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Total Phenolic Content and Antioxidant Capacity of *Dunaliella salina* Were Cultivated Under Stress Conditions on Salt Field Media

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Abstract: *Dunaliella salina* (*D. salina*) is a unicellular green microalga with a high β-carotene content and bioactive compounds that is essential for any study of the application of microalgae. The current research aimed to evaluate total phenolic content, and antioxidant capacity of *D. salina* CCAP 19/18 strain in RM1 and RM2 salt field media under various stress conditions including natural light, high salinity, and nitrogen starvation. As a result, after 17 days of being enrichment, in the RM1 medium, there was no significant difference in phenolic content under natural light, high salinity, and nitrogen starvation stresses, with $p = 0.305$. Similarly, the antioxidant capacity had no significant difference, which was $p = 0.105$ (natural light with high salinity), and $p=0.428$ (nitrogen starvation with high salinity). While in RM2 medium, the phenolic content and antioxidant capacity under nitrogen starvation stress far exceeded that of natural light and high salinity, with respective figures being 157.147 fg acid gallic/cell and 600.443%/cell and these figures had a significant difference ($p<0.05$). As a consequence, we could choose an appropriate medium for cultivating and harvesting algae in a large-scale pilot. These true potential extract ingredients in algae are fascinating for cosmetic industries or used for various health benefits such as nutraceuticals and medications against damaging causes, particularly free radicals.

Keywords: Dunaliella Salina, Carotenoid, Total Phenolic and Antioxidant Capacity

1. Introduction

Dunaliella salina (*Dunaliellaceae*) is one of the promising microalgae consumed as food and medicine for many years [1]. Microalgae *D. salina* has immense economic potential because they contain important pharmaceutical substances, such as β-carotene, glycerol, and other pigments [2]. Other microalgal molecules, besides pigments and lipids, such as phenolics, flavonoids, sterols, and tocopherols, have been the subject of numerous recent research due to their antioxidant, anti-inflammatory, and antibacterial effects. More particularly, phenolics are a diverse range of chemical compounds obtained mostly from plants that provide considerable benefits when consumed, including the prevention of numerous health diseases due to their radical scavenging activity [3]. In addition, antioxidants are substances that play a vital role in the prevention of oxidation and oxidative damage to cells by scavenging free (oxygen or hydroxyl) radicals such as superoxide anion radicals (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH), and singlet oxygen ($^1O_2$). Some previous investigations have shown that microalgae can be used as a source of antioxidant chemicals such as lutein in *Muriellopsis* sp., β-carotene in *D. salina*, carotenoids in *Haematococcus pluvialis*, astaxanthin in *H. pluvialis* and *Chlorococcum* sp., and so on. There have been few studies on the antioxidant activity of microalgae, particularly the relation between their antioxidant capacity and phenolic content. Therefore, it is important to evaluate the relationship between these two variables by selecting some sources that are rich in antioxidants from microalgae [4].

Microalgae are photoautotrophic organisms exposed to high oxygen levels and radical stress in their natural habitat.

As a result, they have evolved many effective defense systems against reactive oxygen species and free radicals. Microalgae can produce considerable amounts of high-value substances (antioxidant carotenoids, phenolic compounds, and polyunsaturated fatty acids) under stress conditions such as high light, nitrogen starvation, high salinity, ect [5]. In particularly, under nutrient stress conditions, growth is inhibited, and most of the fixed carbon in algal biomass is converted to secondary metabolite. Nitrate and salt stress both had an unfavorable effect on the cell's osmotic potential, promoting ROS production and eventually inhibiting development. The antioxidative pool, which includes polyphenols and carotenoids, neutralizes ROS [6].

The present study aimed to investigate how high light intensity, high salinity, and nitrogen starvation affect the total phenolic content and antioxidant capacity of *D. salina* CCAP 19/18 on RM1 and RM2 media to harvest biomass efficiently and achieve cost savings on a pilot scale in Vietnam.

2. Materials and Methods

2.1. Dunaliella salina Strains and Medium

The experiments were carried out the strain of *Dunaliella salina* CCAP 19/18 provided by Ph.D. Juergen E. W. Polle, Department of Biology, Brooklyn University, New York, United States.

The algae were grown in 1.5 M MD4 medium containing NPK 0.15 g/L, $MgSO_4$ 1.86 g/L, EDTA 8.76 mg/L, $FeCl_3$ 0.49 mg/L, $MnCl_2$ 1.89 mg/L, pH = 7.5 at 25°C under light intensity of 50 µmol photons.m^{-2}. s^{-1} with a frequency of 12:12 light/ dark cycle.

2.2. Experimental Design

D. salina was cultivated in 1.5M MD4 medium include two stages:

Growth phase: *D. salina* strain cultured in 1.5M MD4 medium with continuous aeration and illumination at 100 µmol photons.m^{-2}. s^{-1} with a frequency of 12:12 light/ dark cycle.

Inhibition phase: after 17 days of being enrichment, *D. salina* was transferred to different stress conditions. High salimity, natural light and nitrogen starvation were carried out with continuous aeration. The experiements were cultivated in two mediums: RM1 and RM2 and were repeated three times.

RM1 medium [7]: salt field water diluted with disstilled water to reach a salinity of 1.5M (90‰), then nutrient supplied similar to MD4 medium.

RM2 medium [7]: salt field water (380‰) diluted with seawater to reach salinity 1.5M (90‰), then nutrient supplied similar to MD4 medium.

2.3. Determination of Cell Density

Take 100µl of algae suspension immobilized with Lugol's iodine solution (5% iodine and 10% potassium iodide mixed in distilled water). The cell density in the culture was determined by direct cell counting using a Neubauer Haemocytometer, the chamber depth is 0.1 mm, and the main grid is made up of nine large 1 mm × 1 mm squares. The following formula determined the cell number [8]:

Number of cells/ml = total cells counted x 10^4 x dilution factor.

2.4. Total Phenolic Content

One milliliter aliquot of the algal suspension was centrifuged at 10000 rpm for 5 minutes. After centrifuging, the pellet was extracted with 1 ml of methanol, and the mixture was vigorously shaken and incubated for around 30–60 minutes and centrifuged again at 10000 rpm for 5 minutes. Take 500 µL from extract mixed with 500 µL of Folin–Ciocalteu reagent and allow to stand at room temperature for 5–10 minutes. Next, 500 µL sodium bicarbonate solution (30%) was added to the mixture. After incubation for 90 minutes at room temperature, the absorbance was measured at 750 nm. Total phenolics were calibrated against gallic acid standard solutions (10-200 mg/L) and are expressed as mg gallic acid equivalent (G. A. E.) biomass [9].

Calibration curve for standard gallic acid: y = 30,263x – 0,0638; R^2 = 0,9948.

2.5. Antioxidant Capacity

Reagent solution DPPH: About 0.004 g DPPH (1,1-diphenyl-2 picrylhydrazyl) reagent was dissolved in 100 ml methanol [10].

One milliliter aliquot of the algal suspension was centrifuged at 10000 rpm for 5 minutes. After extracting the pellet with 1 ml of methanol, the mixture was rapidly shaken and incubated in the showcase cooler (4°C, 4 hours) before being centrifuged again at 10000 rpm for 5 minutes. 500 µL of test samples was mixed to 1 ml of 0,004% DPPH methanolic solution. The mixture was vortexed for 1 minute before being left at room temperature for 30 minutes in the dark. The absorbance of all the sample solutions was measured at 517 nm [11]:

Radical scavenging activity was expressed as percentage inhibition of DPPH radical and was calculated by following equation:

$$\text{Percentage inhibition (I\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

2.6. Statistical Analysis

Data were processed using Microsoft Excel 2019 software and SPSS 20.0 software's one-way analysis of variance (ANOVA). Whether or not an observed correlation is statistically significant was evaluated by P values (significant when $P \leq 0.05$). The data were presented as the mean ± standard error of mean (SEM). All treatments were evaluated three times.

3. Results and Discussion

3.1. The Total Phenolic Content of D. salina

The total phenolic content of *D. salina* in RM1 and RM2

media under different stress conditions was shown in (Figure 1 and Table 1). The phenolic content of RM1 medium under natural light, high salt concentration (4M NaCl), and nitrogen starvation had no significant difference (p=0.305), with the figure standing at 56.328 fg acid gallic/ cell, 51.698 fg acid gallic/ cell, 50.478 fg acid gallic/ cell respectively. In contrast, under nitrogen starvation conditions in RM2 medium, there was a significant difference (p< 0.05) compared to the other two stress conditions. In addition, the phenolic content of nitrogen starvation (about 157.147 fg acid gallic/ cell) was far higher than that of the natural light and high salinity, which was 37.967 fg acid gallic/ cell and 52.589 fg acid gallic/ cell respectively. In conclusion, there was no change in three stress conditions in RM1 medium. However, in RM2 medium, the optimal stress condition was nitrogen starvation.

Andriopoulos, V etal. (2022) reported that the cell density of *D. salina* reduced considerably after three days of N_2 deprivation. After three days of stress treatment, the cell density of *D. salina* was reduced by 64% compared to the control. The cumulative stress reduction in cell density in *D. salina* showed a less noticeable reduction in cell density than the single stress reduction. Total phenolics in *D. salina* rose considerably when exposed to N_2-deprivation stress. Furthermore, cumulative stress exposure of tested algae resulted in a highly significant increase in total phenolic compounds. Because of the impact of N_2-deprivation on the ability to increase osmotic pressure. This result suggests that limiting the nitrogen source while controlling the temperature may be a promising way to acquire a greater phenolic content for biofuel fabrication [3].

Kaumeel Chokshi *et al*. (2017) reported that polyphenols were the substrates for the H_2O_2-scavenging enzyme peroxidase and prevent ROS from expanding by changing the peroxidation rate and reducing the fluidity of the cell membrane. The accumulation pattern of total polyphenols was precisely identical to the pattern of proline synthesis. The largest accumulation of total polyphenols was seen in the nitrogen-starved culture after 2 days, specifically, 246.75 ± 4.19 µg/g FW, which was about 1.7-fold greater than the control culture1.7-fold greater than the control culture (143.47 ± 5.47 µg/g FW). Despite the polyphenols' claimed antioxidant qualities and the possibility of microalgae as a source of polyphenols, there has been little research on the accumulation of polyphenols by microalgae [12].

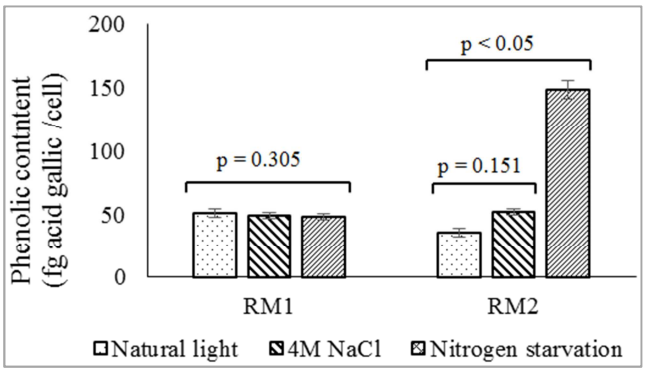

Figure 1. *Phenolic content of D. salina under different stress conditions in RM1 and RM2 media.*

3.2. The Antioxidant Capacity of D. salina

The antioxidant capacity of *D. salina* in RM1 and RM2 media under different stress conditions was shown in (Figure 2 and Table 1). The figures for the antioxidant capacity under natural light and nitrogen starvation were slightly higher than high salinity, which were 223.804%/cell and 238.282%/cell, compared to 199.635%/cell of high salinity in RM1 medium. Meanwhile, in RM2 medium, the antioxidant capacity of *D. salina* under nitrogen starvation was highest, at 600.443% /cell, and had a significant difference (p<0.05), followed by 171.858% /cell (natural light) and 171.667 858% /cell (high salinity). In conclusion, in RM1 medium, natural light and nitrogen starvation gave a high phenolic content and antioxidant capacity, while in RM2 medium, nitrogen starvation reached an optimal phenolic content and antioxidant capacity.

Algal cells activated several defense systems for scavenging the ROS generated in different cellular compartments. Under unfavorable conditions, the generation rate of ROS exceeded their scavenging rate, and the excess ROS caused oxidative injury to the cell. To counter ROS toxicity, cells employed innate defense responses. This included an array of enzymatic antioxidants like SOD, CAT, APX, and various non-enzymatic scavengers like proline, and polyphenols. The increase in the activities of antioxidant enzymes SOD, CAT, peroxidases, glutathione reductase, etc., have been reported previously in the microalgae grown under a different nutrient limitations or starvation conditions including nitrogen starvation [12].

Mostafa *et al* (2012) reported that the reduction in nitrate content induced a stress condition and an increase in carbon skeleton compounds (as phenolics) as a result of metabolic modifications under these stress conditions, as well as a decrease in nitrogen skeleton compounds as phycobilin pigment synthesis. Because of the synergistic effects of the phycobilin pigment and the phenolic compounds produced in excess under stress nitrate conditions, which had high redox potentials, the antioxidant activity remained comparable to or even higher than the control. The reported antioxidant activity (comparable to that observed in the presence of high nitrate content (6-9 g/L) on nitrogen deprivation) was mostly due to the high synthesis of carbon skeleton compounds (phenolic compounds), which had significant antioxidant activity [13].

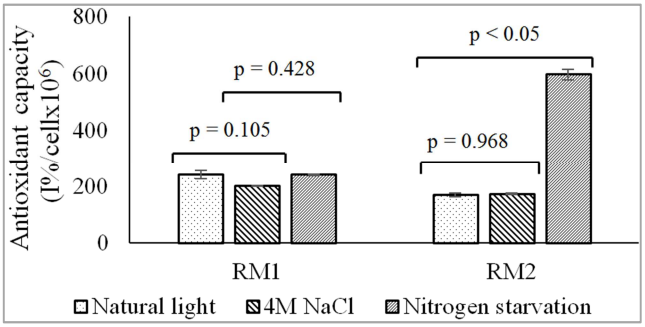

Figure 2. *Antioxidant capacity of D. salina under different stress conditions in RM1 and RM2 mediums.*

Table 1. *Phenolic content and antioxidant capacity of D. salina under different conditions in RM1 and RM2 mediums.*

Stress conditions	Phenolic content (µg acid gallic/ ml)		Antioxidant capacity (I%)	
	RM1 medium	RM2 medium	RM1 medium	RM2 medium
Natural light	56.328±3.535[a]	37.967±3.167[a]	223.804±13.728[ab]	171.858±5.718[a]
Salt stress (4M NaCl)	51.698±2.292[a]	52.589±2.232[a]	199.635±1.739[a]	171.667±2.257[a]
Nitrogen starvation	50.478±2.264[a]	157.147±7.463[b]	238.282±2.640[b]	600.443±18.452[b]

a, b: Means for groups in homogeneous subsets are displayed

4. Conclusion

Antioxidant capacity and total phenolic content of *D. salina* changed in different stress conditions such as light stress (natural light), salinity stress (4M NaCl), and nitrogen starvation was cultivated in two media, RM1 and RM2. Among these stress conditions, nitrogen starvation in both mediums indicated that *D. salina* could reach a higher phenolic content and antioxidant capacity than the other conditions. Based on this study, we could raise the microalgae on a large scale, such as in a plastic bag bioreactor or even 1000 liter because it saves material, extracts more antioxidant substances, and makes a pharmaceutical product that helps people against free radicals that have severe effects on our lifestyle.

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