

ACCUMULATION OF CAROTENOID AND LIPID IN MICROALGAE *DUNALIELLA BARDAWIL* DCCBC 15 CULTIVATED UNDER NITROGEN STARVED CONDITIONS

ABSTRACT

Dunaliella bardawil is a unicellular green microalgae that can accumulate large amounts of carotenoids under adverse culture conditions, so it has high commercial value and is used as a functional food. Nutrient starvation, especially nitrogen starvation, induces carotenoid and lipid production in algal cells. *Dunaliella bardawil* cells were grown in MD4 medium after 12 cultural days reaching maximum cell density (1.4×10^6 cells/mL) that changed to four stress conditions—nitrogen replete, nutrient starvation, and half and fully nitrogen-depleted conditions—to investigate the carotenoid and lipid contents of *D. bardawil* microalgae. The results showed that the cell density *D. bardawil* decreased significantly under cultural conditions. High amounts of carotenoids were detected under fully nitrogen-depleted conditions (14.631 pg/cell; car/chl: 12.346). *D. bardawil* was able to overproduce lipids under both nitrogen-free (351.580 pg/cell; lipid/chl: 219.792) and fully nitrogen-free (373.136 pg/cell; lipid/chl 321.401) conditions. Therefore, fully nitrogen-depleted conditions of cultivation stimulate significantly *D. bardawil* cells accumulating a large amounts lipid and carotenoid.

Keywords: *Dunaliella bardawil*, carotenoid, lipid, nitrogen starvation

1. Introduction

Dunaliella salina var. *bardawil* (*D. bardawil*) is considered a valuable source of carotenoids, polyunsaturated fatty acids, antioxidants, and vitamins. Under favorable conditions, both *D. salina* and *D. bardawil* can produce a substantial amount of β -carotene, exceeding 10% of their dry weight, which is significantly greater than that of carrots and tomatoes [1]. The beta-carotene derived from these algae has applications in the cosmetics and pharmaceutical industries as an additive, coloring agent, immune system enhancer, antioxidant, and anticancer agent and in supporting the treatment of cardiovascular diseases [2], [3]. *D. salina* can accumulate lipids, serving as a source of fatty acids for food and aquaculture and as a potential alternative green fuel to fossil fuels [4].

The economic and commercial significance of *D. salina* lies in its capacity to accumulate high-value products such as carotenoids, glycerol, and fatty acids [5]. Nitrogen is an essential macroelement for the growth and development of algae. Nitrogen deficiency significantly affects carotenoid accumulation. Restrictive conditions decrease biomass production while enhancing the production of secondary products. Nitrogen depletion is considered an effective strategy to maximize lipid accumulation in algae, reaching up to 90% [6]. Numerous studies have suggested that inducing oxidative stress through nitrogen deficiency can help *D. salina* cells increase lipid accumulation for biofuel production [6]. According to Pisa and Lele (2005), under nitrogen deficiency, *D. salina* ceases growth and reduces chlorophyll synthesis because chlorophyll is a nitrogen-rich molecule, and the formation of free radicals leads to a fourfold increase in β -carotene content. Furthermore, under nutrient-depleted conditions, *D. salina* can accumulate significantly more lipids and carotenoids than under high-salt conditions of 3.5 M [7].

Nitrogen deficiency disrupts physiological processes, leads to a decrease in the content of the main photosynthesis pigments and disruption of the functioning of

photosystems (PSI, PSII) in microalgae [8]. According to Zhu and *et al.*, 2015, a lack of nitrogen changes the direction of carbon flow from protein synthesis lead to carbohydrate or lipid synthesis [9]. In *D. salina*, the growth and photosynthetic rates of cells were decreased during nutrient deprivation, accompanied by lipid globule accumulation and reduced chlorophyll levels. The carotenoid biosynthesis pathway of *D. salina* is regulated at both the transcriptional and posttranscriptional levels and that a complex crosstalk occurs at the physiological and molecular levels in response to the deprivation of different nutrients [10]. Therefore, the accumulation of carotenoids and lipids in *D. bardawil* algae is dependent on the nitrogen concentration in the cultivation environment. This study aimed to assess the extent to which nitrogen depletion in cultivation media leads to the accumulation of high-value secondary products such as carotenoids and lipids in *D. bardawil* algae.

2. Materials and methods

2.1. The *Dunaliella bardawil* strain DCCBC 15 and cultivation conditions

The *Dunaliella bardawil* strain DCCBC15 was cultured and maintained in the Laboratory of Biochemistry and Toxicology, Faculty of Pharmacy, Nguyen Tat Thanh University. Cultivation was carried out in MD4 media with a salinity of 1.5 M under white light conditions at 90 $\mu\text{mol photon/m}^2/\text{s}$, a light-dark cycle of 12:12 hours, and a temperature of $25^\circ\text{C} \pm 2^\circ\text{C}$ [11].

2.2. Methods

2.2.1. Cell density determination

The cell density of the algae was directly measured using a hemocytometer after 21 days of inhibition. A 100 μL algae sample was fixed with Lugol's solution (5% iodine and 10% KI). The hemocytometer had a depth of 0.1 mm and a square area of 1 mm^2 . The cell density per mL was determined using the following formula [12]:

$$D = \frac{n}{i} \times 10^4 \times \text{dilution factor.}$$

Where:

n: Total number of counted cells

i: Counting area

D: Cell density (cells/mL).

2.2.2. Total carotenoid content determination

One milliliter of culture was centrifuged at 10,000 rpm for 5 minutes, and the algae pellet was extracted with 3 mL of ethanol:hexane (2:1 v/v). Four milliliters of hexane and 2 mL of water were added, and the mixture was shaken vigorously. This extract was centrifuged at 10,000 rpm for 5 minutes. The pigment layer with hexane on top was read at wavelengths of 450 nm, 662 nm, and 645 nm. The total carotenoid content was determined by the following formula: Carotenoid ($\mu\text{g/mL}$) = $A_{450} \times 25.2$ [13], [14].

The amounts of chlorophyll *a* and *b* are determined as follows [15]:

$$\text{Chlorophyll a } (\mu\text{g/mL}) = 11.75 (A_{662}) - 2.35 (A_{645})$$

$$\text{Chlorophyll b } (\mu\text{g/mL}) = 18.61 (A_{645}) - 3.96 (A_{662})$$

Total chlorophyll ($\mu\text{g/mL}$) = chlorophyll *a* + chlorophyll *b*

Where A_{645} is the absorbance at 645 nm and A_{662} is the absorbance at 662 nm.

The total carotenoid content is expressed per cell (pg/cell), and the carotenoid/chlorophyll ratio is calculated. The unit per cell evaluates the accumulation of carotenoids in each cell, while the ratio indicates the trend of secondary carotenoid accumulation relative to cell growth.

2.2.3. Total lipid content determination

For the phosphovanillin reagent, 0.06 g of vanillin was dissolved in 2 mL of ethanol, 8 mL of distilled water was added, and the mixture was shaken well. Fifty milliliters of concentrated phosphoric acid was added, and the mixture was shaken well in the dark [16], [17].

To determine the total lipid content, 1 mL of algae culture was centrifuged at 10,000 rpm and 4°C for 10 minutes, and the algae pellet was extracted with 2 mL of concentrated sulfuric acid, boiled in a water bath at 100°C for 10 minutes, and cooled. Five milliliters of phosphovanillin reagent was added, and the mixture was incubated at 37°C with continuous shaking. The sample was measured at 530 nm [16], [17].

Construction of the lipid standard curve: Commercial rapeseed oil (Tuong An brand) was dissolved in chloroform at a concentration of 1 mg/mL, and standard lipid concentrations ranging from 10-150 μg were prepared in test tubes with caps. The sample was incubated at 90°C for 10 minutes to allow chloroform evaporation. Then, 2 mL of concentrated sulfuric acid was added, and the mixture was heated in a water bath at 100°C for 10 minutes and cooled. Five milliliters of phosphovanillin reagent was added, and the mixture was incubated at 37°C with continuous shaking. The absorbance of the sample was measured at 530 nm. The lipid standard curve formula was $y = 0.005x - 0.0531$ ($R^2 = 0.9929$).

The total lipid content is expressed per cell (pg/cell), and the lipid/chlorophyll ratio is calculated. The unit per cell evaluates the lipid accumulation in each cell, while the ratio indicates the trend of lipid accumulation relative to cell growth.

2.3. Experimental design

Dunaliella bardawil DCCBC 15 was cultured in MD4 media supplemented with 1.5 M NaCl (Tran et al., 2014) for two stages:

Growth cultivation stage: *D. bardawil* DCCBC15 was cultured under white light at 90 $\mu\text{mol photon/m}^2/\text{s}$. The starting cell density was approximately 0.15×10^6 cells/mL, the light-dark cycle was 12:12 hours, the temperature was $25^\circ\text{C} \pm 2^\circ\text{C}$, and the sample was shaken 3-4 times/day.

Inhibition stage: After 12 days of growth cultivation, cell density reached about 1.4×10^6 cells/mL, *D. bardawil* was transferred to inhibition conditions, which included the following:

Nitrogen supplementation (+NPK): The initial medium was removed by centrifugation at 6,000 rpm for 5 minutes at 15°C, and a new medium supplemented with 0.1 g/L NPK was added

Nutrient depletion: *D. bardawil* was cultured under initial cultivation conditions.

½ Nitrogen depletion (-½ NPK): The initial medium was removed by centrifugation at 6,000 rpm for 5 minutes at 15°C, and the amount of a new medium was reduced by half the amount of NPK added to the initial medium.

Complete nitrogen depletion (-NPK): The initial medium was removed by centrifugation at 6,000 rpm for 5 minutes at 15°C, and a new medium without NPK was added.

Algal harvesting was performed after 21 days of inhibition by centrifugation at 10,000 rpm for 5 minutes at 15°C using 1 mL of the culture, followed by storage at -20°C. The carotenoid and lipid levels of *D. bardawil* were then analyzed under these experimental conditions.

2.4. Data analysis

The data were processed using Microsoft Office Excel 2019, and one-way ANOVA was conducted using SPSS 25.0 software, with significance set at $p < 0.05$. The results are presented as the mean \pm standard error.

3. Results and Discussion

3.1 Total carotenoid content

After stress (day 12), the cell density of *D. bardawil* decreased rapidly in the conditions of +NPK (about 0.84×10^6 cell/mL at day 21), -1/2NPK (about 0.76×10^6 cell/mL at day 21), -NPK (about 0.54×10^6 cell/mL at day 21) and slightly in this of nutrient depletion (about 13×10^6 cell/mL at day 21). The carotenoid content of *D. bardawil* under -NPK conditions (14.631 pg/cell) was significantly greater than that under the other inhibition conditions ($p < 0.05$). Moreover, the carotenoid contents of *D. bardawil* under nutrient-depleted conditions (9.980 pg/cell), +NPK conditions (11.396 pg/cell), and -½ NPK conditions (11.837 pg/cell) were not significantly different ($p = 0.161$) (Figure 3.1, Table 3.1). Similarly, the carotenoid/chlorophyll ratio under -NPK conditions (12.346) was the highest among the inhibition conditions ($p < 0.05$). Furthermore, the -½ NPK treatment had a greater carotenoid/chlorophyll ratio (9.137) than did the nutrient-depleted (7,309) and +NPK treatments (7.079) ($p < 0.05$) (Figure 3.2, Table 3.1). Thus, under conditions of nitrogen depletion ranging from ½ NPK to complete (-NPK), the accumulation of carotenoids in *D. bardawil* cells may be attributed to the rapid occurrence of nutritional stress under these cultivation conditions. Nitrogen is an essential trace element for the growth and development of algae. Nitrogen deficiency promotes the formation of lipid droplets containing carotenoids in the chloroplast and activates genes related to carotenoid synthesis [18]. *D. bardawil* can accumulate large amounts of carotenoids and lipids under unfavorable cultivation conditions such as nutrient deficiency. Under conditions of nitrogen, sulfate, and phosphate deficiency, *Dunaliella* species exhibit reduced growth

rates, decreased chlorophyll content, and increased β -carotene accumulation [7]. When *D. bardawil* cells are stressed, changes in cell structure, physiological activity, metabolic processes, growth reduction, chlorophyll content, and carotenoid and lipid synthesis are enhanced [19]. Under conditions of complete nitrogen deprivation, reduced growth rates, excess energy, and carbon from photosynthesis are strongly stored as nitrogen-free molecules such as β -carotene and triglycerides [20].

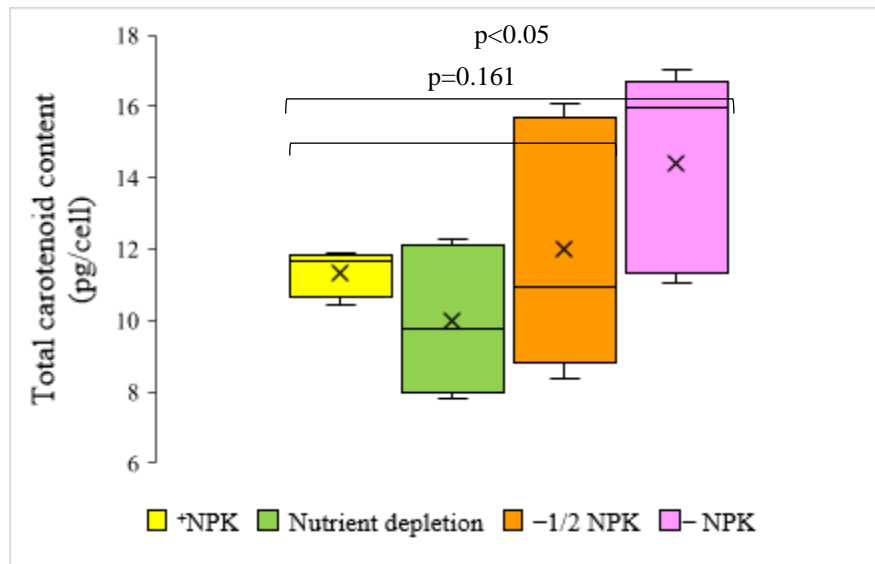


Figure 3.1. Carotenoid content of the microalga *D. bardawil* DCCBC15 under nitrogen-depleted conditions.

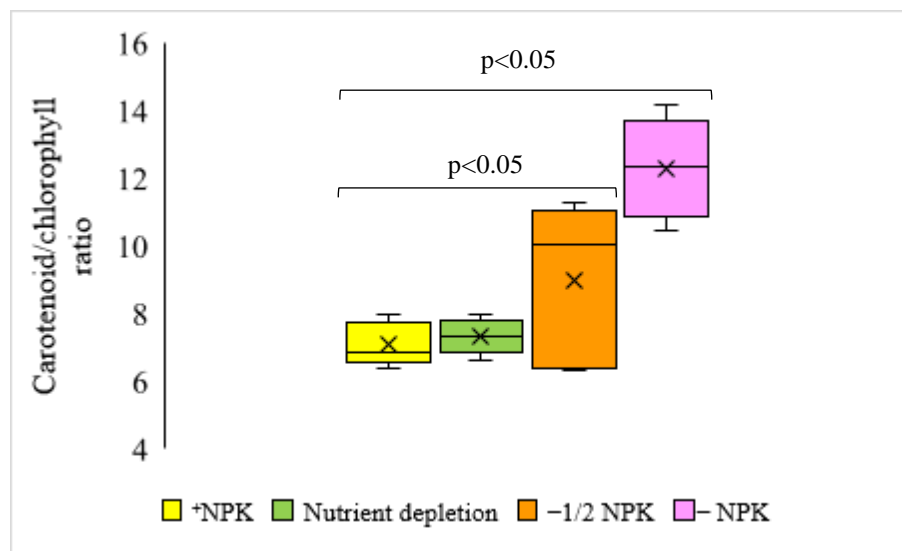


Figure 3.2. Carotenoid/chlorophyll ratio of the microalga *D. bardawil* DCCBC15 under nitrogen-depleted conditions.

3.2 Total lipid content

The previous researchs described that in *D. bardawil*, secondary carotenoids and lipids may be synthesized simultaneously under growth-inhibiting conditions such as nitrogen deficiency. Under conditions of complete nitrogen depletion, *D. bardawil* accumulates two types of lipid droplets: primary lipid droplets (β C-plastoglobuli) and secondary lipid droplets in the cell matrix (CLDs). After nitrogen depletion inhibition, both CLDs and β C-plastoglobuli are formed [21]. When nitrogen is deficient, the photosynthetic process of algae may be altered. Instead of utilizing energy to transform simple organic products into complex products to support growth, algae may prioritize storage over consumption, such as increasing lipid reserves within the cells [6]. Algae can accumulate large amounts of lipids in the stationary and declining phases rather than in the growth phase [22].

The investigation resulted that the lipid content of the microalga *D. bardawil* DCCBC15 under -NPK (373.136 pg/cell) and +NPK conditions (351.58 pg/cell) was not significantly different ($p=0.160$) from that under the two conditions of $-1/2$ NPK (324.196 pg/cell) and nutrient depletion (96.991 pg/cell) ($p<0.05$) (Figure 3.3, Table 3.1). Similarly, the lipid/chlorophyll ratio (lipid/chl) did not significantly differ between $-1/2$ NPK (274.129) and +NPK (321.401) conditions ($p=0.272$) higher than the nutrient - depleted conditions ($p<0.05$). In addition, the lipid/chlorophyll ratios under +NPK (219.792) and $-1/2$ NPK conditions (274.129) were not significantly different ($p=0.167$) and were greater than those under nutrient-depleted conditions (71.831) ($p<0.05$) (Figure 3.4, Table 3.1). Thus, strong lipid synthesis in *D. bardawil* can occur under two conditions: nutritional media with sufficient nitrogen and complete nitrogen depletion. According to Shi *et al.*, 2020, a linear relationship between nitrogen source concentration and lipid content has been observed in many algal species, where lipid production increased by 93% in *Chlamydomonas reinhardtii*, and *Acutodesmus dimorphus* accumulated 75% of neutral lipids under nitrogen-deficient conditions. Nitrogen deficiency may enhance the lipid synthesis process of algae by influencing other biochemical processes. The production of carotenoids, including β -carotene, astaxanthin, and lutein, has also been successfully enhanced by restricting nitrogen in *Chlorella zofingiensis*, *Dunaliella salina*, *Neochloris oleoabundans*, and *Muriellopsis* sp. [23].

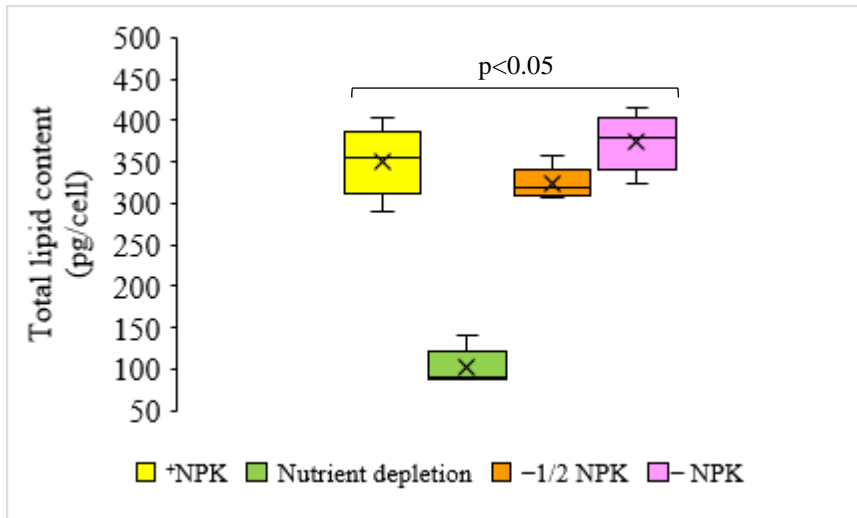


Figure 3.3. Lipid content of the microalga *D. bardawil* DCCBC15 under nitrogen-depleted conditions

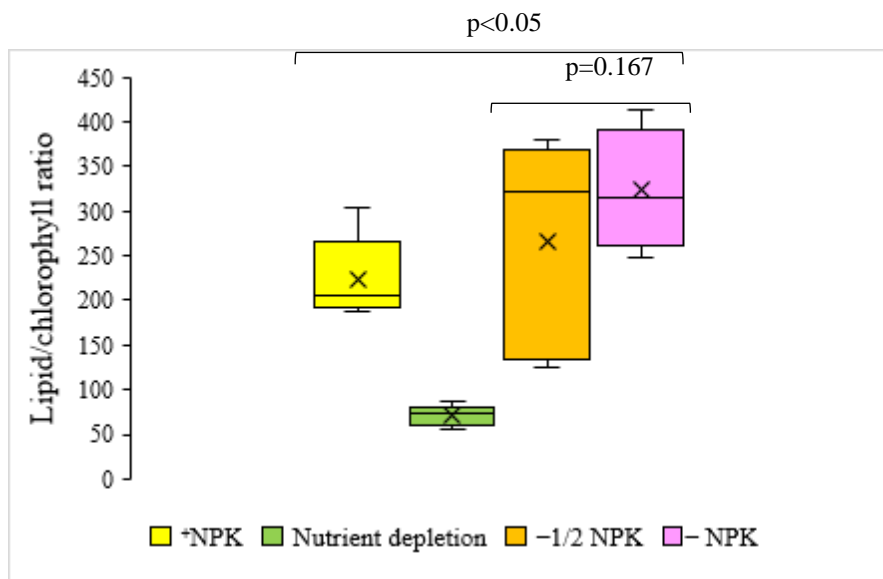


Figure 3.4. Lipid/chlorophyll ratio of the microalga *D. bardawil* DCCBC 15 under nitrogen-depleted conditions.

Bảng 3.1. Carotenoid content, carotenoid/chlorophyll ratio, lipid content and lipid/chlorophyll ratio of *D. bardawil* microalgae under nitrogen-depleted conditions.

Treatments	Carotenoid (pg/cell)	Car/chl	Lipid (pg/cell)	Lipid/chl
+NPK	11.396±0.153 ^a	7.079±0.145 ^a	351.580±10,124^c	219.792±11.047 ^b
Nutrient depletion	9.980±0.516 ^a	7.309±0.124 ^a	96.991±4.474 ^a	71.831±2.700 ^a
-½ NPK	11.837±0.867 ^a	9.137±0.598 ^b	324.196±4.505 ^b	274.129±30.684 ^{bc}
- NPK	14.631±0.698^b	12.346±0.336^c	373.136±7.885^c	321.401±16.096^c

4. Conclusion

D. bardawil algae accumulate a significant amount of carotenoids and lipids under different nitrogen levels under cultivation conditions. Among the four cultivation conditions, the -NPK inhibition condition led to the greatest accumulation of carotenoids in *D. bardawil* algae (14.631 pg/cell). However, substantial lipid accumulation in *D. bardawil* occurred under both +NPK (351.580 pg/cell) and -NPK inhibition (373.136 pg/cell) conditions.

CONFLICT OF INTERESTS

No conflict of interest.

Disclaimer (Artificial intelligence)

Option 1:

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

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