

Optimizing Lipid and Astaxanthin Accumulation in *Chromochloris zofingiensis* Through Nitrogen Limitation and Phosphorus Enrichment

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Abstract

Chromochloris zofingiensis is a promising microalga capable of accumulating both lipids and astaxanthin, two high-value bioproducts relevant to biofuel, aquaculture, and nutraceutical industries. This study aimed to investigate how nitrogen limitation combined with varying phosphorus enrichment affects the growth, pigment composition, lipid content, and astaxanthin accumulation of *C. zofingiensis* under photoautotrophic conditions. A batch culture experiment was conducted with four treatments: standard nitrogen and phosphorus as the control (N & P), nitrogen limitation with normal phosphorus levels (N_{lim} & P), nitrogen limitation with moderate phosphorus enrichment (N_{lim} & P₊), and nitrogen limitation with high phosphorus enrichment (N_{lim} & P₊₊). The major hypothesis is that an elevated phosphorus supply helps alleviate the negative impacts of nitrogen deprivation on growth, while simultaneously promoting the biosynthesis of lipids and astaxanthin. Results showed that nitrogen and phosphorus sufficiency led to the highest growth rate and chlorophyll content, while nitrogen deprivation severely limited photosynthetic pigment and protein synthesis. However, high phosphorus supplementation (N_{lim} & P₊₊) under nitrogen-limited conditions mitigated biomass suppression, leading to biomass yields statistically comparable to the control. This treatment also induced the highest lipid content (34.11% DW) and lipid productivity (33.16 mg L⁻¹ d⁻¹) and significantly elevated astaxanthin concentration (1.81 mg L⁻¹). Moderate phosphorus enrichment (N_{lim} & P₊) maximized per-cell astaxanthin content, highlighting a trade-off between cell-specific pigment accumulation and volumetric productivity. These findings demonstrate that nutrient modulation can redirect metabolic fluxes, enabling dual enhancement of lipids and carotenoids in *C. zofingiensis*. The synergistic effects of nitrogen limitation and phosphorus enrichment offer a practical nutrient engineering strategy for optimizing microalgal bioprocesses aimed at producing biofuels and high-value compounds.

Keywords: astaxanthin production, *Chromochloris zofingiensis*, excessive phosphorus, lipid biosynthesis, nitrogen limitation

1. Introduction

Microalgae are photosynthetic organisms that are recognized as a renewable and sustainable feedstock for biofuel production, animal feed, and high value biochemicals due to their high photosynthetic efficiency, rapid growth, and ability to accumulate a diverse range of compounds, including lipids and pigments (Babu *et al.*, 2022; Ezhumalai *et al.*, 2024). *Chromochloris zofingiensis* is particularly noteworthy among microalgae strains due to its unique capability to co-accumulate substantial amounts of lipids and astaxanthin, a high value ketocarotenoid with potent antioxidant activity (Mao *et al.*, 2018). This species has demonstrated superior adaptability under diverse cultivation regimes, including photoautotrophic, heterotrophic, and mixotrophic conditions, making it a promising candidate for integrated biomass valorization (Wood *et al.*, 2024; Zhang *et al.*, 2019). However, despite

its potential, large-scale application remains constrained by low biomass and product yields under standard cultivation conditions (Wood *et al.*, 2024). Enhancing the biosynthetic capacity of *C. zofingiensis* through nutrient modulation strategies thus remains a critical area of investigation for advancing sustainable microalgal bioprocessing.

A commonly adopted approach for improving microalgal lipid and astaxanthin productivity involves the induction of stress conditions, particularly nitrogen deprivation (Mao *et al.*, 2018; Zhu *et al.*, 2015), which commonly arises in large-scale batch or semi-batch cultivation as nutrients are rapidly exhausted at high cell densities and is also deliberately imposed to redirect cellular metabolism toward high-value metabolite accumulation (Yadav *et al.*, 2025). Nitrogen starvation has been shown to redirect cellular metabolism toward synthesizing storage lipids and secondary carotenoids, at

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the cost of inhibited cell division and diminished biomass accumulation (Maltsev *et al.*, 2023). This metabolic trade-off poses a significant limitation, as the reduction in biomass can offset the gains in product concentration, thereby constraining overall productivity. In *C. zofingiensis*, nitrogen limitation has been reported to induce lipid content and concurrent astaxanthin accumulation, yet biomass yields decline sharply under such conditions (Mao *et al.*, 2018; Zhu *et al.*, 2015).

Research has increasingly focused on phosphorus repletion as a combinatorial nutrient strategy to mitigate the adverse effects of nitrogen limitation on microalgal growth and lipid production. As an essential macronutrient, phosphorus plays a critical role in energy metabolism, nucleic acid synthesis, and membrane stability (Bossa *et al.*, 2024), and its supplementation under nitrogen-deficient conditions has been shown to enhance lipid accumulation without severely compromising biomass productivity (Chu *et al.*, 2014; Zhu *et al.*, 2015). This effect is largely attributed to luxury phosphorus uptake, leading to intracellular polyphosphate accumulation that functions as an energy reserve to sustain anabolic processes during nitrogen starvation (Zhu *et al.*, 2015). Empirical evidence supports this mechanism, as phosphorus repletion under nitrogen deprivation significantly increased lipid productivity and upregulated oxidative phosphorylation-related genes in *Chlorella vulgaris* (Chu *et al.*, 2019).

Beyond lipid enhancement, recent attention has also focused on the relationship between phosphorus availability and carotenoid synthesis. Astaxanthin production in *C. zofingiensis* has been linked to oxidative stress responses and energy-demanding biosynthetic pathways (Zhang *et al.*, 2019). Given that polyphosphate reserves can act as high-energy molecules akin to ATP (Chu *et al.*, 2013), it is plausible that phosphorus repletion may provide the metabolic energy required for sustained astaxanthin synthesis under nitrogen stress.

Although the individual impacts of nitrogen limitation and phosphorus enrichment on lipid and other biomass components have been investigated in other microalgal species (Chu *et al.*, 2019; Fu *et al.*, 2017), their combined effects in *C. zofingiensis* remain largely unexplored. Given the species' unique ability to simultaneously produce lipids and astaxanthin, along with its potential for high-value biomass generation, there is a clear need to evaluate the interactive roles of these two macronutrients systematically. Moreover, previous work has largely focused on heterotrophic or mixotrophic systems (Fu *et al.*, 2017; Shen *et al.*, 2016), leaving a critical knowledge gap regarding photoautotrophic cultivation, which is more sustainable and scalable for outdoor applications.

This study addresses the aforementioned research gap by examining the effects of phosphorus repletion on lipid and astaxanthin production in *C. zofingiensis* under nitrogen-limited, photoautotrophic conditions. The major hypothesis is that a high supply of phosphorus helps reduce the negative impacts of nitrogen deprivation on growth while at the same time promoting the biosynthesis of lipids and astaxanthin. To confirm the hypothesis, we examined different phosphorus concentrations in nitrogen-deficient BG-11 media and then monitored growth, biomass, photosynthetic pigments, protein content, lipid production, and astaxanthin level. The novelty of this

research lies in its focus on the dual enhancement of lipid and astaxanthin productivity in *C. zofingiensis* under strictly photoautotrophic regimes, which has not been systematically studied to date. The outcomes of this work are expected to offer new insights into nutrient engineering strategies for microalgal bioprocess optimization, particularly for strains capable of producing multiple high-value bioproducts.

2. Materials and Methods

2.1. Strain and cultivation conditions

The microalga *C. zofingiensis* UTEX 32 was provided from University of Texas Culture Collection of Algae. This strain was selected for this study due to its recognized potential for concurrent lipid and astaxanthin production. Under static photoautotrophic conditions, stock cultures were maintained in 250 mL Erlenmeyer flasks containing 150 mL of standard BG-11 medium (Stanier *et al.*, 1971). Illumination was provided continuously using cool white, fluorescent light at an intensity of 110 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at 25 °C.

The experimental design employed a batch culture system with four treatment groups: (1) sufficient nitrogen and phosphorus (N & P; 240 mg L⁻¹ NO₃-N and 5.56 mg L⁻¹ phosphorus), (2) nitrogen-deficient and sufficient phosphorus (N_{lim} & P; 8 mg L⁻¹ NO₃-N and 5.56 mg L⁻¹ phosphorus), (3) nitrogen-deficient and moderate phosphorus repletion (N_{lim} & P₊; 8 mg L⁻¹ NO₃-N and 27.8 mg L⁻¹ phosphorus), and (4) nitrogen-limitation and high phosphorus repletion (N_{lim} & P₊₊; 8 mg L⁻¹ NO₃-N and 55.6 mg L⁻¹ phosphorus). The concentrations for sufficient nitrogen and phosphorus were based on the standard BG-11 formula, while the nitrogen-deficient condition was set at 3.3% of the standard BG-11 nitrate concentration. Phosphorus levels were varied up to 1,000% of the original concentration (Table 1).

Pre-cultivation was performed for 4 days to establish exponential growth. Algal cells were harvested by centrifugation at 1,800g for 15 min, washed twice with sterile distilled water to remove residual nutrients, and resuspended to an initial OD₆₈₀ of 0.123 for all treatments. Each culture was maintained in 500 mL flasks with 250 mL working volume, under constant aeration at a flow rate of 0.4 L/min with sterilized air and illumination at 110 $\mu\text{mol m}^{-2} \text{s}^{-1}$ under continuous light period. Cultures were incubated at 25 °C for 5 days.

Table 1. Initial nitrogen and phosphorus concentrations in the modified BG-11 medium

	N & P	N _{lim} & P	N _{lim} & P ₊	N _{lim} & P ₊₊
Nitrogen (NO ₃ -N) (mg L ⁻¹)	240 (100%)	8 (3.3%)	8 (3.3%)	8 (3.3%)
Phosphorus (PO ₄ ³⁻ -P) (mg L ⁻¹)	5.56 (100%)	5.56 (100%)	27.8 (500%)	55.6 (1000%)

*Values inside parenthesis show the ratio of the changed nitrogen/phosphorous concentrations to those in the original BG-11 medium.

2.2. Growth and biomass determination

Growth was monitored daily by measuring optical density at 680 nm (OD₆₈₀) using a GENESYS 20 UV-Vis spectrophotometer (Thermo Scientific, USA). Specific growth rate (μ) was calculated according to standard logarithmic formulas:

$$\mu \text{ (d}^{-1}\text{)} = \frac{[\text{Ln}(\text{Nt}) - \text{Ln}(\text{N0})]}{\Delta t} \quad (1)$$

Nt and N0 are final and initial OD₆₈₀ values, respectively, and Δt is the time interval in days.

For biomass estimation, 25 mL of culture was filtered through 1.2 μm GF/C filters (Whatman, USA), rinsed with deionized water, and dried at 105 °C for 2 hours until constant weight was achieved. Filters were cooled in a desiccator and weighed to determine dry biomass in g L⁻¹ (Fakhri *et al.*, 2021).

2.3. Chlorophyll analysis

Chlorophyll *a* and *b* (Chl *a+b*) of *C. zofingiensis* were extracted using 90% methanol, with samples collected at the end of the experimental period (Fakhri *et al.*, 2024). Two mL of algal culture was centrifuged at 1,800g for 15 min, washed with distilled water, and resuspended in 2 mL of 90% methanol. The suspension was sonicated for 10 min and incubated in a water bath at 70 °C for 10 min. After centrifugation, absorbance readings were taken at 652 and 665 nm. Pigment concentrations were calculated using the following equations (Ritchie, 2006):

$$\text{Chl } a \text{ (}\mu\text{g mL}^{-1}\text{)} = 16.5169 \times A_{665} - 8.0962 \times A_{652} \quad (2)$$

$$\text{Chl } b \text{ (}\mu\text{g mL}^{-1}\text{)} = 27.4405 \times A_{652} - 12.1688 \times A_{665} \quad (3)$$

2.4. Lipid determination

Lipid content was determined gravimetrically based on extraction with a chloroform-methanol (2:1 v/v) mixture following the Bligh and Dyer (1959) method. After 5 days of cultivation, microalgae were harvested and oven-dried at 65 °C for 30 h. Approximately 30 mg of dry biomass (M1) was extracted overnight, and lipids were separated, dried, and weighed. Lipid content was expressed as % of dry weight (% DW), while lipid productivity was determined as mg L⁻¹ d⁻¹:

$$\text{Lipid content (\% DW)} = \frac{\text{M3-M2}}{\text{M1}} \times 100\% \quad (4)$$

M1 refers to the total dry biomass, M2 represents the weight of the empty vial, while M3 corresponds to the weight of the vial containing the extracted lipid.

$$\text{Lipid productivity (mg L}^{-1}\text{ d}^{-1}\text{)} = \frac{\text{W2-W1}}{\Delta t} \times \text{lipid content} \quad (5)$$

W1 is designated as the biomass measured at the initiation of the cultivation process, whereas W2 denotes the biomass obtained at the termination of cultivation.

2.5. Astaxanthin analysis

Astaxanthin content was analyzed using a spectrophotometric method adapted from Boussiba and Vonshak (1991). 10 mL of algal culture was harvested by centrifugation at 1,800g for 15 min. The resulting cell pellet was washed with 5 mL of distilled water to remove residual culture medium. To remove chlorophyll, the pellet was resuspended in 5 mL of 5% (w/v) potassium hydroxide (KOH) prepared in 30% (v/v) methanol. The mixture was vortexed for 30 sec, then incubated in a water bath at 75 °C for 10 min. After incubation, the sample was centrifuged again at 1,800g for 15 min, and the supernatant

was discarded. If the supernatant still exhibited a green color, indicating the presence of chlorophyll, the dechlorophyllation process was repeated until the supernatant became colorless. The dechlorophyllated pellet was then treated with 25 μL of acetic acid and 5 mL of DMSO to facilitate astaxanthin extraction. The sample was vortexed thoroughly and subjected to sonication using an ultrasonic cleaner for 15 min. After sonication, the mixture was incubated in a water bath at 75 °C for another 10 min to enhance pigment release. The final extract was centrifuged, and the supernatant containing astaxanthin was collected. The absorbance of the extract was measured at a wavelength of 530 nm using a spectrophotometer. The astaxanthin concentration, expressed in mg L⁻¹, and cellular astaxanthin content, expressed in mg g⁻¹, were calculated using the following equation (Li *et al.*, 2012).

$$\text{Astaxanthin concentration (mg L}^{-1}\text{)} = \frac{A_{530} - 0.0107}{0.1556} \quad (6)$$

$$\text{Cellular astaxanthin content (mg g}^{-1}\text{)} = \frac{\text{Astaxanthin concentration}}{\text{Biomass concentration}} \quad (7)$$

2.6. Statistical analysis

All experiments were conducted in triplicate. Results are presented as means $\pm$ standard deviation (SD). Statistical significance among treatments was assessed using one-way analysis of variance (ANOVA) followed by Duncan's post-hoc test ($p < 0.05$) using SPSS 25.0 software.

3. Results and Discussion

3.1. Growth and biomass of *C. zofingiensis*

The growth profile (OD₆₈₀) of *C. zofingiensis* under the four culture conditions (N & P, N_{lim} & P, N_{lim} & P₊, and N_{lim} & P₊₊) in the photoautotrophic system is shown in Figure 1A. On day 1, a slight increase in OD₆₈₀ was observed in all groups, with no noticeable divergence among treatments, suggesting comparable initial growth responses. By day 2, OD₆₈₀ values continued to rise gradually across all conditions, though the N & P group began to show a marginally higher increase compared to nitrogen-limited treatments. From day 3 onward, distinct growth patterns became more apparent. The N & P culture showed a steeper rise in OD₆₈₀ compared to nitrogen-limited conditions, indicating a more robust exponential growth phase supported by sufficient nitrogen availability. On day 5, *C. zofingiensis* under the N & P condition reached its highest OD₆₈₀ (1.14). In contrast, all N-deficient treatments attained much lower OD₆₈₀ values (0.870 to 0.932), suggesting that *C. zofingiensis* growth was clearly constrained by nitrogen limitation. In a similar study, Fakhri *et al.* (2025) found that the growth of *C. pyrenoidosa* was severely inhibited under nitrogen deficiency and P repletion. In addition, further increasing concentration of phosphorus in the media could not promote the growth of microalgae.

Biomass concentration, however, showed a different response (Figure 1B). The N & P condition produced the highest biomass at 0.53 $\pm$ 0.07 g L⁻¹, while N_{lim} & P and N_{lim} & P₊ produced lower values of approximately 0.38 $\pm$ 0.05 and 0.40 $\pm$ 0.06 g L⁻¹, respectively. Interestingly, N_{lim} & P₊₊ yielded 0.49 $\pm$ 0.03 g L⁻¹ of biomass, which was statistically not different from the N & P group ($p > 0.05$).

This outcome contradicts the assumption that all N-deficient treatments uniformly suppress biomass and suggests that phosphorus enrichment at sufficiently high levels (as in N_{lim} & P_{++}) can partially offset the adverse effects of nitrogen deprivation. In accordance with our study, Fu *et al.* (2017) reported that phosphorus supplementation up to 45 mg L⁻¹ under nitrogen-limited conditions can alleviate growth inhibition and support biomass accumulation of *Chlorella regularis*.

These observations suggest a synergistic, though not compensatory, effect of phosphorus under nitrogen-limited conditions. Nitrogen is essential for synthesizing cellular macromolecules such as proteins, nucleic acids, and chlorophyll; thus, its deficiency is expected to constrain biomass production regardless of other nutrient availability (Nagappan & Kumar, 2021). The enhanced biomass in the N_{lim} & P_{++} treatment may result from improved energy metabolism or maintenance of homeostasis enabled by

luxury phosphorus uptake and polyphosphate accumulation, as described by Zhu *et al.* (2015) and Chu *et al.* (2019). In such scenarios, excess phosphorus could support ATP generation and modulate enzymatic functions, enabling modest growth despite persistent nitrogen stress.

Nonetheless, the biomass recovery in N_{lim} & P_{++} was incomplete and did not reach the level of nitrogen-sufficient control, reinforcing that phosphorus cannot fully substitute for nitrogen. The data, therefore, supports a nuanced conclusion: while nitrogen remains the critical driver of algal growth, high phosphorus availability can modestly mitigate the impact of nitrogen starvation, thereby enhancing biomass accumulation in *C. zofingiensis* under suboptimal nitrogen conditions.

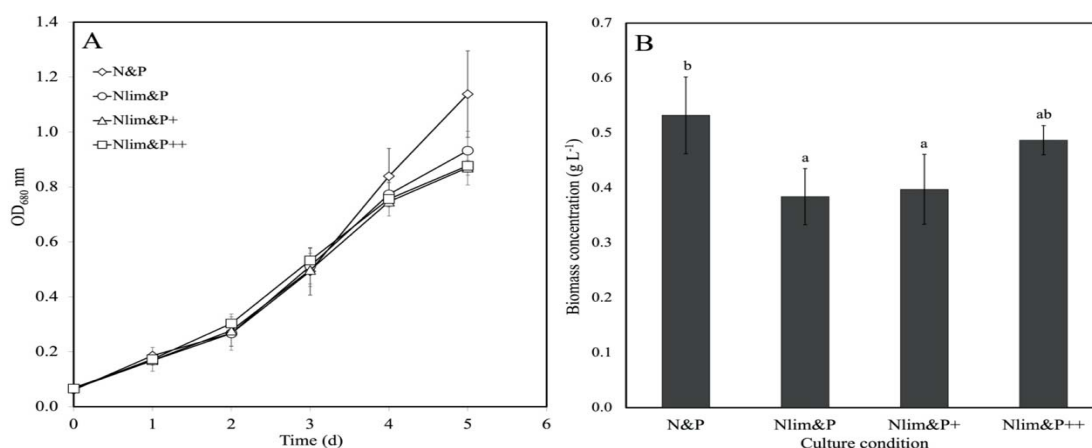


Figure 1. Growth of the *Chromochloris zofingiensis* cells: A) changes in optical density at 680 nm (OD₆₈₀); and B) biomass concentration of *C. zofingiensis* grown in different culture conditions. Error bars demonstrate the standard deviations from the triplicate experiments (n = 3). Same letters show that there was no difference between treatments (p > 0.05)

3.2. Chlorophyll and protein content of *C. zofingiensis*

Nitrogen limitation profoundly affected photosynthetic pigments and protein content (Figure 2). Chl *a+b* concentrations were highest in the N & P control, reflecting robust photosynthetic capacity under nutrient-replete conditions. By contrast, N-deficient cultures exhibited dramatically lower chlorophyll: for example, the N_{lim} & P treatment contained only 5.77 µg mL⁻¹ Chl *a+b* at day 5, versus 18.96 µg mL⁻¹ in N & P. The drop is more than threefold and was statistically significant (Figure 2A). Supplementing excess P in the N-deficient media did not increase chlorophyll levels of *C. zofingiensis*. This phenomenon was also observed in our previous investigation of *C. pyrenoidosa*. Such reductions are expected because nitrogen is a major constituent of chlorophyll and associated proteins (Fakhri *et al.*, 2025). Limited nitrogen causes the destruction of nitrogenous compounds such as chlorophyll and the degeneration of chloroplasts, resulting in a loss in photosynthetic performance (Liu *et al.*, 2022).

Protein content exhibited a similar nitrogen-dependent pattern (Figure 2B). Protein, which is rich in nitrogen, was highest in the N & P treatment and lowest in N_{lim} & P_{++} . N-limited cultures rely on internal protein reserves and accumulate less new protein, so protein per cell or biomass fell sharply. Additional phosphorus did not reverse this effect. This result is consistent with some studies reporting that nitrogen scarcity constrains protein synthesis in microalgae (Liu *et al.*, 2022; Zarrinmehr *et al.*, 2020). Importantly, phosphorus is essential for nucleic acids and energy metabolism, but it plays a less direct role in protein production. Thus, in our data, the P-repleted (N_{lim} & P_{++} , N_{lim} & P_{++}) cultures did not improve protein relative to N_{lim} & P. Our findings align with the study in *Tetraselmis subcordiformis* Yao *et al.* (2018). Despite the abundance of phosphorus, there is no chlorophyll or protein regeneration, implying that energy or substrate availability from polyphosphates is inadequate to resume biosynthetic processes without nitrogen.

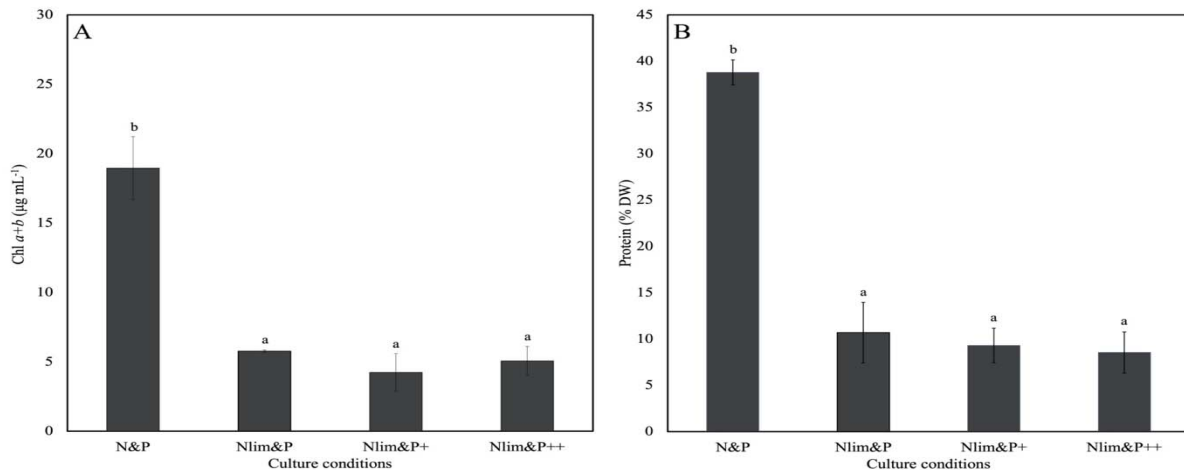


Figure 2. Chl *a+b* of *Chromochloris zofingiensis* cultivated in different culture conditions (A). Protein content of *C. zofingiensis* cultivated in different culture conditions (B). Error bars demonstrate the standard deviations from the triplicate experiments ($n = 3$). At a 95% confidence level, different letters show that there was a remarkable difference between treatments

3.3. High phosphorus triggered enhanced lipid accumulation

Figure 3 illustrates the effects of different nitrogen and phosphorus regimes on lipid accumulation and lipid productivity in *C. zofingiensis*. As shown in Figure 3A, lipid content varied significantly across treatments ($p < 0.05$). The control culture (N & P) exhibited the lowest lipid content at approximately $18.11 \pm 0.19\%$ DW. Under nitrogen-limiting conditions with normal phosphorus supplementation (N_{lim} & P), lipid content markedly increased to about 30.56% DW, representing a significant improvement compared with the control ($p < 0.05$). This finding aligns with well-documented responses in microalgae where nitrogen deprivation triggers reallocation of carbon flux toward storage lipids, particularly triacylglycerols (TAGs) (Huang *et al.*, 2019; Maltsev *et al.*, 2023). Nitrogen stress upregulates the malic enzyme, generating NADPH that favors fatty acid synthesis. Under limited nitrogen conditions, these enzymes would drive carbon into lipids (Ma *et al.*, 2018).

A similar lipid content was observed in N_{lim} & P_+ cultures, indicating that moderate phosphorus enrichment did not further enhance lipid accumulation. However, the highest P treatment (N_{lim} & P_{++}) yielded the greatest lipid levels of all (34.11% DW), which corresponds to an 88% increase over the N & P control and an 11.64% increase over N_{lim} & P (Figure 3). This progressive enhancement is consistent with the notion that luxury phosphorus uptake can be channeled into storage products under nutrient stress (Chu *et al.*, 2013; Fu *et al.*, 2017). For example, Chu *et al.* (2013) reported that *C. vulgaris* with ample P under N starvation achieved the highest lipid accumulation of any condition tested. In their study, phosphorus taken up by the cells was stored as polyphosphate and accompanied by increased lipid yield. Similarly, our *C. zofingiensis* cultures likely use extra phosphorus to fuel energetic and reductive processes for fatty acid synthesis. Phosphorus repletion may have enhanced this effect by providing additional ATP and phosphate donors; indeed, microalgae can rapidly assimilate surplus P when starved of N (Chu *et al.*, 2019; Chu *et al.*, 2013). The excess polyphosphate and associated metabolic energy can indirectly promote fatty

acid assembly. We also note that simply increasing P in N-replete cultures does not usually raise lipids, but in the context of N deficiency, it seems to prime the cells for even greater TAG storage. Thus, our data demonstrates a synergistic effect: nitrogen limitation sets the stage for lipid accumulation, and phosphorus repletion further enhances it.

Lipid productivity of *C. zofingiensis* exhibited a parallel pattern. The control treatment yielded approximately $19 \text{ mg L}^{-1} \text{ d}^{-1}$. Nitrogen-limited treatments with normal (N_{lim} & P) and moderate phosphorus (N_{lim} & P_+) achieved similar values ($23\text{--}25 \text{ mg L}^{-1} \text{ d}^{-1}$), with no significant differences ($p > 0.05$). Conversely, N_{lim} & P_{++} strongly improved lipid productivity to $33.16 \text{ mg L}^{-1} \text{ d}^{-1}$, a statistically significant enhancement relative to all other groups ($p < 0.05$). This outcome highlights the critical role of phosphorus in sustaining cellular metabolism under nitrogen stress. Whereas nitrogen limitation tends to suppress cell division and biomass accumulation, high phosphorus availability counterbalances this limitation, enabling the cells to maintain higher growth rates while still channeling carbon into lipid synthesis. The net effect is a synergistic increase in biomass and lipid content, culminating in maximal lipid productivity.

From a bioprocessing perspective, these results are highly relevant. For industrial biofuel production, lipid productivity is a more critical metric than lipid content (% DW), since it integrates the fraction of lipids and the biomass accumulation rate (Senousy & Ellatif, 2020). Although nitrogen limitation reduced growth relative to the nutrient-replete control (N & P), the N_{lim} & P_{++} condition achieved the highest lipid productivity among all treatments, exceeding not only other nitrogen-limited conditions but also the control culture. Specifically, lipid productivity under N_{lim} & P_{++} reached $33.16 \text{ mg L}^{-1} \text{ d}^{-1}$, representing a 41.7% increase compared with N_{lim} & P and a substantial improvement over N & P. This outcome indicates that high phosphorus availability can effectively compensate for nitrogen-induced growth suppression by sustaining sufficient biomass formation while simultaneously enhancing lipid biosynthesis.

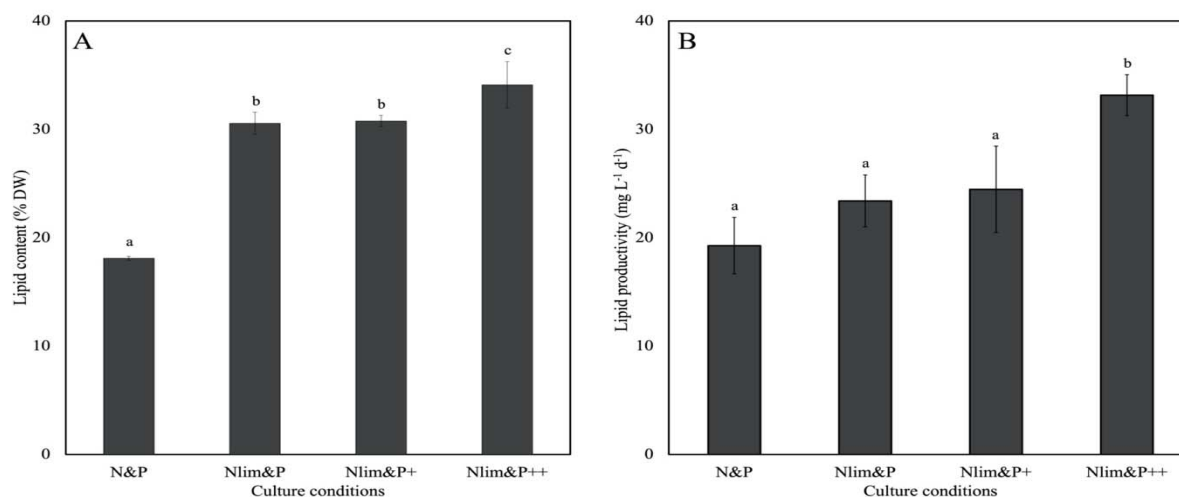


Figure 3. (A) Lipid content of *Chromochloris zofingiensis* under different phosphorus and nitrogen supply; (B) Lipid productivity of *C. zofingiensis* under different phosphorus and nitrogen supply. Error bars demonstrate the standard deviations from the triplicate experiments ($n = 3$). At a 95% confidence level, different letters show that there was a remarkable difference between treatments.

3.4. Astaxanthin production was triggered by nitrogen deficiency and phosphorus supply

Figure 4 illustrates the effects of nitrogen limitation and varying phosphorus supply on astaxanthin accumulation in *C. zofingiensis*. Astaxanthin concentration in the control (N & P) was the lowest, averaging 0.45 mg L^{-1} (Figure 4A). Under $N_{\text{lim}} \& P$ condition, astaxanthin concentration increased significantly to approximately 1.29 mg L^{-1} ($p < 0.05$). In a similar study, Li *et al.* (2022) demonstrated that *Haematococcus pluvialis* exposed to nitrogen starvation exhibited a marked increase in astaxanthin accumulation. A further increase was observed under moderate and high phosphorus supplementation ($N_{\text{lim}} \& P_{+}$ and $N_{\text{lim}} \& P_{++}$), with concentrations about 1.81 mg L^{-1} in the latter. The cellular astaxanthin content followed a similar trend (Figure 4B). The control culture accumulated 1.0 mg g^{-1} , whereas $N_{\text{lim}} \& P$ increased the content to 3.3 mg g^{-1} . The maximum value of 4.3 mg g^{-1} was obtained under $N_{\text{lim}} \& P_{+}$, with a slight decline under $N_{\text{lim}} \& P_{++}$ (3.7 mg g^{-1}), although still significantly higher than the control.

Our study confirms that nitrogen limitation is a strong inducer of astaxanthin biosynthesis in *C. zofingiensis*. Nitrogen deprivation reduces chlorophyll synthesis and photosynthetic proteins, creating oxidative stress that stimulates carotenoid accumulation as a protective mechanism (Shi *et al.*, 2020). Certain proteins undergo biodegradation, and the resulting carbon skeletons are partially redirected into the biosynthetic pathway of astaxanthin (Juneja *et al.*, 2013; Ren *et al.*, 2021). The 3–4-fold increase in cellular astaxanthin under nitrogen limitation highlights the metabolic shift from growth-oriented processes toward secondary metabolite synthesis.

Phosphorus availability further modulated this response. Moderate phosphorus supplementation ($N_{\text{lim}} \& P_{+}$) yielded the highest per-cell astaxanthin content, suggesting phosphorus supports metabolic activity required for carotenoid biosynthesis. Phosphorus plays essential roles in ATP production, nucleic acid metabolism, and redox balance, which may enhance precursor availability for the carotenoid pathway. However, excessive phosphorus ($N_{\text{lim}} \& P_{++}$) slightly reduced per-cell astaxanthin, although volumetric concentration continued to rise, likely due to sustained

biomass production. This indicates a trade-off: higher phosphorus supports biomass growth and volumetric pigment yield, while moderate supplementation optimizes per-cell astaxanthin accumulation.

The pigmentation switches in *C. zofingiensis* are directly related to astaxanthin production, as evidenced by the significant increase in astaxanthin content under phosphorus-enriched conditions (Figure 4C), which quantitatively demonstrates the stimulatory effect of phosphorus supply on astaxanthin accumulation. When provided with nutrient-adequate (N & P) culture medium, the cells showed a rich green pigmentation indicative of elevated chlorophyll production and low induction of carotenoid pigments. However, the nitrogen-deficient treatments showed a vivid yellow–orange pigmentation indicative of chlorophyll breakdown and the production of secondary carotenoid pigments such as astaxanthin. This pigmentation change reflects the visible manifestation of cells undergoing the process of reprogramming the rate at which the cell produces photosynthetic pigments and channeling the flow of carbon for the production of astaxanthin. Microscopic observations revealed changes in cellular appearance accompanying the yellow–orange pigmentation. Together with the quantitative astaxanthin measurements, these phenotypic changes are consistent with enhanced secondary carotenoid accumulation under nitrogen-limited conditions.

3.5. Biotechnological Implications and Future Directions

The capacity of *C. zofingiensis* to co-accumulate astaxanthin and lipids under nitrogen stress combined with phosphorus optimization distinguishes this species from most commercially exploited microalgae, which typically specialize in single target metabolite. For example, *Haematococcus pluvialis*, the dominant industrial source of natural astaxanthin, can accumulate exceptionally high astaxanthin contents but exhibits slow growth, high sensitivity to stress, and negligible lipid productivity, leading to elevated production costs (Shah *et al.*, 2016). Conversely, lipid-oriented strains such as *Nannochloropsis* spp. and *Chlorella vulgaris* are widely applied for biofuel and aquafeed production due to their robust growth and high triacylglycerol yields, yet they lack the capacity to

synthesize high-value ketocarotenoids like astaxanthin (Cecchin *et al.*, 2022). In this context, *C. zofingiensis* offers a unique “double-duty” platform capable of producing both astaxanthin and lipid-rich biomass, which confers a substantial biotechnological advantage within integrated biorefinery frameworks, where astaxanthin recovery can offset the costs associated with biofuel or feedstock production.

Future research should focus on metabolic engineering to improve astaxanthin biosynthetic pathways, particularly control of central enzymes β -carotene ketolase and

hydroxylase. It may also be possible to upgrade the cultivation strategy by utilizing two-stage processes, such as photobioreactor design or nutritional stress mixing. The integration of process engineering and the implementation of the toolbase of omics will be the most critical factors in achieving the organism's metabolic potential. Lastly, the future of *C. zofingiensis* as a foundation organism for sustainable biotechnology lies very strongly at the intersection of renewable energy, the aquaculture industry, and human health.

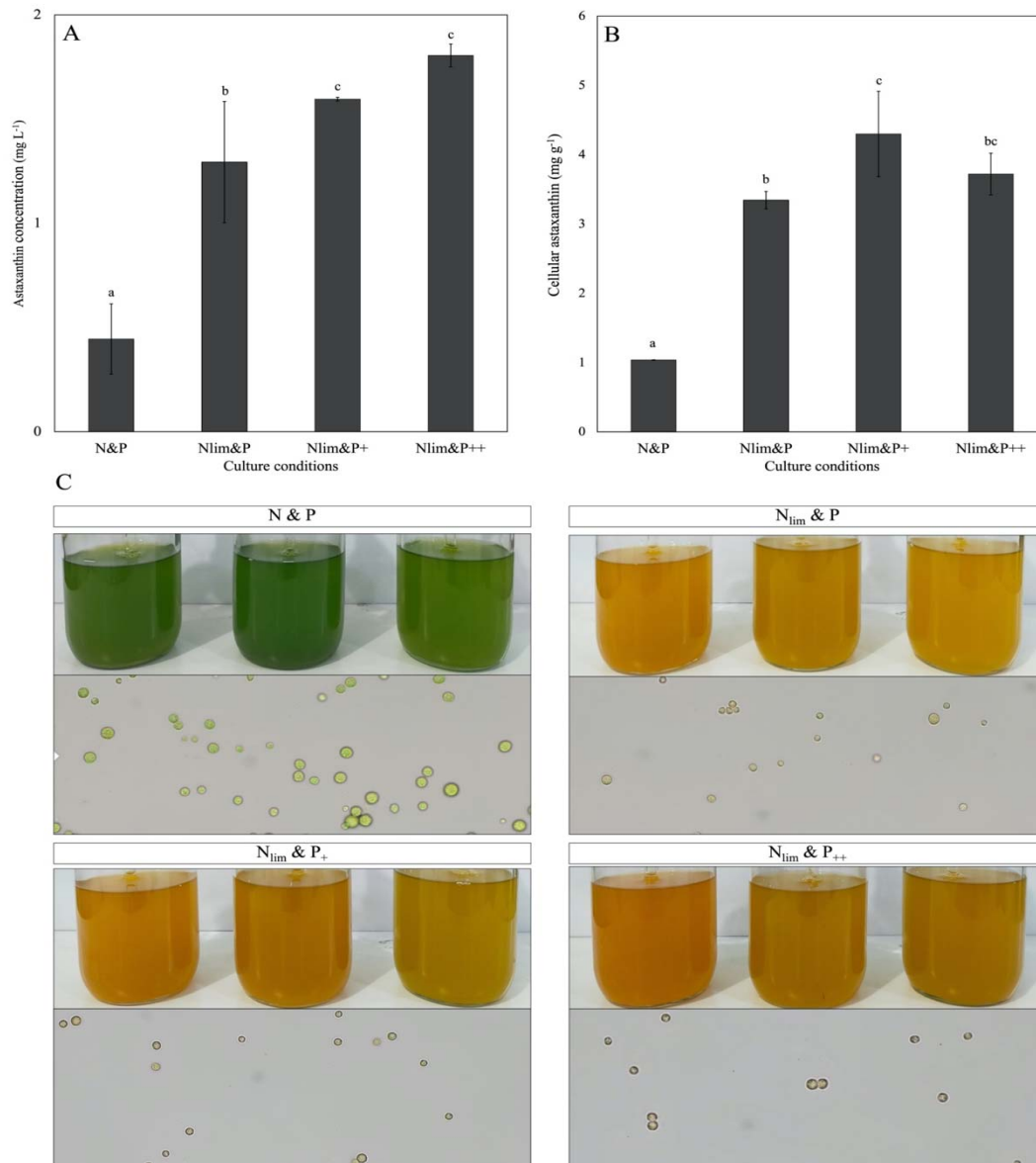


Figure 4. (A). Astaxanthin concentration (mg/L) of *Chromochloris zofingiensis* under different phosphorus and nitrogen supply; (B). Cellular astaxanthin (mg g⁻¹) of *C. zofingiensis* under different phosphorus and nitrogen supply; (C). Changes in coloration of *C. zofingiensis* cultures at day 5. Error bars demonstrate the standard deviations from the triplicate experiments (n = 3). At a 95% confidence level, different letters show that there was a remarkable difference between treatments.

4. Conclusions

This study demonstrated that the strategic manipulation of nitrogen and phosphorus availability effectively enhances the dual production of lipids and astaxanthin in *Chromochloris zofingiensis* under photoautotrophic conditions. Nitrogen limitation alone suppressed growth

and photosynthetic pigment biosynthesis but triggered metabolic reprogramming toward storage lipid and carotenoid accumulation. When combined with phosphorus enrichment, particularly at high levels (N_{lim} & P₊₊), this stress condition significantly increased lipid productivity and astaxanthin accumulation, while partially restoring biomass levels. Notably, the highest astaxanthin concentration and lipid productivity were achieved in the

N_{lim} & P₊₊ treatment, suggesting phosphorus can provide the metabolic energy and cofactors necessary to support biosynthesis under nitrogen-deficient conditions. These results confirm that phosphorus repletion under nitrogen stress synergistically improves both volumetric and per-cell yields of target bioproducts. This dual-stimulus strategy provides a promising route for optimizing high-value compound production without severely compromising growth—a common bottleneck in microalgal bioprocesses. The findings contribute to the growing knowledge on nutrient stress responses in microalgae and offer practical insights for scalable applications in biofuel, aquaculture, and pharmaceutical sectors. Future studies should explore long-term cultivation strategies, integrate multi-stage cultivation systems, and apply transcriptomic or metabolomic tools to unravel further the regulatory pathways involved. Overall, this research establishes *C. zofingiensis* as a robust microalgal platform for sustainable biotechnology, bridging the fields of renewable energy and natural antioxidant production.

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