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 under four culture conditions—nitrogen replete, nutrient starvation, and half and fully nitrogen-depleted conditions—to investigate the carotenoid and lipid contents of D. bardawil microalgae. 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*

UNDER PEER REVIEW

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]. 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 µmol photon/m^2/s, a light-dark cycle of 12:12 hours, and a temperature of 25°C ± 2°C [8].

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 mm2. The cell density per mL was determined using the following formula [9]:

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

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 (µg/mL) = $A_{450} \times 25.2$ [10], [11].

The amounts of chlorophyll *a* and *b* are determined as follows [12]:

Chlorophyll a (µg/mL) = 11.75 (A_{662}) – 2.35 (A_{645})

Chlorophyll b (µg/mL) = 18.61 (A_{645}) – 3.96 (A_{662})

Total chlorophyll (µg/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.

UNDER PEER REVIEW

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 [13], [14].

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 [13], [14].

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 µmol photon/m^2/s. The starting cell density was approximately 0.15 x 10^6 cells/mL, the light-dark cycle was 12:12 hours, the temperature was 25°C ± 2°C, and the sample was shaken 3-4 times/day.

Inhibition stage: After 12 days of growth cultivation, *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.

UNDER PEER REVIEW

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 ± standard error.

3. Results and Discussion

3.1 Total carotenoid content

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 1, Table 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 2, Table 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 is an environmental factor that strongly affects carotenoid accumulation. Nitrogen deficiency promotes the formation of lipid droplets containing carotenoids in the chloroplast and activates genes related to carotenoid synthesis [15]. *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 [16]. 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 [17].

UNDER PEER REVIEW

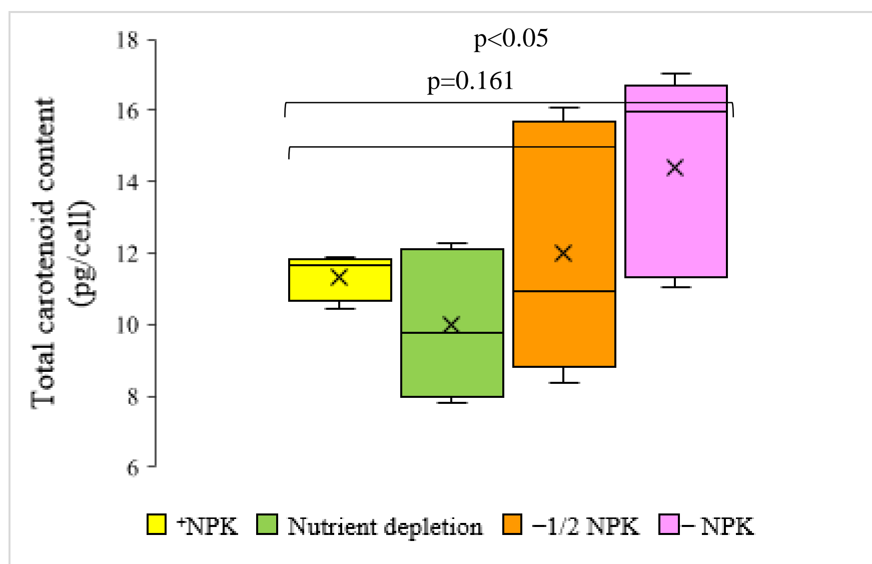

Figure 1. *Carotenoid content of the microalga D. bardawil* DCCBC15 *under nitrogen-depleted conditions.*

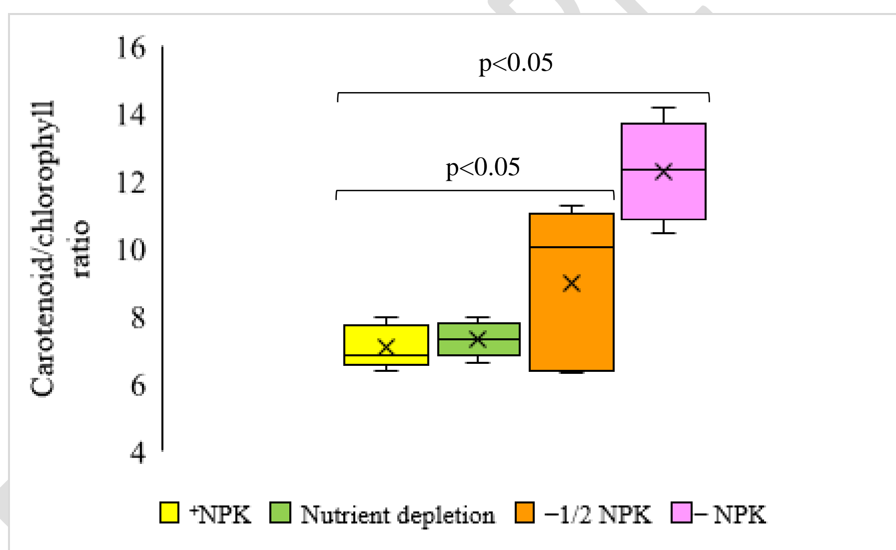

Figure 2. *Carotenoid/chlorophyll ratio of the microalga D. bardawil* DCCBC15 *under nitrogen-depleted conditions.*

3.2 Total lipid content

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 -½ NPK (324.196 pg/cell) and nutrient depletion (96.991 pg/cell) ($p<0.05$) (Figure 3, Table 1). Similarly, the lipid/chlorophyll ratio (lipid/chl) did not significantly differ between -½ 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 -½ NPK conditions (274.129) were not

UNDER REVIEW

significantly different ($p=0.167$) and were greater than those under nutrient-depleted conditions (71.831) ($p<0.05$) (Figure 4, Table1). Thus, strong lipid synthesis in *D. bardawil* can occur under two conditions: nutritional media with sufficient nitrogen and complete nitrogen depletion.

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 (βC-plastoglobuli) and secondary lipid droplets in the cell matrix (CLDs). After nitrogen depletion inhibition, both CLDs and βC-plastoglobuli are formed [18]. 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 [19].

Different species of *Dunaliella* contain substantial amounts of lipids, carbohydrates, and β-carotene, which can be utilized for the production of various liquid/gaseous biofuels and different health-enhancing bioproducts. Lipids, especially fatty acids such as TAG, are raw materials converted into fatty acid methyl esters (FAME) or biodiesel. Many algae have been reported to have the capacity to accumulate a high lipid content (5–77%). Lipid-rich algae containing high levels of TAG are suitable for the sustainable production of biodiesel [20]. 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 *Muriell*opsis sp. Nitrogen deficiency may also promote the simultaneous accumulation of both lipids and the antioxidant pigment astaxanthin in algae [21].

UNDER PEER REVIEW

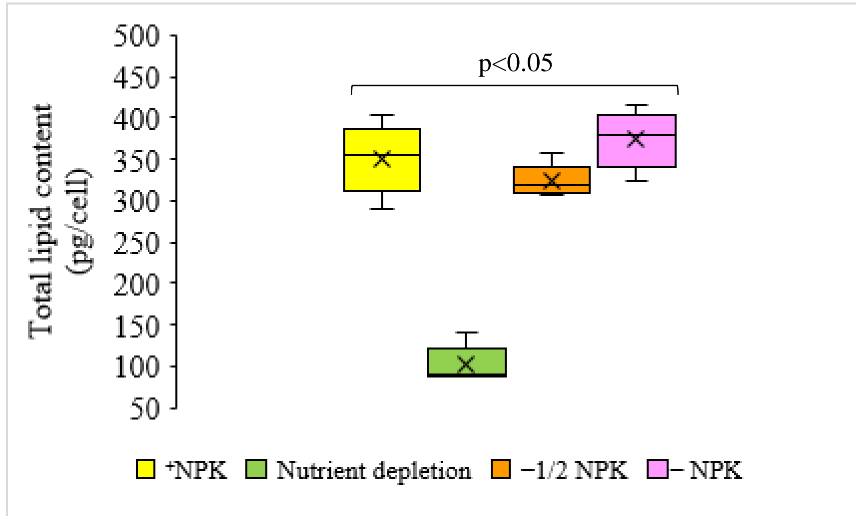

Figure 3. *Lipid content of the microalga D. bardawil* DCCBC15 *under nitrogen-depleted conditions*

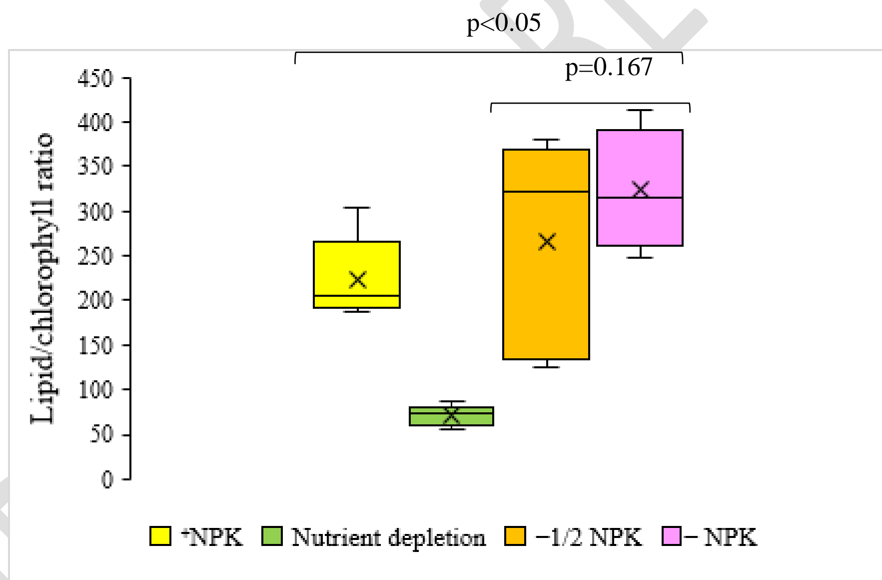

Figure 4*. Lipid/chlorophyll ratio of the microalga D. bardawil* DCCBC 15 *under nitrogen-depleted conditions.*

UNDER REVIEW

Table 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.

REFERENCES

1. Klausner, A., *Algaculture: food for thought.* Bio/technology, 1986. **4**(11): p. 947-953.
2. Chavoshi, Z.Z. and M. Shariati, *Lipid production in Dunaliella bardawil under autotrophic, heterotrophic and mixotrophic conditions.* Brazilian Journal of Oceanography, 2019. **67**.
3. Hosseini Tafreshi, A. and M. Shariati, *Dunaliella biotechnology: methods and applications.* Journal of applied microbiology, 2009. **107**(1): p. 14-35.
4. Ryckebosch, E., K. Muylaert, and I. Foubert, *Optimization of an analytical procedure for extraction of lipids from microalgae.* Journal of the American Oil Chemists' Society, 2012. **89**(2): p. 189-198.
5. Singh, P., M. Baranwal, and S.M. Reddy, *Antioxidant and cytotoxic activity of carotenes produced by Dunaliella salina under stress.* Pharmaceutical biology, 2016. **54**(10): p. 2269-2275.
6. Yilancioglu, K., et al., *Oxidative stress is a mediator for increased lipid accumulation in a newly isolated Dunaliella salina strain.* PloS one, 2014. **9**(3): p. e91957.

UNDER PEER REVIEW

7. Vo, T., et al., *Effect of osmotic stress and nutrient starvation on the growth, carotenoid and lipid accumulation in Dunaliella salina A9.* A9, Research in Plant Sciences, 2017. **5**(1): p. 1-8.
8. Tran, D., et al., *Growth, antioxidant capacity and total carotene of Dunaliella salina DCCBC15 in a low cost enriched natural seawater medium.* World Journal of Microbiology and Biotechnology, 2014. **30**: p. 317-322.
9. Guillard, R.R. and M.S. Sieracki, *Counting cells in cultures with the light microscope.* Algal culturing techniques, 2005: p. 239-252.
10. Prieto, A., J.P. Cañavate, and M. García-González, *Assessment of carotenoid production by Dunaliella salina in different culture systems and operation regimes.* Journal of biotechnology, 2011. **151**(2): p. 180-185.
11. Shaish, A., A. Ben-Amotz, and M. Avron, *[41] Biosynthesis of β-carotene in Dunaliella*, in *Methods in enzymology*. 1992, Elsevier. p. 439-444.
12. Lichtenthaler, H.K. and A.R. Wellburn, *Determinations of total carotenoids and chlorophylls a and b of leaf extracts in different solvents*. 1983, Portland Press Ltd.
13. Mishra, S.K., et al., *Rapid quantification of microalgal lipids in aqueous medium by a simple colorimetric method.* Bioresource technology, 2014. **155**: p. 330-333.
14. Park, J., et al., *Easy and rapid quantification of lipid contents of marine dinoflagellates using the sulpho-phospho-vanillin method.* Algae, 2016. **31**(4): p. 391-401.
15. Byrd, S.M. and J.M. Burkholder, *Environmental stressors and lipid production in Dunaliella spp. II. Nutrients, pH, and light under optimal or low salinity.* Journal of Experimental Marine Biology and Ecology, 2017. **487**: p. 33-44.
16. Phadwal, K. and P. Singh, *Effect of nutrient depletion on β-carotene and glycerol accumulation in two strains of Dunaliella sp.* Bioresource technology, 2003. **90**(1): p. 55-58.
17. Lamers, P.P., et al., *Carotenoid and fatty acid metabolism in nitrogen-starved Dunaliella salina, a unicellular green microalga.* Journal of biotechnology, 2012. **162**(1): p. 21-27.
18. Davidi, L., et al., *Origin of β-carotene-rich plastoglobuli in Dunaliella bardawil.* Plant physiology, 2014. **164**(4): p. 2139-2156.

UNDER PEER REVIEW

19. Byrd, S.M., J.M. Burkholder, and P.V. Zimba, *Environmental stressors and lipid production by Dunaliella spp. I. Salinity.* Journal of Experimental Marine Biology and Ecology, 2017. **487**: p. 18-32.
20. Goswami, R.K., K. Agrawal, and P. Verma, *Microalgae Dunaliella as biofuel feedstock and β-carotene production: An influential step towards environmental sustainability.* Energy Conversion and Management: X, 2022. **13**: p. 100154.
21. Shi, T.-Q., et al., *Stresses as first-line tools for enhancing lipid and carotenoid production in microalgae.* Frontiers in bioengineering and biotechnology, 2020. **8**: p. 610.

UNDER PEER REVIEW