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Effect of Osmotic Stress and Nutrient Starvation on the Growth, Carotenoid and Lipid Accumulation in *Dunaliella salina* A9

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Abstract *Dunaliella salina* A9 is unicellular green alga isolated from the saltern, Khanh Hoa province, Viet Nam. The effect of halostress and nutrient starvation was studied in this alga to estimate the growth, chlorophyll content and capacity of carotenoid and lipid accumulation. The results showed decrease in cell number and chlorophyll content as *Dunaliella salina* in response to a change from the optimal medium 1.5M NaCl to hypo-osmotic medium (0.5M NaCl) and hyper-osmotic medium (3.5M NaCl). We also observed decrease in cell count in nutrient starvation after 9 days of culture in MD4 medium. Salinity stress has more severe effect on the growth of *Dunaliella salina* A9 with greater decrease in cell number compared to nutrient starvation. The stress induced increasing carotenoid and lipid accumulation in cells. However the carotenoid and lipid accumulation in hypo-osmotic stress and the nutrient starvation were higher than in hyper-osmotic stress. The results suggested negative relationship between the growth rate, chlorophyll content and carotenoid, and lipid accumulation of *Dunaliella salina* under stress conditions.

Keywords: *Dunaliella, Dunaliella salina A9, carotenoid, lipid, sulfo-phospho-vanillin reagent*

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1. Introduction

Unicellular green algae of the genus *Dunaliella* is a halophillic eukaryote, ovoid form, flagellated and lack a rigid polysaccharide wall and thus have been found to be able to rapidly change their volume and shape in response to changes in the extracellular hypo-or hyper-osmotic pressure [2,29,33]. The osmotic response process of *Dunaliella* under salinity stress include the changes of cell volume, intracellular ions concentration, intracellular glycerol concentration, and the expression of some salt-induced genes [13].

The algal genus *Dunaliella* possess the unique ability to accumulate large amounts of β-carotene both in nature or adverse growth conditions such as high light intensity, high salt concentration, extreme temperatures or nutrient deficiency. β-carotene accumulation was concentrated in intra-chloroplastic lipoidal globules [4]. The green alga *D. salina* became one of the most important biological sources of β-carotene [17]. *D. salina* could accumulate β-carotene up to 50 $mg.g^{-1}$ of dry weight. Under the conditions of high light, high salinity, and nutrient deprivation, up to 10% of the dry weight in *D. salina* could be β-carotene [4,11].

In Dunaliella salina, the contents of total lipids, unsaponifiables and fatty acid composition were basically depend on NaCl and nitrogen concentration in the culture. Increasing NaCl combined with decreasing N levels in the growth medium increased the total unsaturated fatty acids (TU) at the expense of total saturated fatty acids [15]. This study aimed to estimate ability of carotenoid and lipid accumulation under stress conditions such as hypo-hyper osmotic stress and nutrient starvation in *Dunaliella salina* A9 isolated from the saltern, Khanh Hoa province, Viet Nam.

2. Materials and Methods

2.1. *Dunaliella salina* Strain and Medium

The strain used in this study was *D. salina* A9 isolated from the saltern, Khanh Hoa province, Viet Nam. The alga was grown in 1.5 M MD4 medium containing NPK 0.1g/l, $MgSO_4$ 1.86g/l, EDTA 0.00876g/l, $FeCl_3$ 0.00049g/l, $MnCl_2$ 0.00189g/l, $NaHCO_3$ 50mM, pH = 7.5 [39],

at 25℃ under continuous light intensity of 50 µmol photon/m^2/s.

2.2. Experiment Design

The alga *D. salina* A9 was grown in 1.5M NaCl MD4 medium to exponential phase (after 9 days) and then stressed with two salinities: hypo-hyper osmotic stress by transferring to 0.5M (hypo-osmotic stress) and 3.5M (hyper-osmotic stress) growth medium, and nutrient starvation. For hypo-osmotic stress, this algal culture centrifuged at 3000 rpm for 5 min, then pellets were transferred to 0.5M MD4 medium. For hyper-osmotic stress, NaCl added to 1.5 MD4 medium to obtain 3.5M NaCl concentration. Chlorophyll content, carotenoid and lipid accumulation determined before and every three days after the stress. The experiments were triplicated.

2.3. Growth Analysis

100 µl algal suspension were stopped movement by Lugol solution (5% iodine and 10% potassium iodide). Cell density was determined by direct counting every three days, using a light microscope with 0.1 mm deep counting chamber (Neubauer Haemocytometer). Cell number was determined by following formula: Number of cells/ml = total cells counted x 10^4 x dilution factor.

2.4. Total Carotene and Chlorophylls

One milliliter aliquot of algal suspension was centrifuged at 5000 rpm for 5 min and the pellet extracted with 3ml of ethanol:hexane 2:1 (v/v). Two milliliters of water and 4ml hexane were added and the mixture vigorously shaken and centrifuged again at 5000 rpm for 5 min. The hexane layer was separated and its absorbance at 450nm, 662nm and 645nm. Total carotene: A_{450} × 25.2 equal the micrograms of carotene in sample [32,36]. Chl *a* and Chl *b* contents were estimated according to Lichtenthaler and Wellburn [20]:

$$\mathbf{Chl}a\,(\mu g/ml) = 11.75\ (A662) - 2.35(A645)$$

$$\mathbf{Chl}b\,(\mu g/ml) = 18.61\ (A645) - 3.96(A662)$$

$$\mathbf{Total\ chlorophyll} = \mathbf{Chl}a + \mathbf{Chl}b$$

Where: Chl *a* is chlorophyll a, Chl *b* is chlorophyll b

2.5. Sulfo-phospho-vanillin Assay for Lipid Accumulation

Phosphovanillin reagent was prepared by initially dissolving 0.06 g vanillin in 2 ml absolute ethanol; 8 ml deionized water and stirred continuously. Subsequently 50 ml of concentrated phosphoric acid was added to the mixture, and the resulting reagent was stored in the dark until use. To ensure high activity, fresh phospho-vanillin reagent was prepared shortly before every experiment run [26].

For SPV reaction of the algal culture for lipid quantification, One mL of algal suspension was centrifuged at 5000 rpm for 5 min and the pellet was extracted with 2 mL of concentrated (98%) sulfuric acid was added to the sample and was heated for 10 min at 100℃, and was cooled for 5 min in ice bath. 5 mL of freshly prepared phospho-vanillin reagent was then added, and the sample was incubated for 15 min at 37℃ incubator shaker at 200 rpm. Absorbance reading at 530 nm was taken in order to quantify the lipid within the sample [26].

2.6. Data Analysis

Data was processed in Excel 2013 and analyzed by one-way ANOVA using SPSS software version 20.0. All significant levels were set at $p < 0.05$.

3. Results and Discussion

3.1. The Growth of *Dunaliella salina* A9

Dunaliella salina A9 was grown in 1.5M MD4 medium with the growth rate of 0.29 cell/ml/day in exponential phase (from day 0 to day 9). Cell density decreased markedly as salinity in the culture medium increased from 1.5M growth medium to 3.5M or decreased to 0.5M. Compared to nutrient starvation condition, altered salinity affects cell density more severely. Cell survival recovered in 3.5M NaCl stress from day 19 to day 21 (89 cells/ml and 107 cells/ml, respectively), but recovery was not observed in hypo-osmotic salt stress. *Dunaliella salina* A9 cells adapted with the hyper-osmotic condition after 6 days of stress (Figure 1).

According to Al-Hasan *et al.* [1] there were some correlation among halostress-induced changes in the growth rate, pigmentation, chloroplast structure and lipid composition of *D. salina*. *Dunaliella salina* has an impressive capacity to grow in different salt concentrations. Upon hypo-saline shock conditions (cells were transferred from 9 % NaCl growth media to 3 or 6 % NaCl growth media), cells transiently and rapidly (within minutes) increased in sizes and slowly over hours acquired their original size and volume. Upon hyper-saline shock by transferring from 9 % NaCl to 12 or 15 % NaCl growth media, cells transiently decreased in size (within minutes) and then slowly within hours regained the original size. Cell population grown in lower concentration of NaCl was smaller and more uniform in size than those grown in the condition with higher concentrations of NaCl. This property reflects that *D. salina* has a potential to regulate the cell volume, thereby allowing the cells to tolerate large variations in the salt concentration of growth media [41].

Dunaliella, particular *Dunaliella salina* can accumulate a larger amount of carotene and lipid when they cultured under adverse conditions such as high light intensity, high salt concentration, extreme temperatures and nutrient deficiencies. Phadwal and Singh [30] was evaluated that under sulphate, nitrate and phosphate limitation *Dunaliella* decreased in the growth rate and chlorophyll content but, increased in the beta-carotene content. *Dunaliella* cell number decreased and increased in cell sizes after salinity stress. Cells transferred from green to yellow or orange in the color [38]. Upon the stress, *Dunaliella salina* A9 cells were changed in the structure, physiology and metabolism with the reduced growth and chlorophyll content, but the enhanced carotenoid and lipid biosynthesis.

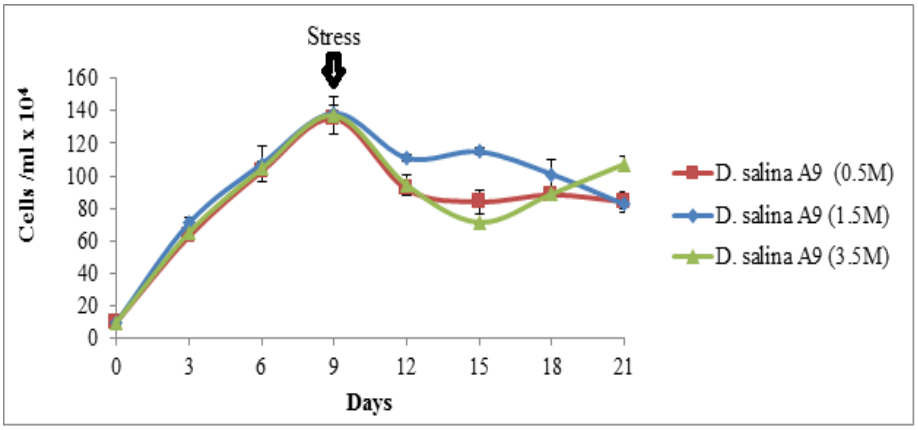

Figure 1. The growth curve of *Dunaliella salina* A9 under the different stress

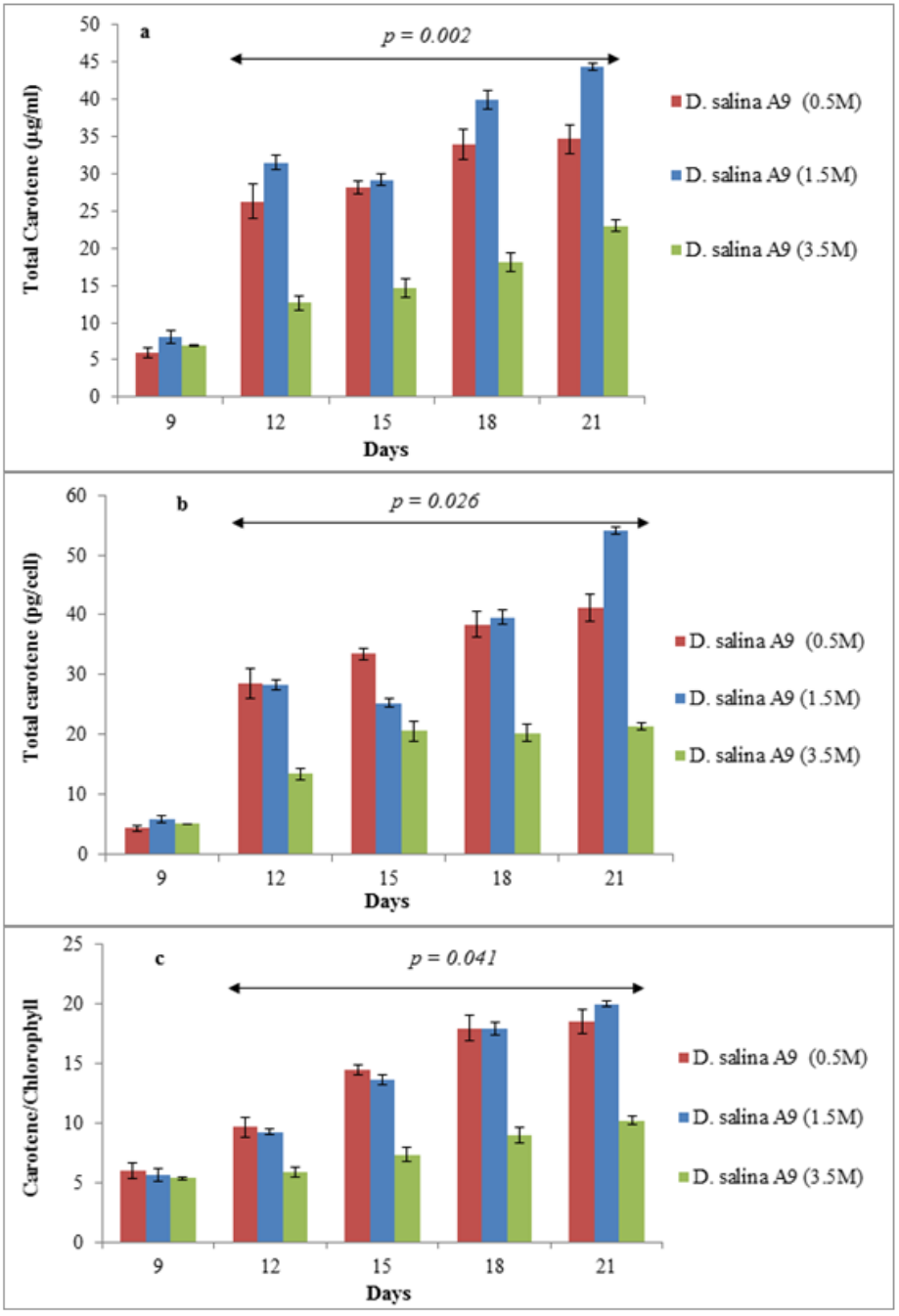

Figure 2. Carotenoid content of *D. salina* A9, carotenoid per volume (a), carotenoid per cell (b) and carotenoid to chlorophyll ratio (c) under the different stress

3.2. Carotenoid Content of *Dunaliella salina* A9

Carotenoid content of *D. salina* A9 increased as the salt stress and the nutrient starvation. This increase in the hypo-osmotic stress and the nutrient starvation were more than in the hyper-osmotic stress. There were no significant differences in carotenoid content between the hypo-osmotic stress and the nutrient starvation, carotenoid per volume ($p = 0.367$), carotenoid per cell ($p = 0.972$) and carotenoid to chlorophyll ratio ($p = 1.000$) (Figure 2). Carotenoid content per cell was no significant difference in hypo-osmotic stress from day 15 ($p = 0.074$) (Figure 2b). The carotenoid to chlorophyll ratio in the hypo-osmotic stress and the nutrient was higher than in the hyper-osmotic stress. There was significant difference in the carotenoid to chlorophyll ratio after 6 days stress under the hyper-osmotic stress ($p = 0.028$) (Figure 2c).

Changes in culture conditions, nutrients deficiency, physical modifies and regimen growth are developed as strategies in microalgae cultures to increase compounds of interest to different process [9,21,28,34]. In *D. bardawil*, increasing light intensity and light period or inhibiting growth by various stress conditions such as nutrient deficiency or high salt concentration caused a decrease in the content of chlorophyll per cell and an increase in the amount of β-carotene per cell and the β-carotene-to-chlorophyll ratio [4]. β-carotene accumulation of *Dunaliella salina* is enhanced up to 10% of the alga dry weight under several conditions: high irradiance, stress temperatures, high salt concentration and/or nutrient deficiency [7,32].

When increasing salinity the *Dunaliella salina* cells became enriched with carotenoid globules that were concentrated at the cell periphery. The chloroplast was degenerated as halostress and the carotenoid globules increased in size. At higher salt concentrations the lamellae degenerated into smaller, less compact units dispersed in the cytoplasm. However, intact thylakoids were still recognized [1].

The adaptation of *D. salina* to salt stress can enhance rapidly the intracellular concentration of glycerol and glycine betaine, neutral lipid biosynthesis and carotenoid overaccumulation [33]. Fluctuations in medium osmolarity result in changes of plasma membrane lipid order, thus triggering activation of a protein kinase cascade and ultimately leading to conversion of starch into glycerol in the chloroplast [31]. In addition to the salt stress were responsible for changes in gene expression events through an as yet unidentifed transcription factor or factors followed by de novo protein biosynthesis. Known salt stress-induced gene products include transcripts and proteins involved in carbon and iron assimilation as well as carotenoid biosynthesis [14,16,33]. Our previous results documented that decreased in cell growth is accompanied by carotenoid biosynthesis and lipid content of *Dunaliella salina* after 9 days nutrient stress [22].

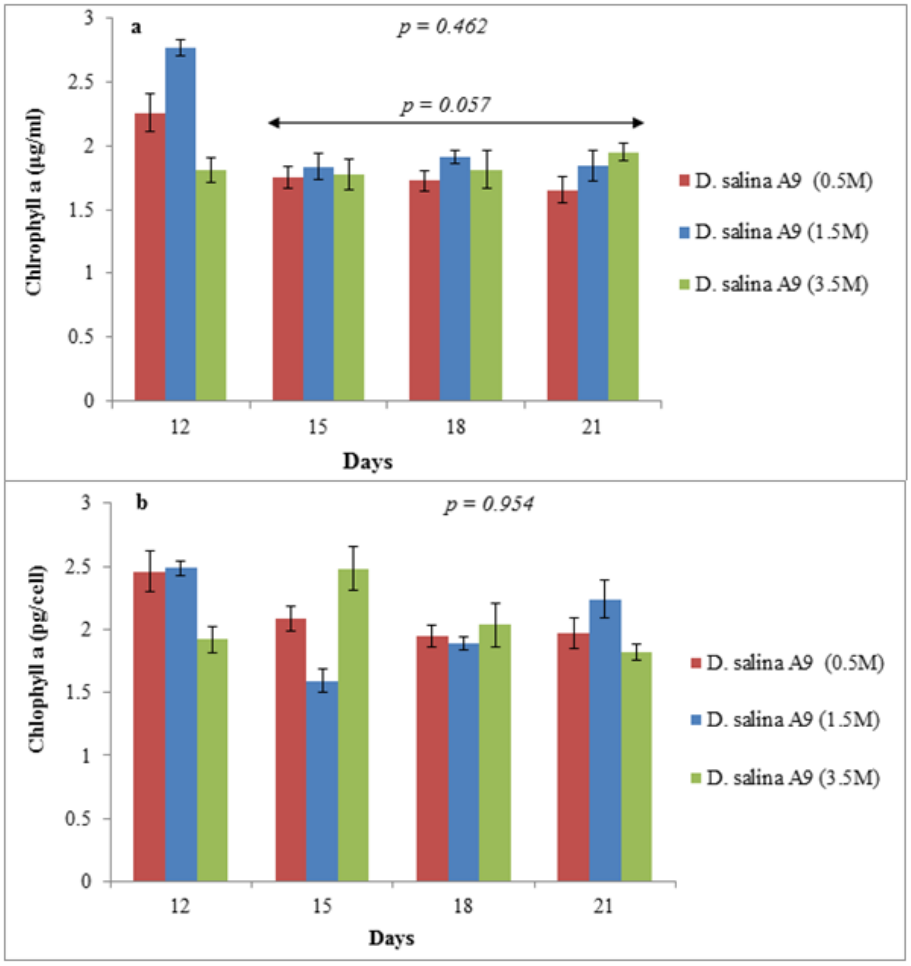

Figure 3. Chlorophyll a content, chlorophyll a per volume (a) and chlorophyll a per cell (b) under the different stress

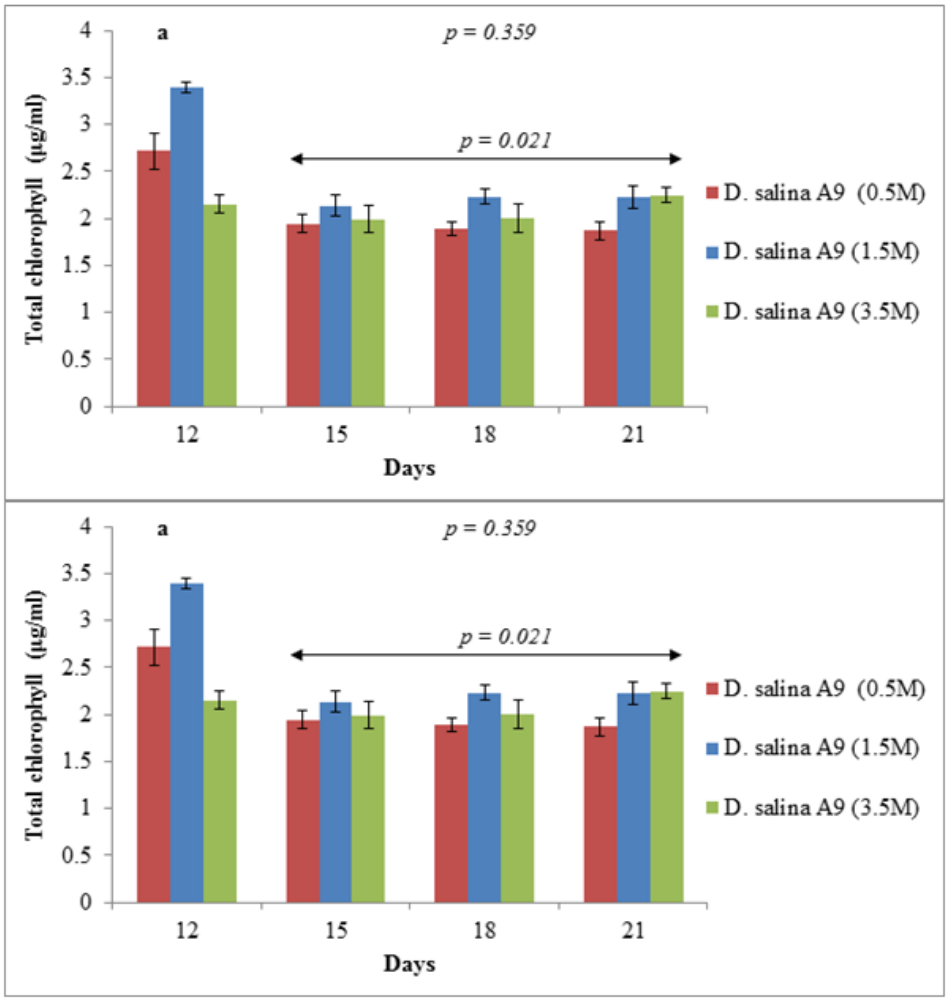

Figure 4. Total chlorophyll content, total chlorophyll per volume (a) and total chlorophyll per cell (b) under the different stress

3.3. Chlorophyll Content of *Dunaliella salina* A9

Chlorophyll a and total chlorophyll per volume of *D. salina* A9 decreased after 3 days stress and no significant difference after 6 days stress (Figure 3a, Figure 4a). Chlorophyll *a* and total chlorophyll per cell decreased strongly under condition of the hypo-osmotic stress and nutrient starvation for 6 days after the stress. While chlorophyll content per cell was not change under the hyper-osmotic stress (Figure 3b, Figure 4b). The decrease in chlorophyll content of *Dunaliella salina* triggered decrease in growth (Figure 1) and induced increase in carotenoid biosynthesis (Figure 4), and lipid accumulation (Figure 5).

Nitrogen and phosphorous are important elements for life and acquired for optimal growth and production of biomass in microalgal culture. Chlorophyll is a nitrogen-rich compound and is easily accessible, it is utilized as an intracellular nitrogen pool to support further cell growth and biomass production as the nitrogen in the media becomes depleted [19]. In marine phytoplankton, nitrogen limitation affects photosynthesis by reducing the efficiency of energy collection due to loss of chlorophyll and increases in non-photochemically active carotenoid pigments. It also directly affects photochemical energy conversion because of a decrement in protein synthesis that appears to affect chloroplastic proteins (and thus the proteins of SI and PSII reaction centers) more strongly than cytoplasmic proteins [10]. No significant difference in chlorophyll content for 6 days after the stress (Figure 3, Figure 4) reflected adaption of *Dunaliella salina* A9 to the stress conditions with increasing the cell number (Figure 1).

The chlorophyll content of *D. salina* A9 decreased as nutrient starvation (Figure 4). Growth of cells depleted the nitrogen in the media, chlorophyll was degraded to reutilize the nitrogen for growth with chlorophyll a and b levels decreasing [9]. In *Neochloris oleoabundus* a rapid decrease in chlorophyll a was observed after 2 days of culture in low-nitrogen treatments. The chl a content started to decrease by day 4 in intermediate nitrogen treatment and did not decrease in higher nitrogen treatments [19].

Halostress injures the chloroplast and elevates the carotene to chlorophyll ratio, apparently to combat this injury [6]. This caused the reduced growth rates (Figure 1) and changes in biochemical compositions of *Dunaliella salina* cells such as chlorophyll (Figure 3, Figure 4), carotenoid content (Figure 2) and lipid accumulation (Figure 5).

3.4. Lipid Accumulation of *Dunaliella salina* A9

Figure 5 indicate the effect of the osmotic stress and nutrient starvation on lipid accumulation of *D. salina* A9.

Lipid accumulation increased after the stress, while the hypo-osmotic stress and the nutrient starvation were higher lipid concentration than the hyper-osmotic stress (Figure 5). Lipid accumulation of *D. salina* A9 increased immediately and remained at about the same after the hypo-osmotic stress. While in the nutrient starvation lipid accumulation increased slowly and the highest after 9 days stress (Figure 5b).

Salinity is an intricate stress factor affecting net lipid productivity in microalgal cells [25]. Ben-Amotz *et al*. [8] documented the relationship between the chemical composition of eukaryotic algae and environmental conditions and species specific response with a general trend to protein decrease and carbohydrate increase on nitrogen starvation. Many algae accumulate lipids under nitrogen deficient conditions, and a few, such as *Chlorella* and contain 70 % of the algal organic weight lipid [5].

Dunaliella species have been found to have unusually high contents of total lipids, carotenoids and polyunsaturated fatty acid (PUFA) such as 16:4 as well as 18:3 [6,24]. The patterns of constituent fatty acids of the total lipids varied according to the NaCl concentration. When the salinity was raised from 2.5 to 20% there was an increase in the proportion of the polyunsaturated fatty acid 18:3 [1]. Currently, lipid signal can be used as an additional marker for large detection of *Dunaliella salina* strains, with the support of morphology and salinity tolerance ability, lipid droplets can be seen in the inner part of the cells and increased after salinity stress [38].

Neochloris oleoabundans is a green microalga well known for the ability to increase its fatty acid (FA) content when it is cultured under nitrogen depletion. When nitrogen was consumed, cell division stopped, even though biomass accumulation continued for several days. The new biomass was composed mostly of lipids and storage oils [19]. The results demonstrated that there were positive relationship between carotenoid content and lipid accumulation of *D. salina* A9 after the stress.

Azachi *et al.* [3] found that the gene *Kcs* encoding for β-ketoacyl-CoA synthase is upregulated in salt stress, resulting in shifting of C16 fatty acids to C18 fatty acids component in microsomes and plasma membrane, and probably an increase in substrate for desaturation. The cells grown in 3.5 M NaCl contained a considerably higher ratio of C18 (mostly unsaturated) to C16 (mostly saturated) fatty acids compared with cells grown in 0.5 M salt. The salt-inducible *Kcs* may play a role in adapting intracellular membrane compartments to function in the high internal glycerol concentrations balancing the external osmotic pressure.

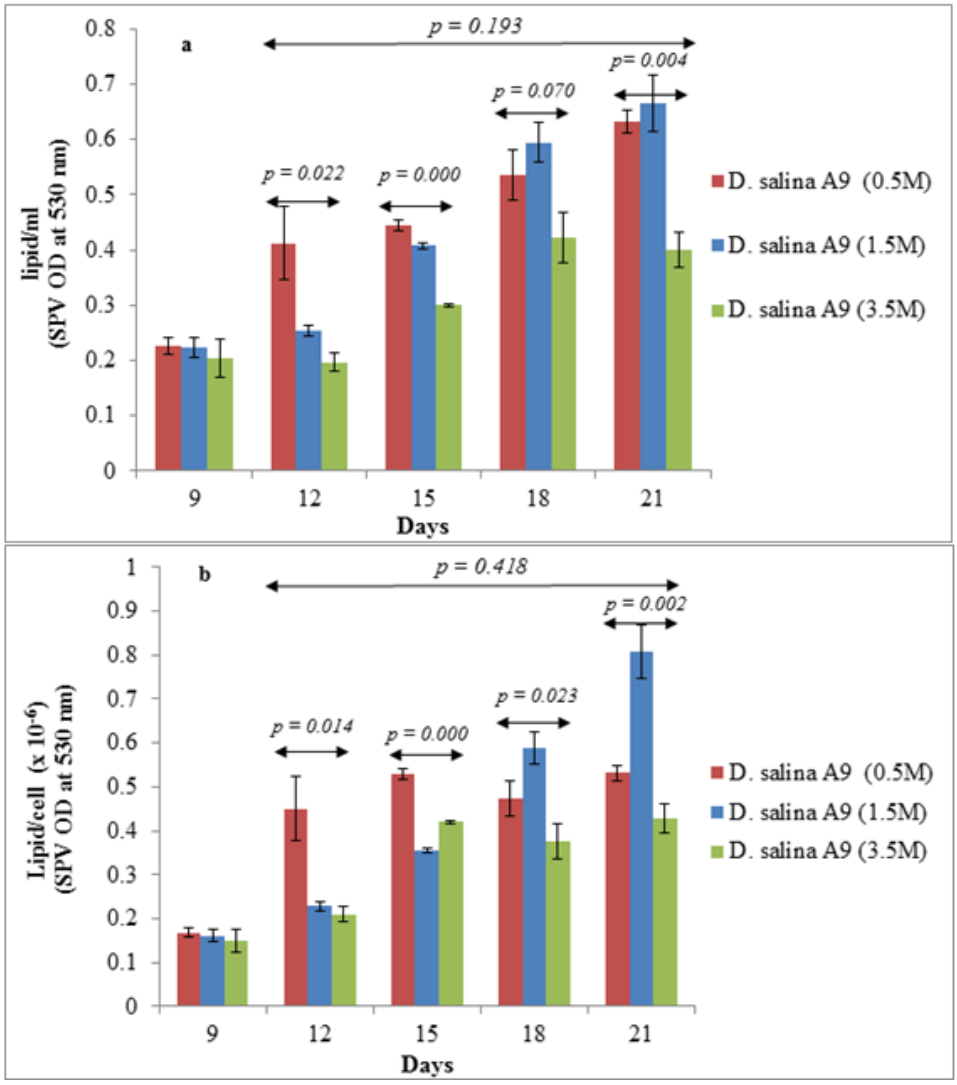

Figure 5. Lipid accumulation of *D. salina* A9, lipid per volume (a) and lipid per cell (b) under the different stress

4. Conclusion

Dunaliella is unicellular green microalga, expecially *Dunaliella salina* can accumulate a larger amount of carotenoid under adverse conditions such as high irradiance, salinity, nutrient starvation. *Dunaliella salina* A9 isolated from the saltern, Khanh Hoa province, Viet Nam, used to investigate the growth, capacity of carotenoid and lipid accumulation under the osmotic stress and nutrient starvation. The results demonstrated that decrease in the growth rate, chlorophyll content and increase in carotenoid and lipid accumulation as stressed cells. The lower growth obtained with the salinity stress, while chlorophyll content was not significant difference after 3 days stress. However carotenoid and lipid accumulation of *Dunaliella salina* A9 were the highest with the hypo-osmotic stress (0.5M) and nutrient starvation. Previous studies demonstrated the antioxidant [27,35,37,40], antimicrobial [12,23] and anticancer [18] properties of the extract from *Dunaliella salina* under the stress conditions. *Dunaliella salina* A9 can apply to functional food and biofuel in Viet Nam, because of capacity of higher carotenoid and lipid accumulation under stress conditions. We will continue to study capacity of antioxidant, antibiotic and anticancer from extract of *Dunaliella salina* A9 under the stress conditions.

Conflict of Interests

The author(s) declare(s) that there is no conflict of interest regarding the publication of this article.

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