

Accumulation of lipid in *Dunaliella salina* under Nutrient Starvation Condition

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Abstract The effect of nutrient starvation on lipid accumulation of *Dunaliella salina* A9 was studied. In nutrient starvation, cell colour changed from green to yellow (or orange) and cell growth reached stationary phase after 9 days of the culture. The study showed that under nutrient stress, decreased in cell growth is accompanied by carotenoid biosynthesis and lipid content of *Dunaliella salina*. The results of this study can be used to increase carotenoid and lipid production in microalgae for functional food and biofuel in the future.

Keywords: *Dunaliella salina* A9, *Dunaliella bardawil* and Sulfo-phospho-vanillin reagent

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1. Introduction

Dunaliella currently belongs to the order of Chlamydomonadales, family Dunaliellaceae, according to NCBI database [28]. Almost 200 years from its discovery in 1838 by Michel Felix Dunal, the genus has received great taxonomic treatment with 22 marine and halophilic species found and described. The genus is well characterized in term of habitat and cell cycle [6]. Later, *D. salina* is the most well-studied species in this genus, enjoying great attention as it has been considered an economically-efficient method to produce β -carotene, capable of accumulating more than 10% of cells dry biomass under optimal stress-inducing conditions [7]. Other than β -carotene, the species can accumulate good amount of secondary metabolites in industrial scale culture [20]. These secondary metabolites are produced as the organism response to different stress condition, for example glycerol is produced after salt-stress induction [12,20,24], neutral lipids and antioxidants (e.g. β -carotene) in response to macronutrient starvation [9,21,22,41] and high light intensity [2,13,18]; production of carotenoids, especially α -carotene and 9-cis β -carotene [10,17,25] in low temperature, and can intensify in the presence of multiple stresses [1,2].

Of different stressors, macronutrient limitation appears to be the main regulatory factor controlling neutral lipid accumulation [33,35,40]. In nutrient depletion condition, *D. salina* accumulates intra- or extra-plastidic lipid bodies composed of both TAGs (triacylglycerides) and carotenoids [2,14]. Previous finding has indicated that β -carotene synthesis and lipid deposition is inter-dependent; biosynthesis

of β -carotene is suppressed when lipid metabolism pathway is inhibited [30]. Bonnefond *et al.* [5] found that β -carotene, in particular, has a positive relationship with TAGs synthesis when N:C ratio in medium decreases. Currently, TAGs are targets for biodiesel production [11,16,19,34]. Production of biofuel from algae is dependent on microalgal biomass production rate and lipid content. Both biomass production and lipid accumulation are limited by several factors, of which nutrients play a key role. In earlier studies, we were interested in screening Vietnam's marine algae flora in search for a potential producer of β -carotene, TAGs and other valuable compounds [37,38,39] and compare their production to *D. bardawil*, a global source for bioactive compounds production [37,39]. We have found that a local strain named *D. salina* A9 has considerable concentration of β -carotene per volume culture and higher growth rate compared to *D. bardawil* [39], which may confer its competitive lipid producer. This study is a continuation of previous effort to examine the strain's efficacy in lipid production.

2. Materials and Methods

2.1. *Dunaliella salina* Strains and Experiments

The experiments were carried out on 2 *Dunaliella salina* strains including *Dunaliella salina* A9 isolated at Department of Algal Biotechnology, International University, Viet Nam and *Dunaliella salina* var. *bardawil* DCCBC 15 (*D. bardawil*) kindly provided by Dr. E.W. Polle, Department of Biology, Brooklyn College of CUNY Brooklyn, NY (USA).

The algae were grown in the MD4 medium (1.5M NaCl) according to Tran *et al.* [36]. Briefly, the medium contained natural seawater, and was added with NPK 0.1 g/L, MgSO₄ 1.86 g/L, EDTA 0.00876 g/L, FeCl₃ 0.00049 g/L, MnCl₂ 0.00189 g/L, NaHCO₃ 50mM, pH = 7.5. *Dunaliella* strains were cultivated at light intensity 50 μmol photon/m²/sec and continuous light in 50 ml flasks at 25°C temperature during 15 days. Each strain was triplicate in each experiment, and all experiments were repeated at least twice.

2.2. Growth Analysis

Lugol solution (5% iodine and 10% potassium iodide mixed in distilled water) was used to stop algae movement. Cell density was estimated by cell count 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 10⁴ x dilution factor.

2.3. Sulfo-phospho-vanillin Assay for Lipid Estimation

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 H₂SO₄ 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 [23].

For SPV reaction of the algal culture for lipid quantification, 1 mL of algal suspension was centrifuged at 5000 rpm for 5 min and the pellet was extracted with 2 mL of concentrated (98%) H₂SO₄. The mixture was then heated for 10 min at 100°C, and 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°C incubator shaker at 200 rpm. Absorbance reading at 530 nm was taken in order to quantify lipid content of the sample [23].

2.4. Data Analysis

Data was processed in Excel and analyzed by one-way ANOVA using SPSS software. All significant levels were

set at p < 0.05.

3. Results and discussion

3.1. Cell Morphology

Dunaliella salina cells were green in exponential growth phase from day 0 to day 9, however they turned yellow or orange and cell size increased significantly in stationary growth phase (after 9 days of culture (Figure 1). Microscopy showed carotenoid accumulation of *D. salina* increased under condition of nutrient starvation in the stationary phase. *Dunaliella* cells may change shape with changing conditions, often becoming spherical under unfavourable conditions. Cell size may vary to some degree with growth conditions and light intensity. Chloroplast can accumulate large quantities of β-carotene within oily globules in the inter-thylakoid spaces, thus cell becoming orange-red rather than green. The β-carotene globules of *Dunaliella salina* were found to be composed of practically only neutral lipids, more than half of which were β-carotene [2].

3.2. The Growth and Lipid Accumulation of *Dunaliella salina*

Dunaliella salina A9 reached stationary phase of growth after 9 days (Figure 2). Lipid accumulation (lipid per ml and lipid per cell) increased during culture. In particular, significantly higher lipid content was obtained after 12 days of growth (Figure 2), corresponding to the observation of carotene-rich globules (Figure 1). For *Dunaliella bardawil*, maximum cell number obtained at day 6 and then decreased (Figure 3). However, lipid accumulation increased significantly after 9 days (Figure 3). The nutrient starvation in the stationary phase led to lipid accumulation and carotenoid in chloroplast of cells earlier in *D. bardawil* than in *D. salina* A9.

The lipid content in microalgae varies from 1-85% of dry weight and is effect by the nutritional composition of the medium. Lipid accumulation in algae typically occurs during periods of environmental stress as the nutrient deficient condition. *Dunaliella* species respond to nitrogen starvation by increasing lipid production [8].

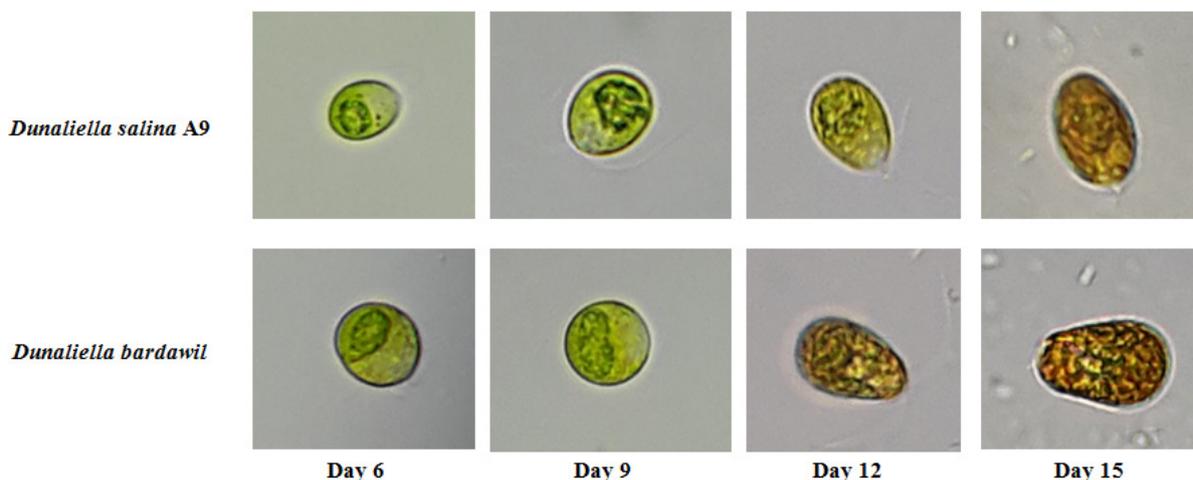


Figure 1. Morphology of *Dunaliella salina* cells under nutrient starvation

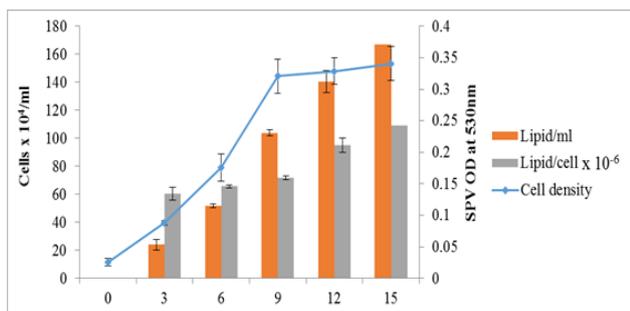


Figure 2. The growth curve and lipid accumulation of *Dunaliella salina* A9 in nutrient starvation condition

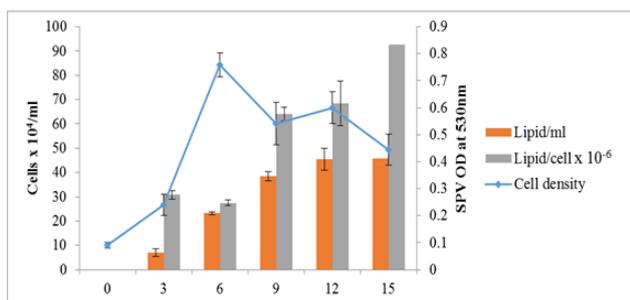


Figure 3. The growth curve and lipid accumulation of *Dunaliella bardawil* in nutrient starvation condition

Vanitha *et al.* 2007 compared lipid profiles between the *D. salina* and *D. bardawil* and found that they are very different. While neutral lipids do not differ significantly between the two, polar lipids in *D. bardawil* is significantly lower than *D. salina*, comprising of 24.2 % compared to 42.52% of total lipids in *D. salina*, while glycerolipids content in *D. bardawil* is much higher (40.42% total lipid compared to 28.4 % in *D. salina*). This may suggest that the two species behave differently under the same stress condition, shifting their lipid composition to serve alternative counter-stress mechanisms. Comparing lipid/cell and lipid/mL of the two cultures, are they significantly different?

In this study, we found that increase in β -carotene synthesis in *D. salina* A9 under stress condition corresponds to increase in total lipid concentration, which agrees with previous studies [5,30,37]. Other research has found that TAGs abundance over total lipid increases with Nitrogen depletion and β -carotene synthesis, while polar lipids composition decreases [5]. This might be explained by new findings about new roles of TAGs besides storage organelles, and as constitutors of intermediary products in biosynthesis of more complex molecules such as glycolipids and phospholipids [15]. In the meantime, abundance of polar lipids correspond to the minimal availability for cell and organelle membranes [5].

4. Conclusion

D. salina can accumulate higher carotenoid and lipid contents under stress conditions, such as nutrient starvation, salinity and light. Lipid from microalgae is supplied in food, feed and biofuel production. *Dunaliella* species have been used as global source of lipids production for biodiesel and carotenoids [3]. Previously, a strain of *D. salina* collected from salt pan in Bombay,

India was reported to have prominent concentration of eicosapentanoic acid (EPA) [4] as high as 21.4%. EPA is part of a series of bioactive polyunsaturated fatty acids (PUFAs). Commonly known PUFAs such as eicosapentanoic (EPA), docosahexanoic (DHA), arachidonic acid (AA), α -linoleic (ALA) and γ -linoleic (GLA), also loosely referred to as omega-3 and omega-6 fatty acids, have anti-inflammatory and inflammatory related diseases, as potent vasodilators or anti-coagulation properties [29], hypertension, diabetes, coronary heart disease, skin diseases, etc. [26,27,31,32]. As a result of this study, we were able to demonstrate that *Dunaliella salina* A9 increased lipid production under nutrient starvation condition. Our future direction would be to explore the lipid content of this strain under different stress conditions for novel bioactive compounds and their maximal capacity.

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