% decrease was observed when the salinity decreased to 0.5 M in comparison to the control on the 15th day. As well as, the total omega-3 content increased 58% compared to day 0 under hyper osmotic shock. As illustrated in Fig. 6B, the NaCl concentration of 200 mM caused the increase of the total omega-3 value to 25.67 mg. g dw^{-1} in *C. vulgaris*, which is equivalent to 123% as compared with the control on day 15. Salt stress increased the total omega-3 content about 69% after 15 days as well.

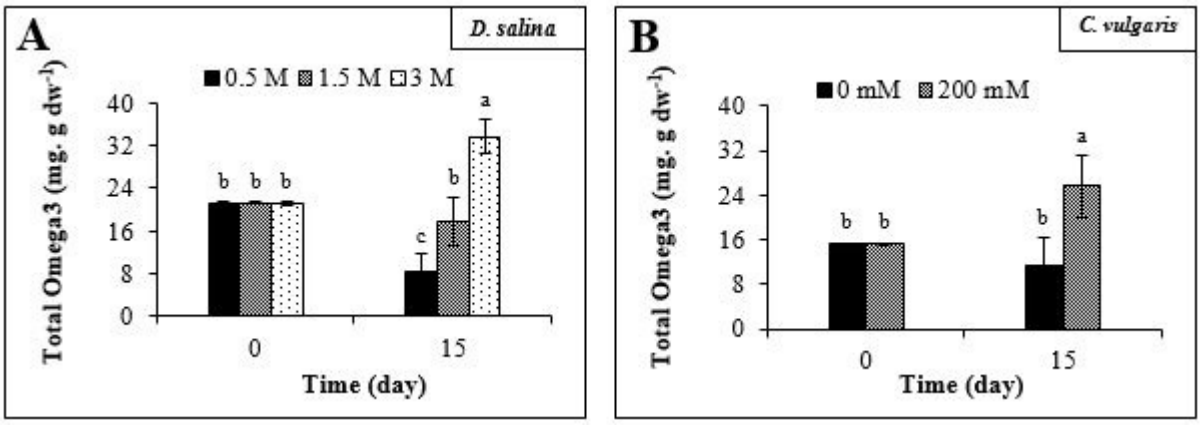

Figure 6- Effect of salt stress on the total omega-3 fatty acid content per unit of biomass dry weight in *D. salina* (A) and *C. vulgaris* (B). The data are expressed as mean ± standard deviation of three replicates. Different letters indicate significant differences between various concentrations in each species at $p<0.05$ according to Duncan test.

Quantitative analysis of fatty acid methyl ester profiles (percent of total fatty acid) under salt stress

As illustrated in Fig. 7A, B and C, the EPA and DHA exist in the trace amounts in *D. salina* and *C. vulgaris*. The 3 M concentration of NaCl, simultaneously increased the percentage of the ALA, EPA, DHA (omega-3 types) and some omega-3 fatty acid biosynthetic intermediates including the oleic acid and stearic acid amounts of *D. salina*. While, the linoleic acid and palmitic acid percentages decreased on 15 days after supplementation (Fig. 7A). Moreover, at 0.5 M of NaCl, ALA, EPA, DHA and total omega-3 contents were markedly decreased on the 15th day of cultivation so that DHA was not detected. As well as, the oleic acid content of *D. salina* cells was decreased noticeably and stearic acid, linoleic acid and palmitic acid of them was increased (Fig. 7B). Although, 200 mM of NaCl had no significant effect on the percentage of DHA of *C. vulgaris* on the 15th day, increased both ALA and EPA percentages which, accompanied by the increase in the total omega-3 fatty acid percentage. Furthermore, increasing NaCl concentration resulted in the concurrent increase of the palmitic acid, stearic acid, and linoleic acid amounts but decreased the oleic acid percentage (Fig. 7C).

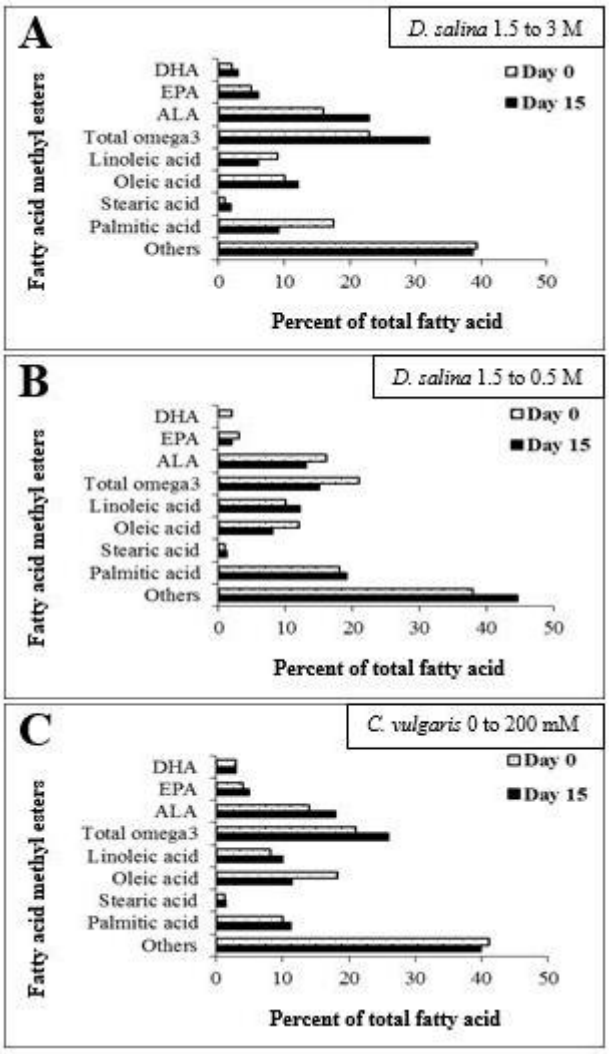

Figure 7- Quantitative analysis of fatty acid methyl ester profiles, in terms of fatty acids (percent of total fatty acid) in *D. salina* under hyper osmotic stress from 1.5 to 3 M of NaCl (A), hypo osmotic stress from 1.5 to 0.5 M of NaCl (B) and *C. vulgaris* under salt stress of 200 mM NaCl (C). The data are expressed as means of three replicates.

DISCUSSION

The results of the present study clearly indicated that the proper growth of *D. salina* merely occurred at the NaCl concentration of 1.5 M but lower or higher NaCl concentrations than 1.5 M inhibited cell growth. There are some reports that the increasing NaCl concentration, reduced the growth rate of *D. salina* while, increased its glycerol content [27]. It appears that the part of metabolic energy presumably expended in producing glycerol caused the decreased growth [28]. The growth of *C. vulgaris* also decreased under salt stress. It has been reported that some organic osmolytes e.g. proline accumulates in expense of metabolic energy in some species of chlorophyceae family such as *Chlorella* under salt stress [21]. It seems that such mechanism resulted in the reduced growth of *C. vulgaris*. Results suggest that the high NaCl concentration led to the enhancement in the lipid production of *D. salina*, despite of the reduction of cell number. Conversely, the low NaCl concentration decreased its lipid pool. As well as, the increase of lipid production observed in the cultures of *C. vulgaris* under salt stress. It seems that increase of lipid content is an adaptive response to salt stress, which is probably due to glycerol production as an organic osmolyte and fatty acid sources. However, relationship between NaCl

concentration and lipid accumulation remains unknown [11]. The increase in the degree of unsaturation and enhancement of the long chain fatty acid contents such as omega-3 types consist of DHA, EPA and specially ALA is probably due to dual role of NaCl in increase of activity of enzymes responsible for elongation and unsaturation of fatty acids in *D. salina* in response to salt stress [29-31]. In omega-3 biosynthetic pathway, palmitic acid is converted to stearic acid, oleic acid and linoleic acid respectively. Linoleic acid in turn might be diverted to ALA, EPA and then DHA with elongation and unsaturation performance of special enzymes [32]. It seems that in our experiment, salt stress finally resulted in the decrease in palmitic acid which in turn increased the ALA, EPA and DHA production. Therefore, the considerable decrease in palmitic acid caused the increase in the total omega-3 content. It could be concluded that unsaturation of fatty acids, especially those constituent cell membrane related to their critical role in cell membrane stability and adaptive response to unfavorable environmental conditions such as salt stress [31]. Our investigation in *C. vulgaris* revealed that salt stress, increased the level of ALA and EPA while not affected the DHA content, which resulted in the increase of total omega-3 fatty acid value but not as much as *D. salina*. The increase of some omega-3 types such as ALA and EPA contents was higher in *D. salina*. The increase of EPA content has been reported in several microalgae, including *Nitzchia laevis* and *Chlorella minutissima* under salt stress [33-34]. These findings emphasize on similar function of different microalgal species in response to salt stress. It appears that increase of PUFAs like ALA and EPA contents have key role in survival of *D. salina* and *C. vulgaris* under salt stress. Biomass concentration and biosynthesis of lipid and omega-3 of individual cells are both important in affecting total lipid and omega-3 contents of microalgae. In spite of the reduction in cell numbers, increase of the total lipid and mostly total omega-3 contents was observed in both species, along with the increase of salt concentration in the medium suggesting that lipid metabolism was influenced by salt stress directly.

CONCLUSION

When, two different types of microalgae, *D. salina* as a halotolerant and *C. vulgaris* as a fresh water microalga were subjected to the different intensities of salt stresses, lipid and omega-3 productions were stimulated in both of them. This was higher in *D. salina* than the other one. *D. salina* has two special characteristics: non existence of cell wall which made it suitable for easy lipid extraction and high amount of lipid and omega-3 production under salt stress in comparison with *C. vulgaris*. Accordingly, *D. salina* is preferred for simple obtaining of high lipid amount and its applications for biodiesel and omega-3 (ALA, EPA, DHA) productions in the purpose of reducing environmental pollutions and pharmaceutical consumptions. It is well known that cell population has an important role in biotechnological applications of microalgae. As *D. salina* has a low growth rate in response to salt stress, It suggested that salt stress should be applied after increasing of cell biomass.

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Erratum

In Article “Changes of the Total Lipid and Omega-3 Fatty Acid Contents in two Microalgae Dunaliella Salina and Chlorella Vulgaris Under Salt Stress”, with the number of DOI: http://dx.doi.org/10.1590/1678-4324-2017160555, published in journal Brazilian Archives of Biology and Technology, vol. 60, the 01 page.

That read:
“http://dx.doi.org/10.190/1678-4324-2017160555”

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“http://dx.doi.org/10.1590/1678-4324-2017160555”