Southeast-Asian J. of Sciences, Vol. 9, No. 2 (2022) pp. 124-130

TOTAL PHENOLIC CONTENT AND ANTIOXIDANT CAPACITY OF MICROALGAE DUNALIELLA SALINA WERE CULTIVATED UNDER SALINITY STRESS IN SALT FIELD MEDIA

Nguyen Thi Hong Phuc*, Pham Thi Ngoc Dung, Vo Hong Trung

Faculty of Pharmacy,
Nguyen Tat Thanh University, Ho Chi Minh City, Vietnam
e-mail: nthphuc@ntt.edu.vn

Abstract

Dunaliella salina (D. salina), a unicellular green microalga, has *rich sources of natural antioxidants,* particularly β-carotene. In this study, we determined how the different salt concentrations affected the cell density, the total phenolic content, and the antioxidant capacity of D. salina CCAP 19/18 on RM1 and RM2 media. As a result, *D. salina* was exposed to various salt stress concentrations to inhibit after 13 days of enrichment, with salt stress up to 4M showing significantly higher total phenolic content in both RM1 and RM2 media, at approximately 23.617 fg acid gallic/cell and 5.735 fg acid gallic/cell, respectively. Similarly, the figures for the antioxidant capacity in RM1 and RM2 media stood at 217.345 %/cell and 178.090 %/cell, respectively. The outcomes of this investigation can be used to enhance the antioxidant substance production in microalgae for functional food and biofuel in the future.

1. Introduction

Dunaliella, which belongs to the order *Chlamydomonadales,* family *Dunaliel-*

Key words: Dunaliella salina, carotenoid, total phenolic and antioxidant capacity.

laceae, is one of the most promising microalgae and has been consumed as food and medicine for many years due to its large economic potential because it contains substances important in pharmaceuticals such as beta-carotene, glycerol, and other pigments [1]. The ability of cells to survive and flourish in saline environment under the influence of osmotic stress has received considerable attention and *Dunaliella* are recognized as the only eukaryotic and photosynthetic organism, which shows a remarkable degree of adaptation to a variety of salt concentrations, ranging from 0.05M to saturation (5.0M) [2].

Moreover, algae contain a wide range of compounds with free radical scavenging ability and reduce the damage caused by free radicals, both in vitro and in vivo. These molecules include phycobiliproteins like phycocyanin, sulfated polysaccharides, and carotenoids. Phenolic compounds are another type of free radical scavenger found in algae. While algae are reported by several authors as a potential source of natural antioxidants, only recently has the role of phenolics and their antioxidant activity gained significant attention in macroalgae [3] In particular, total phenolic compounds rose as salinity increased. Phenolic compounds are cellular solutes that aid to reduce environmental stress. The activity of ROS scavenging enzymes was dramatically impacted by salinity. Total phenolic compounds rose as salinity increased. Phenolic compounds are cellular solutes that aid to reduce environmental stress. Increasing antioxidant activity is widely known as the key to preventing salt damage, whereas sensitive species generally exhibit no change or a decline in activity. [4].

The present study aimed to analyze in detail the suitable concentrations of salinity stress conditions in RM1 and RM2 media to harvest biomass efficiency and cost savings for D. salina on a Vietnam pilot scale.

2. Materials and methods

2.1 Dunaliella salina strains and medium

The experiments were carried out on 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 1.5M MD4 medium containing NPK 0.15 $g.L^{-1}$, $MgSO_4$ 1.86 $g.L^{-1}$, EDTA 8.76 $mg.L^{-1}$, $FeCl_3$ 0.49 $mg.L^{-1}$, $MnCl_2$ 1.89 $mg.L^{-1}$, pH = 7.5 at 25^0C under light intensity of 50 μmol photons. $m^{-2}.s^{-1}$ with a frequency of 12:12 light/ dark cycle.

2.2. Study design

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

Growth phase: D. salina strain is cultured in 1.5M MD4 medium with continuous aeration and under natural light conditions

Inhibition phase: After 13 days of enrichment, D. salina was transferred to different salt stress concentrations, such as 1M, 1.5M, 3M, and 4M NaCl to inhibit the algae, with 1.5M NaCl as a control sample. The experiments were cultivated in two media: RM1 and RM2, and were repeated three times.

RM1 medium [5]: Salt field water diluted with disstilled water to reach a salinity of 1.5M (90 ‰), then nutrient supplied similar to MD4 medium.

RM2 medium [5]: Salt field water (380‰) diluted with seawater to reach salinity 1.5M (90‰), then nutrient supplied similar to MD4 medium

2.3. Analysis methods

2.3.1. 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 [6]: Number of cells/ml = total cells counted x 104 x dilution factor

2.3.2. Total Phenolic content

One milliliter aliquot of the algal suspension was centrifuged at 10000 rpm for 5 min. After centrifuging, the pellet was extracted with 1 ml of methanol, and the mixture was vigorously shaken and incubated for 30–60 min and centrifuged again at 10000 rpm for 5 min. Take 500 μL from extract mixed with 500 μL of Folin–Ciocalteu reagent and allow to stand at room temperature for 5–10 min. Next, 500 μL sodium bicarbonate solution (30%) was added to the mixture. After incubation for 90 min at room temperature, the absorbance was measured at 750 nm. Total phenolics were calibrated against gallic acid standard solutions (10-200 mg.L-1) and are expressed as mg gallic acid equivalent (G.A.E.) biomass [7].

Calibration curve for standard gallic acid:

$$y = 30,263x–0,0638; R^2 = 0,9948.$$

2.3.2.1. DPPH radical scavenging assay

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

One milliliter aliquot of the algal suspension was centrifuged at 10000 rpm for 5 min. After extracting the pellet with 1 ml of methanol, the mixture was rapidly shaken and incubated in the showcase cooler (4^oC, 4 hours) before being centrifuged again at 10000 rpm for 5 minutes. 500 μL of test samples

were added 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 [8]: Radical scavenging activity was expressed as percentage inhibition of DPPH radical and was calculated by following equation:

$$\text{Percentageinhibition(I\%)} = \frac{\text{Absorbanceofcontrol} - \text{Absorbanceofsample}}{\text{Absorbanceofcontrol}} \times 100$$

2.4. 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. Result and Discussion

3.1. Determination of total phenolic content

The total phenolic content of D. salina in RM1 and RM2 media under different salt stress conditions was shown in (Fig 1 and Table 1). In RM1 medium, there were no changes in total phenolic content at 1 M and 3 M salt concentrations (15.417 fg/cell and 15.702 fg/cell) compared to the control sample at 15.842 fg/cell, and there was no significant difference ($p<0.05$). Whereas a significant increase was observed at 4M salinity, around 1.5 times compared to the control sample. In the RM2 medium, concentrations at 1M and 3M NaCl increased slightly (16.197 fg/cell and 17.773 fg/cell) compared to the control sample at 13.123 fg/cell ($p<0.05$), while concentrations at high salinity (4M NaCl) increased considerably at 23.617 fg/cell, and both of these stress conditions had a significant difference ($p<0.05$). In conclusion, a salt concentration of 4M NaCl in RM1 medium resulted in a higher total phenolic content than the other concentrations, and levels of NaCl at 3M and 4M in RM2 medium differed noticeably from 1 M and the control sample.

According to Mukherjee et al. (2020), for 21 days, *Cladophora glomerata, Chaetomorpha aerea, Rhizoclonium crassipellitum,* and *Pithophora cleveana* were exposed to growth conditions with salt concentrations ranging from 0 to 1500 ppm. Their studied taxa proved to be rich sources of phenol. In the current experiment, dose-dependent polyphenol content enrichment was seen for salinity stresses in all four algal species. Phenolic levels in thalli exposed to hypersalinity conditions rose (3-4-folds) [9]. Polyphenolic compounds are

produced in response to stress exposure due to their function as active scavengers of the ROS produced by chronic stress. Polyphenolic content increased up to 7 days after stress exposure, followed by a drop in total phenolic content levels. However, the temporal fluctuations in polyphenolic concentrations in each control group were small or insignificant. This continuous rise in polyphenol content under stress conditions might be an adaptive feature for enhancing the turgor pressure necessary to maintain ionic balance during hypersalinity-induced osmotic stress [9].

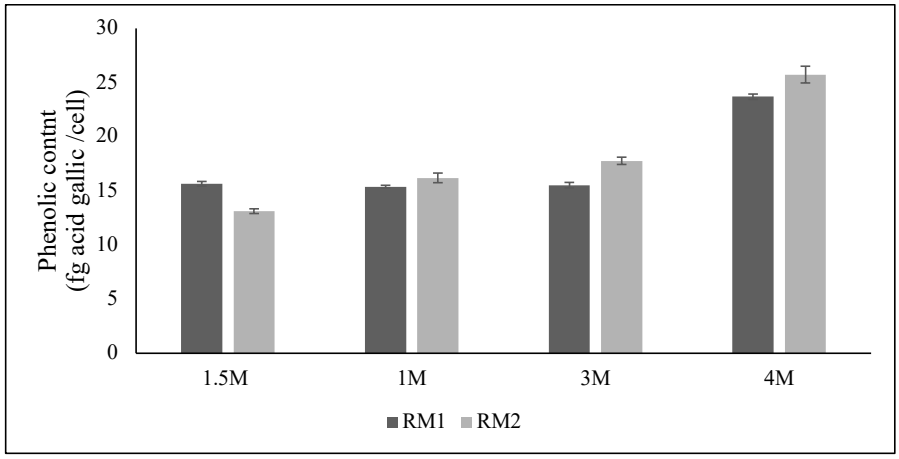

Figure 1. Phenolic content of D. salina under different levels of salt stress in RM1 and RM2 media

3.2. Antioxidant capacity: DPPH radical scavenging The antioxidant capacity of D. salina in RM1 and RM2 medium under different salinity levels was shown in (Fig 2 and Table 1). In the RM1 medium, the results showed that antioxidant capacity under salt concentrations of 1M and 3M NaCl fluctuated about 143.098%/cell and 150.792%/cell, respectively, while at high salt concentrations (4M NaCl), it was more than 1.5 times higher than the control sample (1.5M NaCl) ($p<0.05$). Salinity levels in RM2 medium at 1M and 3M were higher than the control sample at 109.315%/cell and 113.402%/cell, respectively. By contrast, salinity levels at 4M NaCl increased significantly, nearly twofold, compared to the control sample. These figures in both media have a significant difference ($p<0.05$).

As reported by Montazeri-Najafabady et al. (2016), D. salina cells produce the main carotenoid pigment, β-carotene, necessary to protect cells against stress conditions. Under hyper-osmotic conditions, carotene concentration reaches its maximum amount after 2 hours and remains constant up to 24 hours. At hypoosmotic pressure, the corresponding concentration of this pig-

ment decreased and remained constant for 2 hours. It means that D. salina cells need 2 hours to adapt to hypo-osmotic shock, and carbonyl is not a necessary pigment in this condition. Carotenoids are essential to protect *Dunaliella* cells against extreme conditions through different mechanisms [10].

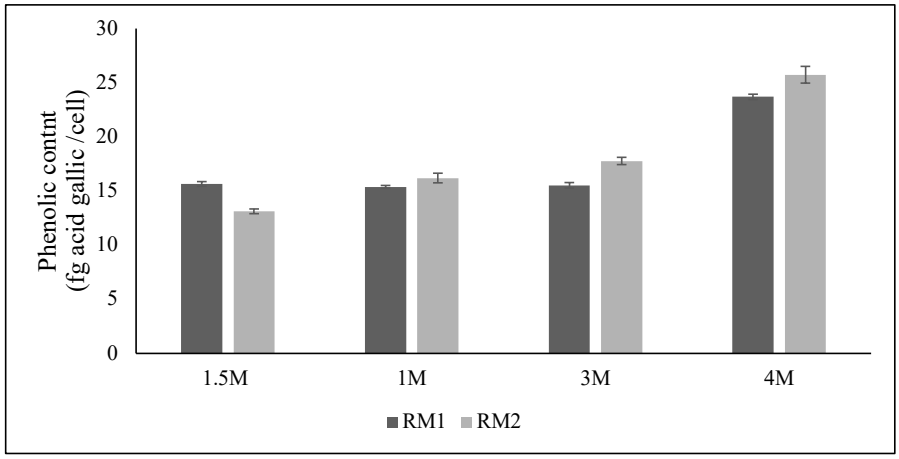

Figure 2. Antioxidant capacity of D. salina under different levels of salt stress in RM1 and RM2 media

Salinity	Phenolic content (fg acid gallic/cell)		Antioxidant capacity (I%/cell)	
	RM1 medium	RM2 medium	RM1 medium	RM2 medium
1.5 M NaCl	15.842±0.216[a]	13.123±0.218[a]	135.088±1.036[a]	87.023±0.591[a]
1.0 M NaCl	15.417±0.129[a]	16.197±0.445[b]	143.098±0.659[b]	109.315±1.018[b]
3.0 M NaCl	15.702±0.254[a]	17.773±0.336[b]	150.792±2.376[c]	113.402±0.826[c]
4.0 M NaCl	23.617±0.247[b]	25.735±0.773[c]	217.345±2.177[d]	178.090±0.920[d]

Table 1. Phenolic content and antioxidant capacity of D. salina under different levels of salt stress in RM1 and RM2 media

4. Conclusion

The results of the experiments showed that D. salina CCAP strain referred to salinities ranging from 3M to 4M for optimal total phenolic content and antioxidant capacity. Based on this optimal salinity, we will apply stress conditions,

especially the effect of osmotic stress for carotene induction on these strains, in our subsequent experiments.

References

[1] Singh, P., Baranwal, M.,& Reddy, S. M. (2016), *Antioxidant and cytotoxic activity of carotenes produced by Dunaliella salina under stress,* Pharmaceutical biology, **54**(10), 2269-2275.

[2] Mishra, A., & Jha, B. (2009), *Isolation and characterization of extracellular polymeric substances from micro-algae Dunaliella salina under salt stress,* Bioresource technology, **100**(13), 3382-3386.

[3] Monteiro, M., et al., *Effect of extraction method and solvent system on the phenolic content and antioxidant activity of selected macro-and microalgae extracts,* **32**(1), 2020, 349-362.

[4] El-Adl, M.F., et al., *Impact of the microalga Dunaliella salina (Dunal) Teodoresco culture and its β-carotene extract on the development of salt-stressed squash,* Cucurbita pepo L. cv. Mabrouka, **28**(4)(2022), 749-762.

[5] Trung, V. H., Anh, N. L. T., & Phúc, N. T. H. , *Môi trường tiết kiệm cho nuôi cấy vi tảo Dunaliella salina quy mô pilot ở Việt Nam,* Tạp chí Khoa học Trường Đại học Sư phạm Thành phố Hồ Chí Minh, **18**(6)(2021), 1006.

[6] Guillard, R. R., & Sieracki, M. S. (2005), *Counting cells in cultures with the light microscope,* Algal culturing techniques, 239-252.

[7] Goiris, K., et al., *Antioxidant potential of microalgae in relation to their phenolic and carotenoid content,* Journal of Applied Phycology, **24**(6)(2012), 1477-1486.

[8] Yaltirak, T., et al., *Antimicrobial and antioxidant activities of Russula delica Fr,* Food and Chemical Toxicology, **47**(8)(2009), 2052-2056.

[9] Mukherjee, P., et al., *Investigation on the effects of nitrate and salinity stress on the antioxidant properties of green algae with special reference to the use of processed biomass as potent fish feed ingredient*, Aquaculture International, **28**(1)(2020), 211-234.

[10] Montazeri-Najafabady, N., et al., *Effects of osmotic shock on production of β-carotene and glycerol in a naturally isolated strain of Dunaliella salina,* Journal of Applied Pharmaceutical Science, **6**(8)(2016), 160-163.