



Impact of Thermal Extremes and Nutrient Availability on the Biochemical Profile and Antioxidant Capacity of the Halophilic Microalga *Dunaliella Salina*

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Abstract

Microalgae are increasingly recognized as a promising biomass feedstock for the production of biofuels, pharmaceuticals, and cosmeceuticals. Among these, *Dunaliella salina*, a halophilic species capable of harnessing solar energy via photosynthesis stands out for its proficiency in synthesizing high-value organic compounds, including carotenoids, lipids, and phenolics. Consequently, this study investigated the microalgal strain *D. salina* CCAP 19/18 to evaluate the accumulation of these metabolites and their associated antioxidant capacity under cold stress and nutrient deprivation. The findings indicate that the most significant accumulation occurred on day 12 post-induction under a combined regime of cold stress (10°C for 6 hours/day) and complete NPK depletion. Conversely, exposure to a thermal stress of 45°C in conjunction with nutrient deficiency proved catastrophic; optical microscopy revealed that cells underwent lysis and clumping, with no signs of viability recorded after three days of stress. These findings provide a scientific foundation for optimizing cultivation strategies of the microalga *D. salina*, aimed at harvesting biomass with elevated contents of bioactive compounds for applications in pharmaceuticals and functional foods.

Keywords: *Dunaliella salina*, Carotenoids, Lipids, Phenolics, Antioxidant Capacity, Temperature.

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Introduction

Microalgae are considered the richest natural sources of carotenoids, for example *Dunaliella salina*, *Haematococcus pluvialis*. One of the sources of carotenoids with potential medical value in *D.salina* microalgae is β -carotene. Dietary supplementation of natural β -carotene offers profound health benefits, primarily attributed to its potent antioxidant properties which neutralize and eradicate deleterious free radicals (Xu et al., 2018). Many studies have been carried out aimed at harvesting large biomass sources in the production of valuable organic substances in *D.salina* microalgae, studies have also shown that

culturing under adverse conditions such as temperature, nutrition, light and salinity directly affect metabolism and production of pigment molecules in microalgae (Colusse et al., 2020).

Maltsev *et al.* (2021) found that nitrogen reduction resulted in an 87.9% increase in total lipid content in *Nephrochlamys yushanlensis* MZ-Ch62 compared to control conditions (Maltsev et al., 2021). According to Almutairi *et al.* (2020), reducing the phosphorus concentration by 50% increases the total lipid content of *Dunaliella salina* by 3 times, and completely removing the phosphorus source increases the total lipid content by about 4 times compared to normal conditions control (Almutairi, 2020). Besides nutritional factors, temperature also affects the synthesis of organic substances in microalgae. Research by Hoffmann et al. (2010) on the microalgae strain *Nannochloropsis salina* showed that TFA and EPA content increased in microalgae low temperature of 17°C compared to 21°C and 26°C (Gaignard et al., 2021). Guo et al. (2022) conducted a study on the effects of bicarbonate concentration and temperature on the microalga *Dunaliella salina* HTBS. The results showed that under the condition of bicarbonate concentration of 0.15M and low temperature of 16°C, lipid, protein, carbohydrate content increased and recorded an increase in carotenoid and chlorophyll content in microalgae (Guo et al., 2022). Trung Vo et al. (2024) studied the influence of phosphorus concentration on growth, pigment and lipid accumulation in the microalga *Picochlorum* sp. The results showed that under the condition of phosphorus concentration of 0.02 g/L and 0.04 g/L, the accumulation of carotenoid and chlorophyll content reached the highest value after 12 days of culture. Meanwhile, lipid content reached its highest value after 9 days of culture at a phosphorus concentration of 0.16 g/L (Nguyen et al., 2024). Consequently, thermal and nutritional variables exerted a positive influence on the biochemical profiles and antioxidant capacity of the microalga *D. salina*. The present study sought to investigate the impacts of thermal and nutritional stress on the accumulation of essential biochemical constituents, specifically chlorophyll, carotenoids, lipids and phenolic compounds, alongside its total antioxidant activity.

Materials and Methods

Materials

The microalgae strain *D. Salina* CCAP 19/18, maintained and stored at the Department of Biochemistry at Nguyen Tat Thanh University, was cultivated in RM2 medium (Vo et al., 2023). This substrate comprised salt field water (380‰) diluted with seawater to achieve a final salinity of 1.5M (90‰), subsequently enriched with a nutrient profile including 0.15 g/L NPK, 1.86 g/L MgSO₄, 8.76 mg/L EDTA, 0.49 mg/L FeCl₃, and 1.89 mg/L MnCl₂. The culture was maintained at a regulated pH of 7.5 and a temperature of 25°C ± 2°C, under a light intensity of 70 μmol photons/m²/s with a 12:12 hour light/dark photoperiod (Trung et al., 2021).

Determination of Cell Density

Cell density was quantified through direct counting using a Neubauer hemocytometer. A 10 μL aliquot of the algal suspension, previously fixed with Lugol's solution (comprising 5% Iodine and 10% KI salt), was introduced into the counting chamber, which featured a depth of 0.1 mm and a 1 mm² grid area. Microscopic observation was subsequently performed under a 10X objective lens. To ensure statistical reliability, the counting procedure was conducted in triplicate for each respective sample (Andersen, 2005).

Cell density per 1 mL was determined according to the formula:

$$D = \frac{n}{i} \times 10^4 \times \text{dilution factor} \quad (1)$$

Where:



n: total number of counted cells

i: counting area

D: cell density (cell/mL)

Total Carotenoid Content

A 1 mL volume of the algal suspension was centrifuged at 6000 rpm for 15 minutes, after which the supernatant was discarded to isolate the biomass. The resulting algal pellet underwent extraction using 3 mL of an ethanol:hexane solvent mixture, gently vortexing. Subsequently, 4 mL of hexane and 2 mL of distilled water were introduced into the mixture, with continuous vortexing maintained until complete decolorization of the biomass was achieved. Following a second centrifugation step, the mixture partitioned into two distinct phases. The pigment-rich organic layer was then recovered and subjected to photometric measurement at specific wavelengths of 450 nm, 645 nm, and 662 nm (Prieto et al., 2011).

Total carotenoid content was determined using the formula (Shaish et al., 1992):

Total carotenoids ($\mu\text{g/mL}$): $A_{450} \times 25.2$

The content of chlorophyll a and b was calculated according to the formula (Dere et al., 1998):

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

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

$$\text{Total chlorophyll } (\mu\text{g/mL}) = \text{chlorophyll a} + \text{chlorophyll b} \quad (2)$$

Where:

A_{645} is the absorbance at wavelength 645 nm

A_{662} is the absorbance at wavelength 662 nm

Determination of Total Lipid Content by Sulfo-Phospho-Vanillin Method

The phosphovanillin reagent was prepared by weighing 0.06 g of vanillin into a 50 mL foil-wrapped volumetric flask. To this, 2 mL of absolute ethanol and 8 mL of distilled water were added, followed by vigorous agitation to ensure complete dissolution. The solution was subsequently diluted to the graduated mark with phosphoric acid (H_3PO_4) and thoroughly homogenized (Park et al., 2016).

A lipid standard curve was established using Canola oil (total lipid content 100 g/100 g) dissolved in chloroform to produce a concentration range of 10–140 $\mu\text{g/mL}$. The mixture was heated at 90°C for 10 minutes in a water bath to achieve complete evaporation of the solvent. Subsequently, 2 mL of concentrated sulfuric acid was added to each test tube, followed by vortexing and incubation at 95°C for 10 minutes. After cooling, 5 mL of phosphovanillin reagent was introduced, and the mixture was vortexed until homogenization was achieved. The absorbance of the samples was then measured photometrically at a wavelength of 530 nm (Park et al., 2016). Based on the resulting data, a linear regression equation ($y=ax+b$) was derived to determine the correlation between optical density (OD, y) and lipid concentration (x, $\mu\text{g/mL}$).

The total lipid content of *D. salina* CCAP 19/18 was quantified by centrifuging a 1 mL aliquot of the microalgal suspension. After the supernatant was discarded, the resulting algal pellet underwent extraction with 2 mL of concentrated sulfuric acid, followed by the analytical procedure described previously. The absorbance of the samples was measured photometrically at a wavelength of 530 nm



(Anschau et al., 2017). The lipid concentration was subsequently calculated using a linear regression equation, where the optical density (OD) was correlated with the total lipid content ($\mu\text{g/mL}$) as follows:

$$\text{Total Lipids } (\mu\text{g/mL}) = (A_{530} - b)/a \quad (3)$$

Where:

x: Total lipid content ($\mu\text{g/mL}$)

y (A_{530}): Optical density measured at 530 nm

a, b: Constants derived from the lipid standard curve equation

Determination of Total Phenolic Content

A gallic acid standard curve was established by dissolving gallic acid in methanol to produce a series of standard concentrations ranging from 1 to 50 mg/L. For each concentration, a 500 μL aliquot was transferred into a 2 mL Eppendorf tube, followed by the addition of 500 μL of Folin-Ciocalteu reagent and thorough vortexing. After a 5-minute incubation period, 500 μL of 10% Na_2CO_3 was introduced slowly, mixed well, and the mixture was subsequently incubated for 90 minutes. Following centrifugation, the absorbance was measured photometrically at a wavelength of 750 nm. Based on these measurements, a linear regression equation ($y=ax+b$) was derived to represent the correlation between optical density (OD, y) and the phenolic concentration expressed as gallic acid equivalents (x, mg/L).

The total phenolic content of *D. salina* CCAP 19/18 was quantified by centrifuging a 1 mL aliquot of the microalgal suspension. After discarding the supernatant, the resulting algal pellet was extracted with 1 mL of absolute methanol and incubated in darkness for 30 minutes. Subsequently, the sample underwent the Folin-Ciocalteu procedure as described previously, followed by centrifugation and photometric measurement of the absorbance at 750 nm (Michiu et al., 2022). The total phenolic concentration was determined using a linear regression equation, where the optical density (OD) was correlated with the phenolic content ($\mu\text{g/mL}$) as follows:

$$\text{Total Phenolic Content } (\mu\text{g/mL}) = (A_{750} - b) / a \quad (4)$$

Where:

x: Total phenolic content ($\mu\text{g/mL}$)

y (A_{750}): Optical density measured at 750 nm

a, b: Constants derived from the phenolic standard curve equation

Determination of Antioxidant Capacity

Reagent Preparation: The DPPH working solution was prepared by weighing 0.004 g of 1,1-diphenyl-2-picrylhydrazyl into a 100 mL foil-wrapped volumetric flask. The reagent was dissolved in absolute methanol and diluted to the graduated mark.

Sample Extraction and Analysis: A 1 mL aliquot of the microalgal suspension was centrifuged, after which the supernatant was discarded. The resulting algal pellet underwent extraction with 1 mL of absolute ethanol, followed by thorough homogenization and incubation at 4°C for 4 hours. Subsequently, the mixture was centrifuged to isolate the extract. A 500 μL volume of this extract was transferred into a



foil-covered 2 mL Eppendorf tube, to which 1 mL of the DPPH reagent was added. The mixture was then incubated in darkness for approximately 30 minutes. The absorbance was subsequently measured at a wavelength of 517 nm (Shah & Modi, 2015). The antioxidant capacity (I%/mL) was determined based on the radical scavenging activity of DPPH according to the following formula (Paixão et al., 2007; Shah & Modi, 2015):

$$I\% = \frac{A_{blank} - A_{sample}}{A_{blank}} \times 100 \quad (5)$$

Where:

I%: The radical inhibition percentage (%).

A_{blank} : The absorbance of the control sample (containing only DPPH reagent and solvent) measured at 517 nm.

A_{sample} : The absorbance of the test solution (containing microalgal extract and DPPH reagent) measured at 517 nm

Experimental Design

Growth Phase: The microalgal strain *Dunaliella salina* CCAP 19/18 was cultivated in RM2 medium at a salinity of 1.5 M, with continuous aeration provided to maintain optimal growth until a cell density of 0.15×10^6 cells/mL was achieved. Following a 12-day cultivation period, the microalga was subjected to various temperature-induced stress conditions.

Stress induction Phase: *D. salina* cultures were exposed to three temperature regimes: 10°C, 25°C, and 45°C ($\pm 2^\circ\text{C}$) (T10, T25 and T45) for daily durations of either 3 or 6 hours (3h or 6h). These thermal treatments were conducted in conjunction with nutrient deprivation strategies, which included 15 treatments with a control group (full nutrients), a 50% NPK reduction ($-\frac{1}{2}$ NPK), and complete NPK depletion (-NPK). Each experimental treatment was performed in triplicate to ensure statistical validity.

Harvesting and Analysis: The stress induction period spanned 12 days. Samples were harvested at specific intervals: initially on day 12 of the growth phase (designated as Before stress), and subsequently on days 3, 6, 9, and 12 during the stress exposure period for further analysis.

Data Analysis

Data were processed using Microsoft office Excel 2016 and analyzed by One Way ANOVA using SPSS 20.0 software with significance error $p \leq 0.05$. All data are presented as: Mean $\pm$ Standard Error (SE).

Results and Discussion

Total Carotenoid Content

The results indicated that the synergistic effect of thermal stress and NPK deprivation significantly enhanced the synthesis and accumulation of carotenoids in *D. Salina* CCAP 19/18 post-induction. Conversely, exposure to a high-temperature regime of 45°C for either 3 or 6 hours daily, in conjunction with nutrient deficiency, proved lethal to the microalgal cultures. This extreme inhibitory environment led to complete cell mortality within three days of stress.

The volumetric carotenoid content ($\mu\text{g/mL}$) of *D. salina* under nutrient-replete (control) and 50% NPK-reduction conditions, coupled with control temperature, was significantly higher than that observed



under complete NPK deprivation ($p \leq 0.05$). Notably, the highest carotenoid concentrations were recorded under two specific regimes: the Control+T25 group on day 9 (10.238 $\mu\text{g/mL}$) and the $-1/2$ NPK+Control group on day 12 (10.252 $\mu\text{g/mL}$), both of which demonstrated statistically significant elevations ($p \leq 0.05$) (Figure 1).

The cellular carotenoid content (pg/cell) of *D. salina* exhibited a marked upward trend following the induction of thermal stress in conjunction with NPK deprivation. Notably, the treatment involving complete NPK depletion combined with the control temperature yielded a significantly higher accumulation of carotenoids compared to nearly all other experimental groups ($p \leq 0.05$). Specifically, the carotenoid content per cell reached its peak value of 38.485 pg/cell on day 12 under the -NPK+Control condition ($p \leq 0.05$) (Figure 2).

Similarly, the carotenoid-to-chlorophyll ratio in *D. salina* under the control temperature regime coupled with complete NPK depletion was significantly higher than that observed in the nutrient-replete and 50% NPK-reduction groups. Specifically, the -NPK+Control treatment yielded the peak carotenoid/chlorophyll ratio of 34.053, representing the highest value among all experimental conditions ($p \leq 0.05$) (Figure 3).

It is evident that NPK deprivation, when coupled with appropriate thermal stress, stimulates the synthesis of carotenoids in *D. salina* CCAP 19/18. In this microalga, carotenoid biosynthesis functions as a pivotal defense mechanism against adverse environmental conditions. Specifically, the accumulation of β -carotene within lipid droplets serves to safeguard the photosynthetic apparatus. Furthermore, the generation of reactive oxygen species (ROS) during suboptimal photosynthesis under stress acts as a biochemical trigger, significantly inducing β -carotene accumulation in *D. salina* (Wu et al., 2020).

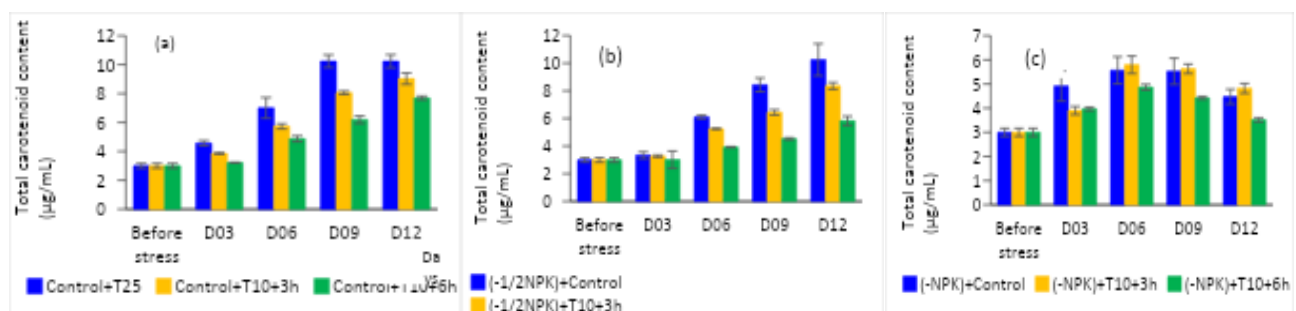


Figure 1. Total Carotenoid Content ($\mu\text{g/mL}$) of *D. salina* CCAP 19/18 under Temperature Inhibited Culture Conditions Combined with NPK Nutrition

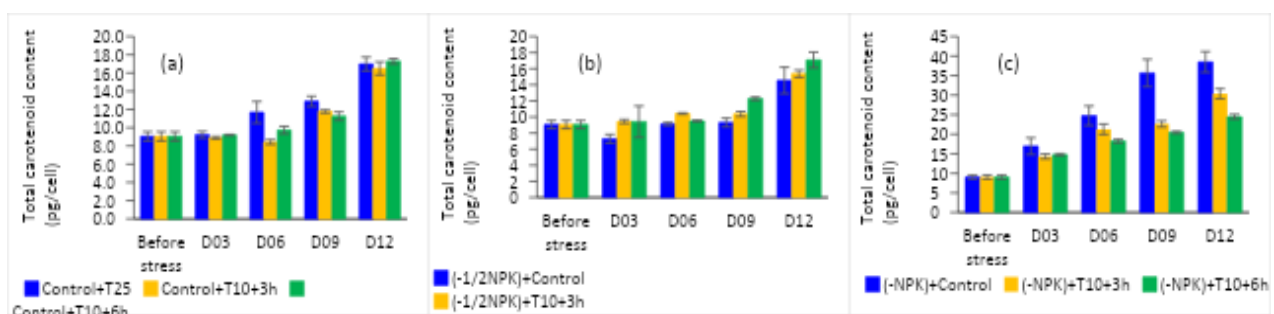


Figure 2. Total Carotenoid Content (pg/cell) of *D. salina* CCAP 19/18 under Temperature Inhibited Culture Conditions Combined with NPK Nutrition



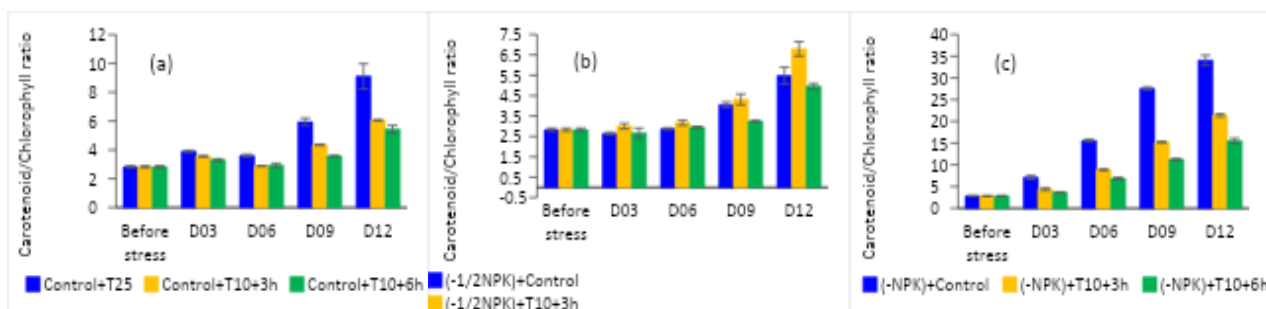


Figure 3. Carotenoid/Chlorophyll Ratio of *D. salina* CCAP 19/18 under Temperature Inhibited Culture Conditions Combined with NPK Nutrition

Total Lipid Content

The volumetric lipid content ($\mu\text{g/mL}$) of *D. salina* CCAP 19/18 exhibited a marked upward trend throughout the stress induction period, peaking on day 12 post-inhibition. Experimental data revealed that lipid accumulation under thermal stress, when coupled with either nutrient-replete (control) or 50% NPK-reduction conditions, significantly outperformed the results obtained from complete NPK deprivation. Notably, the highest lipid concentrations were recorded on day 12 for the Control+T25 and $-1/2$ NPK+Control groups, reaching $246.658 \mu\text{g/mL}$ and $266.966 \mu\text{g/mL}$, respectively ($p \leq 0.05$) (Figure 4).

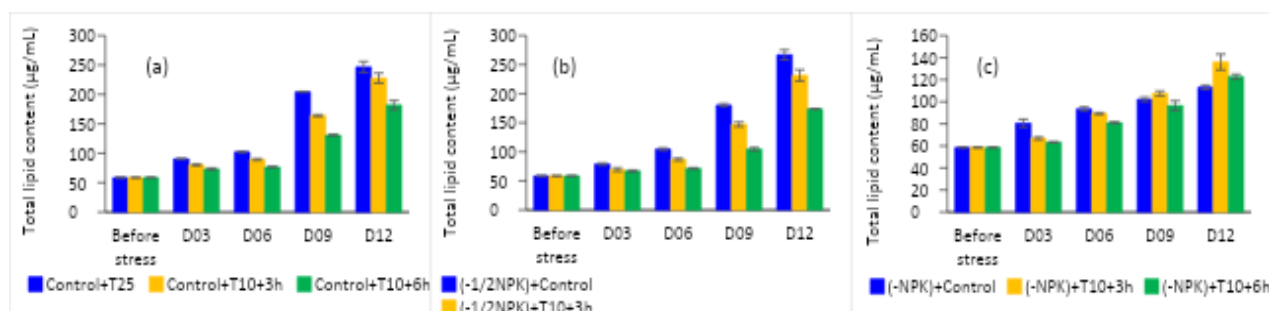


Figure 4. Total Lipid Content ($\mu\text{g/mL}$) of *D. salina* CCAP 19/18 under Temperature Inhibition Conditions Combined with NPK Nutrition

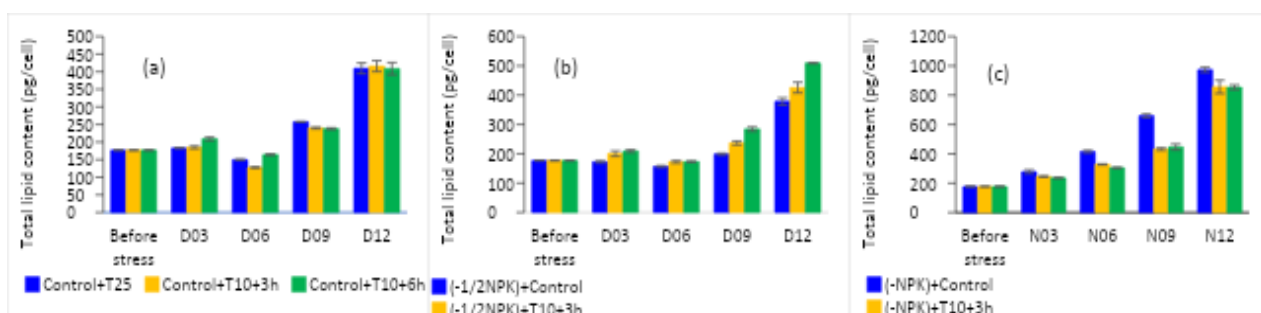


Figure 5. Total Lipid Content (pg/cell) of *D. salina* CCAP 19/18 under Temperature Inhibition Conditions Combined with NPK Nutrition



The cellular lipid content (pg/cell) of *D. salina* under the control temperature regime, coupled with complete NPK deprivation, exhibited significantly higher synthesis and accumulation rates compared to the majority of other experimental stress conditions ($p \leq 0.05$). Notably, the -NPK+Control treatment yielded a peak lipid concentration of 973.238 pg/cell on day 12 post-induction, representing the maximum value recorded across all cohorts ($p \leq 0.05$) (Figure 5). In conclusion, the synergistic application of environmental nutrient stress primarily nitrogen and phosphorus deprivation combined with thermal inhibition (at 10°C and 25°C), significantly enhances the lipid accumulation capacity of *D. salina* CCAP 19/18. Given their versatile biochemical profiles, microalgal lipids represent a high-potential feedstock for diverse industrial applications, ranging from the production of sustainable biofuels and pharmaceuticals to high-value cosmetics and nutritional supplements (Fernandes & Cordeiro, 2021; Nguyen et al., 2024; Vo et al., 2024).

Total Phenolic Content

The total phenolic content of *D. salina* exhibited a marked increase following the induction of thermal stress in combination with NPK deprivation. Specifically, phenolic accumulation ($\mu\text{g/mL}$) under nutrient-replete (control) and 50% NPK-reduction conditions, coupled with temperature inhibition, significantly surpassed the values observed under complete NPK removal. Notably, the Control+T25 regime yielded the highest phenolic concentration, peaking at 3.293 $\mu\text{g/mL}$ on day 9 of the stress exposure period (Figure 6).

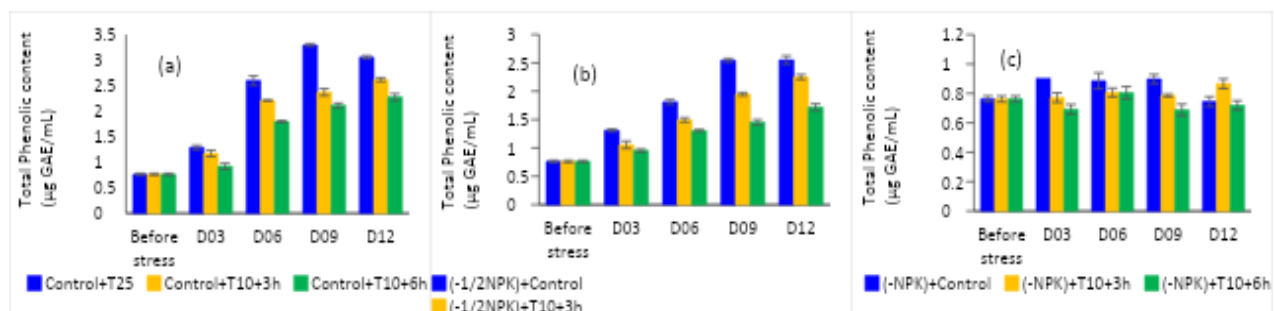


Figure 6. Total Phenolic Content ($\mu\text{g GAE/mL}$) of *D. salina* CCAP 19/18 under Temperature-Inhibited Conditions Combined with NPK Nutrients

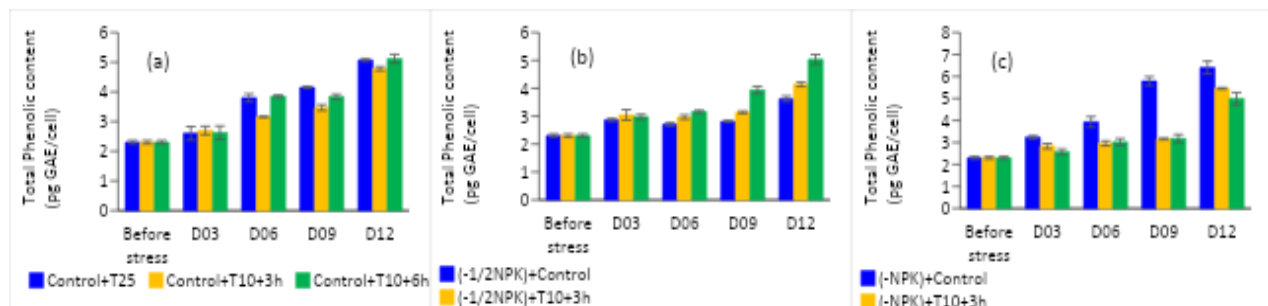


Figure 7. Total Phenolic Content (pg GAE/cell) of *D. salina* CCAP 19/18 under Temperature Inhibition Conditions Combined with NPK Nutrient Depletion

The cellular phenolic content (pg/cell) of *D. salina* under the control temperature regime, in conjunction with complete NPK deprivation, was significantly higher than that observed in nearly all other treatment groups ($p \leq 0.05$). Notably, the highest phenolic concentration was recorded on day 12 post-induction under the -NPK+Control condition, peaking at 6.404 pg/cell ($p \leq 0.05$) (Figure 7). The deprivation of



nitrogen and phosphorus, particularly when synchronized with appropriate thermal stress, exerts a positive influence on the biosynthesis of phenolic compounds, which function as a secondary metabolite defense for cellular protection. These findings align with the research conducted by Trung Vo et al. (2023), which demonstrated a significant elevation in the phenolic content of *D. salina* when cultivated under nitrogen-deficient conditions within an RM2 medium (Vo et al., 2023).

Antioxidant Capacity

Figure 8 illustrates that the volumetric antioxidant capacity (I%/mL) of *D. salina* CCAP 19/18, under the combined influence of thermal stress and NPK deprivation, exhibited a marginal increase post-induction. However, no statistically significant differences were observed between the various culture conditions ($p > 0.05$). In contrast, the cellular antioxidant capacity (I%/cell) showed a pronounced upward trend, with NPK-depleted groups significantly outperforming the control and 50% NPK-reduction treatments. Notably, the -NPK+Control regime yielded the peak antioxidant activity of 336.353% I on day 12 ($p \leq 0.05$), as shown in Figure 9. These findings align with reports suggesting a strong correlation between environmental stress and the accumulation of both enzymatic and non-enzymatic antioxidants. Under such adverse conditions, the homeostatic control of reactive oxygen species (ROS) is disrupted, prompting the overproduction of antioxidant compounds and enhanced enzymatic activity to detoxify and neutralize harmful free radicals that would otherwise cause cellular damage (Mostafa et al., 2024).

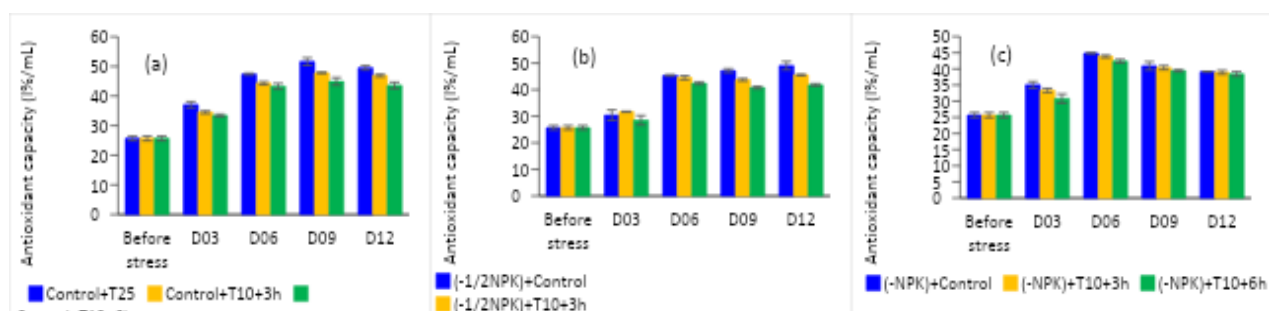


Figure 8. Antioxidant Capacity (I%/mL) of *D. salina* CCAP 19/18 under Temperature Inhibited Conditions Combined with NPK Nutrition

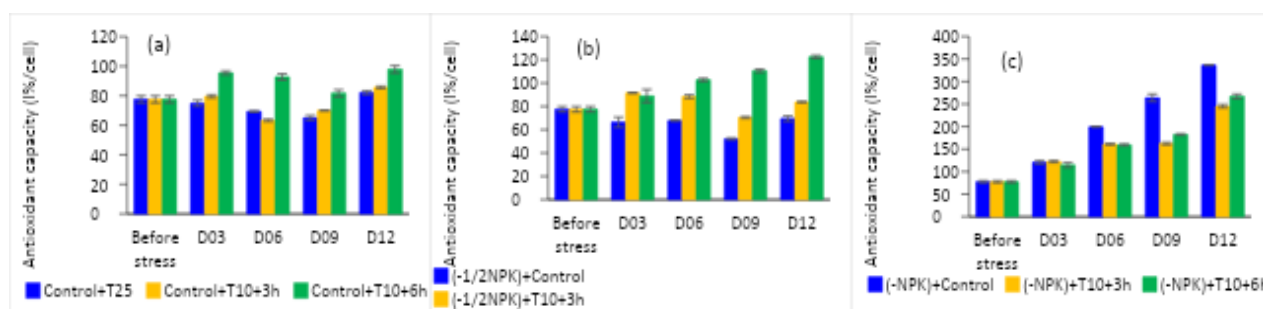


Figure 9. Antioxidant Capacity (I%/cell) of *D. salina* CCAP 19/18 under Conditions of Temperature Inhibition Combined with NPK Nutrition

Conclusion

The biosynthesis and accumulation of bioactive compounds in *D. salina* CCAP 19/18 were significantly enhanced when subjected to NPK deprivation in conjunction with varying thermal stress regimes (10°C, 25°C, and 45°C) within an RM2 medium (1.5 M salinity). Experimental evidence indicates that the synergistic application of complete NPK depletion and a controlled temperature of 25°C yielded the



optimal concentration of carotenoids, lipids, and phenolic compounds, alongside the highest antioxidant capacity. Conversely, exposure to an extreme temperature of 45°C, coupled with nutrient deficiency, proved lethal to the microalgal cultures, with a total loss of cell viability recorded within three days of stress.

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