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Optimizing Cultivation Parameters for Enhanced Chlorophyll and Phycobiliprotein Production in *Nostoc muscorum*

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Abstract

Cyanobacteria are valuable producers of phycobiliproteins, water-soluble pigments with fluorescent, antioxidant, and bioactive properties, widely used as natural colorants and functional bioactive compounds. This research employed a definitive systematic design to systematically evaluate the effects of multiple environmental and nutritional factors, including nitrogen and phosphorus availability, salinity, carbon sources (glucose and acetate), and light/dark cycles (24:0 and 16:8 hours), on the production of phycobiliproteins, namely phycocyanin, allophycocyanin, phycoerythrin, and total phycobiliproteins in *Nostoc muscorum*. Chlorophyll a and phycobiliprotein levels were monitored over a 30-day period across 14 experimental runs. Among them, run 8 characterized by high nitrate (1000 mg/L) and phosphate (40 mg/L), low salinity (0.5% NaCl), low glucose (0.25%), absence of acetate, and a 16:8 h light/dark cycle, achieved the highest levels of chlorophyll a (5.02 ± 0.55 µg mg-1dw), phycocyanin (23.38 ± 1.35 µg mg-1dw), allophycocyanin (8.35 ± 0.44 µg mg-1dw), phycoerythrin (37.86 ± 1.45 µg mg-1dw), and total phycobiliproteins (69.59 ± 2.03 µg mg-1dw). Nitrogen exerted the strongest influence on total phycobiliproteins production ($p = 0.0099$), followed by phosphorus ($p = 0.0154$) and salinity ($p = 0.0105$). Glucose supplementation enhanced pigment synthesis under nutrient-replete conditions, whereas higher acetate concentrations showed an inhibitory effect. Photoperiod significantly affected chlorophyll a content ($p < 0.05$), with a 16:8 h light/dark cycle generally promoting higher accumulation, although its effect on total phycobiliproteins production was not significant ($p = 0.1879$). Overall, these findings provide a solid framework for optimizing cultivation conditions to maximize phycobiliprotein production in *N. muscorum*, and endorsing their application as natural antioxidant pigments and bioactive compounds in industrial biotechnology applications.

Keywords

Cyanobacteria, Pigment, Phycobiliproteins, Optimization, Photoperiod

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Introduction

Cyanobacteria are an ancient monophyletic group of oxygen-producing photosynthetic prokaryotes, with fossil records indicating their presence on Earth for over 3.5 billion years. These microorganisms exhibit significant morphological diversity, appearing as unicellular forms, filaments, or colonies, and can even become macroscopically visible during bloom formations (Pagels et al., 2019). Their widespread distribution across freshwater and marine systems, deserts, polar regions, caves, and various man-made environments highlights their exceptional ecological flexibility (Sudhakar et al., 2024). This adaptability is further demonstrated by their physiological strategies, which allow them to thrive under stressful conditions such as fluctuations in light intensity, temperature, and nutrient availability (Hotos and Antoniadis, 2022). In recent years, interest in cyanobacteria has grown due to their ability to synthesize a wide range of bioactive metabolites, including polysaccharides, polyphenols, carotenoids, and phycobiliproteins (PBPs), which have valuable applications in health, nutrition, and biotechnology (Chen et al., 2022).

Among these bioactive metabolites, PBPs have attracted increasing interest over recent decades. These water-soluble pigments function as essential light-harvesting proteins in cyanobacteria, red algae, and cryptophytes, capturing photosynthetically active radiation (450–650 nm) and transferring it to photosystem reaction centers to support cellular metabolism and primary production in aquatic ecosystems (Jha et al., 2024). Phycocyanin (PC), a blue αβ-type biliprotein containing phycocyanobilin chromophores, absorbs at 610–620 nm and emits near 640–645 nm. Phycoerythrin (PE), characterized by phycoerythrobilin and phycourobilin chromophores, absorbs strongly at 540–570 nm and emits around 575–580 nm, producing intense red fluorescence. Allophycocyanin (APC), primarily located in the phycobilisome core, absorbs in the far-red region at 650–655 nm and emits near 660 nm, facilitating efficient directional energy transfer to chlorophyll a. Recognized as safe by regulatory agencies such as the FDA, phycobiliproteins are increasingly replacing synthetic dyes and have diverse applications in nutraceuticals, pharmaceuticals, cosmetics, food colorants, and biomedical research, reflecting both their functional versatility and growing commercial value (Ma et al., 2024; Tounsi et al., 2023).

Nostoc sp. is a filamentous, nitrogen-fixing cyanobacterium with notable potential for PBP production, especially PC and APC, under diverse growth conditions. In *Nostoc* sp. NK, red light under nitrate-free $BG11_0$ medium yielded the highest C-phycocyanin (C-PC) content (18 % w/w ((Lee et al., 2017). In another study, *Nostoc sphaeroides* reached maximal PBP accumulation under white light at 90 μmol m^{-2} s^{-1}, with blue light enhancing production rates and red light specifically increasing PC and chlorophyll a (Ma et al., 2015). Chromatic adaptation was observed in *Nostoc* sp. at pH 8, 40 μmol m^{-2} s^{-1}, and 35 °C, with PC overexpressed under red light and PE under green light, achieving 0.13 g/g dry weight (Johnson et al., 2014). *Nostoc* strains BTA-61 and BTA-67 produced PE (~128 µg/mg), PC (~168 µg mg^{-1}), and APC (~96 µg/mg) at pH 7.0 (Keithellakpam et al., 2015). It was demonstrated that continuous light and organic carbon supplementation increase PC and APC by 2–2.8-fold (Kovač et al., 2017), while UV radiation and moderate temperature enhanced PBP synthesis in *N. commune* (Llopis et al., 2022). In *Nostoc* sp. PCC 7423, simultaneous addition of glucose (2 g L^{-1}) and sodium nitrate (5 mM) increased biomass and PC content compared to basal BG11$_0$ medium (Cottas et al., 2022). These studies collectively indicate that *Nostoc* sp. adjusts PBP production in response to environmental and nutritional conditions, making it a versatile candidate for biotechnological applications. Previous studies on PBP production in *Nostoc* sp. have primarily examined the individual effects of environmental factors, including light intensity and quality, temperature, and pH, as well as nutritional parameters such as carbon sources. In this study, multiple factors, including nitrogen and phosphorus availability, salinity, carbon sources (glucose and acetate), and light/dark cycles (24:0 and 16:8 h), were systematically evaluated for their effects on PE, PC, APC, and total PBP production in *Nostoc muscorum* using a definitive screening design (DSD). This approach enabled the assessment of the relative contribution of each factor, highlighting those that most strongly influence pigment biosynthesis and providing insight into organism's metabolic responses. The results provide guidance for optimizing cultivation conditions to enhance PBP production and support the development of scalable, sustainable bioprocesses for industrial applications.

Material and Methods

Organism and culture conditions

The cyanobacterium *Nostoc muscorum* was obtained from the algal culture collection of Afagh Negaran Zist Gostar Company (Tehran, Iran). Cultures were cultivated in sterile conditions using a modified nitrate-free BG11 medium (BG11$_0$), which was sterilized through autoclaving at 121 °C for 20 minutes (Hirayama MFG. Corp, Master Switch, Japan). Cultures were maintained at 25 ± 1 °C and pH 8.0, under a light intensity of 75 µmol photons m^{-2} s^{-1} with a 16:8 h light/dark cycle (Sharma et al., 2007). Continuous aeration with filtered air at 0.05 vvm (Atman HP-4000, China) prevented biomass sedimentation and ensured sufficient gas exchange (El Shafay et al., 2021).

Growth monitoring and dry weight measurement

Chlorophyll a was measured every five days to evaluate growth and photosynthetic activity in *Nostoc* sp., as its filamentous and heterogeneous structure complicates accurate cell quantification. For this, a defined volume of culture was centrifuged (Heraeus, Labofuge 6000, Germany), and the pellet was resuspended in 80% acetone. Following an overnight incubation at 4 °C, the mixture was centrifuged again, and the supernatant was spectrophotometrically analysed at 663 nm (Unico, UV-2100PC, China). The concentration pf Chlorophyll a(µg/mL) was calculated according to Eq. 1 (Iranshahi et al., 2014).

$$Chlorophyll = 13.43 \times OD_{663}$$

(1)

Phycobiliprotein extraction method

For PBP extraction, 1 mL of culture was

initially subjected to centrifugation for a duration of 5–10 minutes, after which the supernatant was removed. The resulting pellet was resuspended in 100 µL of pure glycerol, homogenized, and incubated at 4 °C for 48 hours to facilitate gentle cell lysis and osmotic stabilization, ensuring preservation of PBPs and improving extraction efficiency. Subsequently, 900 µL of distilled water was added, and the mixture was vortexed. To adjust the pH and enhance extraction, 66.7 µL of 3 M sodium acetate was incorporated, followed by thorough mixing. The suspension was then maintained at 4 °C until a blue color developed, which typically required 2 h to 2 days. Finally, the mixture was centrifuged for 10 minutes, and the upper layer was carefully collected and transferred to a new container to obtain a homogeneous PBP extract.

PBP quantification analysis

Following extraction, PBP content was quantified by recording the absorbance of the samples at 562, 615, 620, and 652 nm using a spectrophotometer. The concentration of PBPs (µg/mL) were then calculated by the following equations (Bennett & Bogobad, 1973).

$$Phycocyanin\,(PC) = \frac{1000 \times (OD_{615} - OD_{750}) - 474 \times (OD_{652} - OD_{750})}{5.34} \qquad (2)$$

$$Allophycocyanin\,(APC) = \frac{1000 \times (OD_{652} - OD_{750}) - 2.08 \times (OD_{615} - OD_{750})}{5.09} \qquad (3)$$

$$Phycoerythrin\,(PE) = \frac{1000 \times (OD_{562} - OD_{750}) - 2.41 \times (PC) - 0.849 \times (APC)}{9.62} \qquad (4)$$

$$Total\ Phycobiliproteins = PC + PE + APC \qquad (5)$$

OD_{615}, OD_{652}, OD_{562}, and OD_{750} indicate the absorbance values measured at 615 nm, 652 nm, 562 nm, and 750 nm, respectively.

Experimental design

The experimental framework was established using Design-Expert® software (version 13), implementing a Definitive Screening Design (DSD) approach. Fourteen experimental runs were conducted to evaluate the influence of nitrate, phosphate, salinity, glucose, acetate, and light/dark cycles on growth, chlorophyll content, and PBP production in *Nostoc* sp. Factor levels and corresponding results are summarized in Table 1. All experiments were performed in three replicates, and results are reported as mean ± standard error. Zero values for PC, APC, PE, and total PBPs indicate negative concentrations derived from spectrophotometric calculations and were therefore considered non-detectable. Analysis of variance (ANOVA) was applied to assess the significance of the factors for each response variable. Model performance was evaluated using R^2, adjusted R^2, and p-values for individual factors. Detailed statistical data are provided in the Supplementary Material (Table 1).

Results

Chlorophyll content

Figure 1 displays the temporal variation in chlorophyll content over a 30-day cultivation period across the 14 experimental runs. While chlorophyll levels experienced relatively modest fluctuations compared to PBPs, the accumulation patterns and final yields varied significantly among the runs. Notably, Run 8 exhibited the highest chlorophyll accumulation, reaching 5.02 ± 0.55 $\mu g\ mg^{-1}$ dw at day 30. In this run, chloro-

Table 1. Summary of experimental conditions applied for 14 runs and their corresponding response variables

Run	$NaNO_3$ (mg/L)	K_2HPO_4 (mg/L)	NaCl (%)	Glucose (%)	Sodium acetate (%)	L/D cycle (h)	Response 1: Chlorophyll (μg mg^{-1} dw)	Response 2: PC (μg mg^{-1} dw)	Response 3: APC (μg mg^{-1} dw)	Response 4: PE (μg mg^{-1} dw)	Response 5: total PBP (μg mg^{-1} dw)
1	500	40	1.5	0.5	1	16:8	0.45 ± 0.03	0	0	0.91 ± 0.21	0.91 ± 0.21
2	1000	20	0.5	0.5	1	24:0	1.83 ± 0.21	12.43 ± 7.85	8.57 ± 5.41	8.46 ± 5.34	29.47 ± 10.93
3	1000	40	1.5	0	0.5	24:0	0.14 ± 0.01	2.49 ± 0.91	1.94 ± 0.50	2.49 ± 0.44	6.93 ± 1.12
4	0	40	0.5	0	1	24:0	2.28 ± 0.03	6.12 ± 0.92	1.64 ± 0.73	7.06 ± 0.72	14.82 ± 1.38
5	500	20	1	0.25	0.5	24:0	0.02 ± 0.001	0.49 ± 0.22	0.20 ± 0.16	0.75 ± 0.25	1.44 ± 0.37
6	1000	0	1.5	0.5	0	16:8	0.35 ± 0.02	7.22 ± 1.87	3.40 ± 0.99	7.31 ± 1.66	17.93 ± 2.69
7	500	20	1	0.25	0.5	16:8	0.33 ± 0.01	0	0	0	0
8	1000	40	0.5	0.25	0	16:8	5.02 ± 0.55	23.38 ± 1.35	8.35 ± 0.44	37.86 ± 1.45	69.59 ± 2.03
9	0	40	1	0.5	0	24:0	1.28 ± 0.16	5.53 ± 0.55	0.96 ± 0.42	0	6.48 ± 0.69
10	1000	0	1	0	1	16:8	0.17 ± 0.01	0	0	0	0
11	0	20	1.5	0	0	16:8	0.22 ± 0.004	0	0	0	0
12	0	0	0.5	0.5	0.5	16:8	0.64 ± 0.05	0	0	0	0
13	500	0	0.5	0	0	24:0	0.99 ± 0.001	1.76 ± 0.61	1.28 ± 0.53	1.95 ± 0.65	4.99 ± 1.04
14	0	0	1.5	0.25	1	24:0	0.45 ± 0.07	0	0.09 ± 0.002	0	0.09 ± 0.002

Note: The reported values are related to the end of the cultivation (30th day).

Values are presented as mean ± standard error (SE) of three replicates.

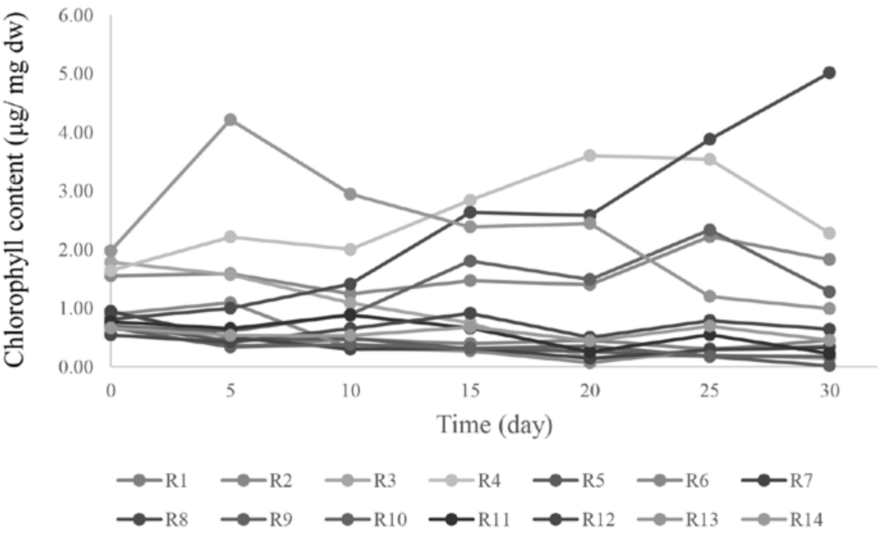


Fig. 1. Variation in chlorophyll content over a 30-day period across 14 experimental runs

phyll content showed a marked increase after day 10 and continued steadily through the end of cultivation. This occurred under conditions of high nitrate and phosphate, low salinity, low glucose supplementation, and a 16:8 h photoperiod. Moderate chlorophyll levels were observed in Runs 2 (1.83 ± 0.21 μg mg^{-1} dw), 4 (2.28 ± 0.03 μg mg^{-1} dw), and 9 (1.28 ± 0.16 μg mg^{-1} dw). These runs exhibited gradual increases during the early and mid-cultivation phases, followed by stabilization or slight decline toward the end. In contrast, several runs accumulated only minimal chlorophyll by day 30. Very low values were recorded in Runs 5 (0.02 ± 0.001 μg mg^{-1} dw), 3 (0.14 ± 0.01 μg mg^{-1} dw), 10 (0.17 ± 0.01 μg mg^{-1} dw), and 11 (0.22 ± 0.004 μg mg^{-1} dw), reflecting conditions. Run 14 also maintained a low chlorophyll content (0.45 ± 0.07 µg mg^{-1} dw). This is consistent with its lack of nitrate and phosphate, both essential for chlorophyll biosynthesis.

Phycocyanin content

Figure 2 presents the variation in phycocyanin (PC) content over a 30-day cultivation period across 14 experimental runs. According to Figure 2, PC accumulation varied notably among the experimental runs, yet the temporal patterns were generally similar, showing a progressive increase over time. No detectable PC was observed in Runs 1, 7, 10, 11, and 12, indicating that *N. muscorum* did not produce measurable amounts of PC under specific environmental and nutritional conditions. The highest PC concentration was observed in Run 8 (23.38 ± 1.35 µg mg-1dw), showing a consistent increasing trend over time. This run was cultivated under high nitrate (1000 mg/L $NaNO_3$) and phosphate (40 mg/L K_2HPO_4) concentrations, low salinity (0.5% NaCl), low glucose (0.25%), no acetate, and a 16:8 h light/dark cycle. Run 2 (12.43 ± 7.85 µg mg^{-1} dw) and Run 6 (7.22 ± 1.87 µg mg^{-1} dw) also showed substantial PC accumulation under high-nitrate conditions with varying phosphate levels and light cycles. Moderate PC levels were recorded in Run 4 (6.12 ± 0.92 µg mg^{-1} dw) and Run 9 (5.53 ± 0.55 µg mg^{-1} dw), which lacked nitrogen but had sufficient phosphate and variable salinity and carbon sources. Lower PC values were observed in Run 3 (2.49 ± 0.91 µgmg^{-1} dw) and Run 13 (1.76 ± 0.61 µg mg^{-1} dw).

Allophycocyanin content

Figure 3 illustrates the 30-day accumulation patterns of allophycocyanin (APC) across 14 experimental runs. APC levels showed substantial variability, with some runs displaying pronounced increases over time, while others remained consistently low. No APC was detected in Runs 1, 6, 7, 10, 11, and 12, likely due to environmental and nu-

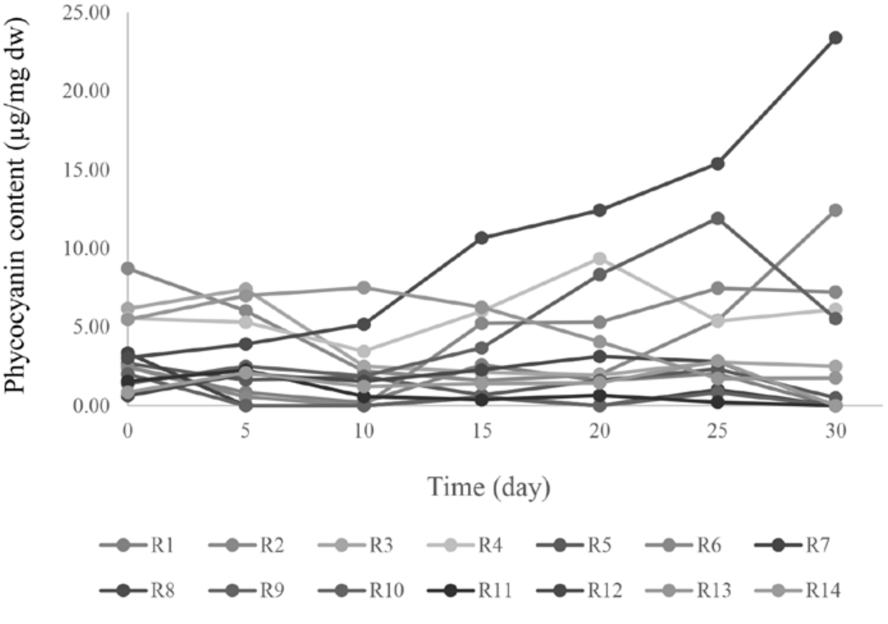


Fig. 2. Variation in phycocyanin (PC) content over a 30-day period across 14 experimental runs

tritional conditions that were unfavorable for APC synthesis by *N. muscorum*. The highest APC contents were recorded in Run 2 (8.57 ± 5.41 µg mg^{-1} dw) and Run 8 (8.35 ± 0.44 µg mg^{-1} dw), both displaying a pronounced upward trend throughout the cultivation period. These runs were characterized by high nitrate levels, moderate phosphate concentrations, low salinity, and limited carbon supplementation. Moderate APC accumulation was observed in Runs 3 (1.94 ± 0.50 µg mg^{-1} dw), 4 (1.64 ± 0.73 µg mg^{-1} dw), and 6 (3.40 ± 0.99µg mg^{-1} dw). In contrast, Run 14, which lacked both nitrate and phosphate, exhibited lower APC content (0.09 ± 0.002 µg mg^{-1} dw).

Phycoerythrin content

Figure 4 displays the changes in phycoerythrin (PE) content over a 30-day cultivation period across 14 experimental runs. PE synthesis varied markedly among the runs. While PE increased noticeably in some runs, others, such as Runs 7, 9, 10, 11, 12, and 14 showed almost no measurable PE during the 30-day period. Run 8 exhibited a rapid and steady rise in PE levels, culminating in the highest value (37.86 ± 1.45 µg mg^{-1} dw) at day 30. This pronounced accumulation occurred under conditions of elevated nitrate and phosphate availability, moderate salinity, relatively low carbon supplementation, and a 16:8 h light/dark cycle. Runs 2 (8.46 ± 5.34 µg mg-1dw), 4 (7.06 ± 0.72 µg mg^{-1} dw), and 6 (7.31 ± 1.66 µg mg^{-1}dw) demonstrated gradual but steady PE accumulation. Runs 1 (0.91 ± 0.21 µg mg-1dw), 5 (0.75 ± 0.25 µg mg-1dw), and 13 (1.95 ± 0.65 µg mg^{-1} dw) showed only limited PE production. Notably, Run 14, which lacked both nitrate and phosphate, failed to produce measurable PE by day 30.

Total phycobiliproteins content

Figure 5 presents the accumulation of total phycobiliproteins (PBPs) over the 30-day cultivation period across 14 experimental runs. PBP production varied widely among the runs, with some showing substantial increases while others remained minimal or undetectable. Run 8 exhibited the highest total PBP accumulation, reaching 69.59 ±

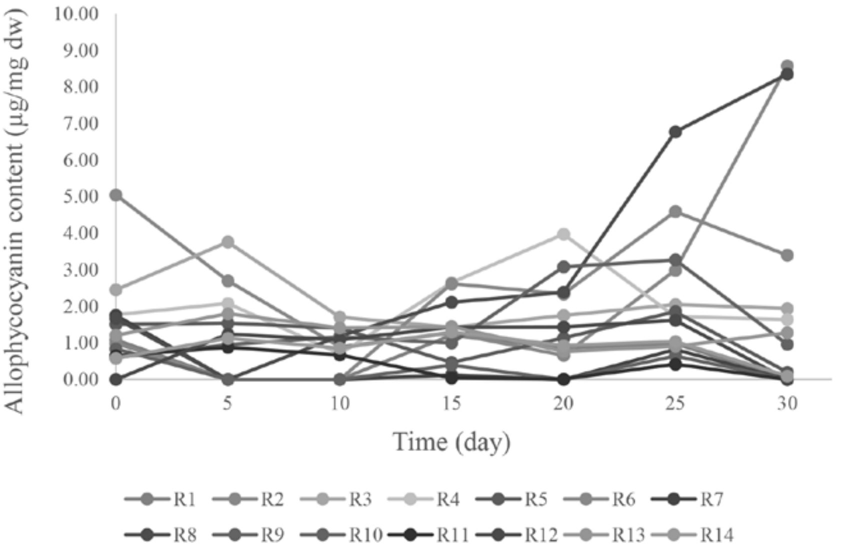


Fig. 3. Variation in allophycocyanin (APC) content over a 30-day period across 14 experimental runs

2.03 µg mg^{-1} dw by day 30 under high nutrient conditions. Substantial but lower accumulation was observed in Runs 2 (29.47 ± 10.93 µg mg^{-1} dw) and 6 (17.93 ± 2.69 µg mg^{-1} dw), each showing a gradual increase over time under high nitrate availability, with variable phosphate levels, salinity and light/dark cycles. Runs 3 (6.93 ± 1.12 µg mg^{-1} dw), 9 (6.48 ± 0.69 µg mg^{-1} dw), and 13 (4.99 ± 1.04 µg mg^{-1} dw) exhibited moderate PBP levels. In contrast, Runs 7, 10, 11, 12, and 14 remained very low or undetectable throughout a 30-day period, reflecting primarily limited nitrate and phosphate availability along with suboptimal carbon and environmental conditions.

Statistical analysis using ANOVA (Table 2) confirmed the observed trends in total PBP accumulation across the experimental runs. Nitrogen (A-N, p = 0.0099), phosphorus (B-P, p = 0.0154), salinity (C-S, p = 0.0105), and acetate (E-A, p = 0.0371) had statistically significant effects on PBP production. In contrast, glucose (D-G, p = 0.1324) and

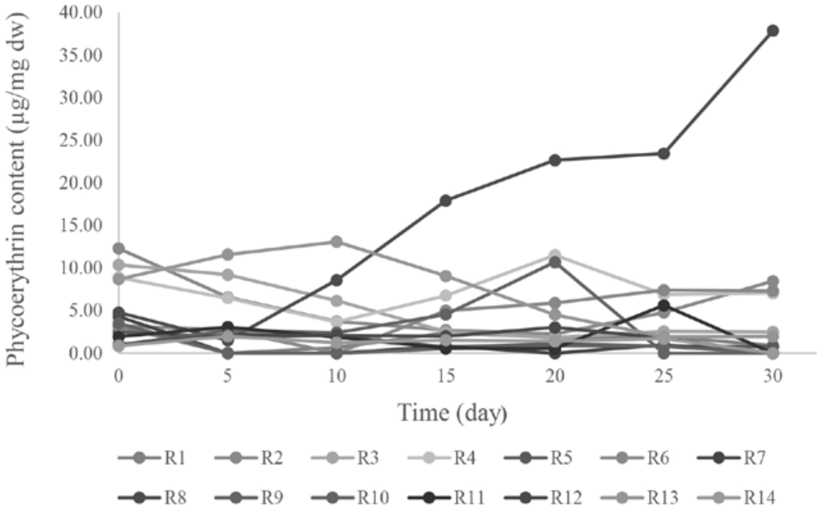


Fig. 4. Variation in phycoerythrin (PE) content over a 30-day period across 14 experimental runs

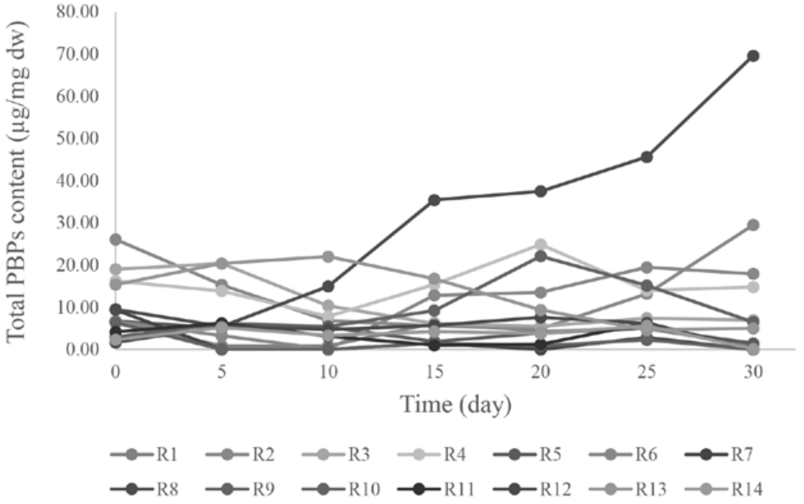


Fig. 5. Variation in total phycobiliproteins content over a 30-day period across 14 experimental runs

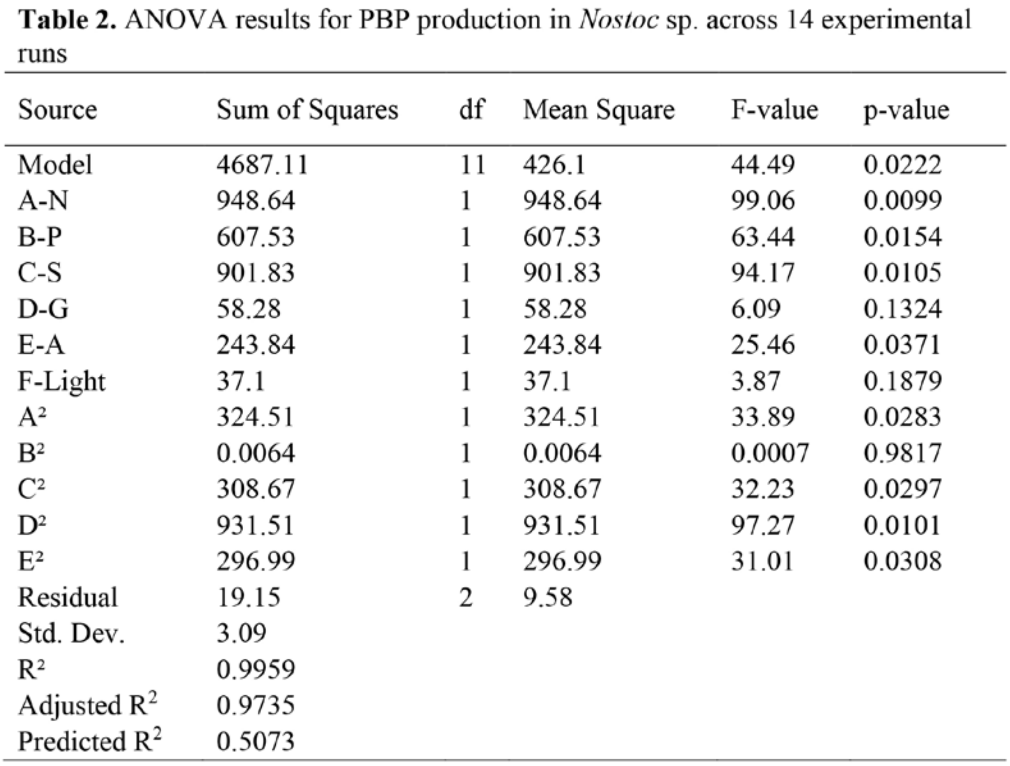


Table 2. ANOVA results for PBP production in *Nostoc* sp. across 14 experimental runs

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	4687.11	11	426.1	44.49	0.0222
A-N	948.64	1	948.64	99.06	0.0099
B-P	607.53	1	607.53	63.44	0.0154
C-S	901.83	1	901.83	94.17	0.0105
D-G	58.28	1	58.28	6.09	0.1324
E-A	243.84	1	243.84	25.46	0.0371
F-Light	37.1	1	37.1	3.87	0.1879
A^2	324.51	1	324.51	33.89	0.0283
B^2	0.0064	1	0.0064	0.0007	0.9817
C^2	308.67	1	308.67	32.23	0.0297
D^2	931.51	1	931.51	97.27	0.0101
E^2	296.99	1	296.99	31.01	0.0308
Residual	19.15	2	9.58		
Std. Dev.	3.09				
R^2	0.9959				
Adjusted R^2	0.9735				
Predicted R^2	0.5073				

photoperiod (F-Light, $p = 0.1879$) were not significant. Significant quadratic effects were observed for nitrogen (A^2, $p = 0.0283$), salinity (C^2, $p = 0.0297$), glucose (D^2, $p = 0.0101$), and acetate (E^2, $p = 0.0308$), indicating non-linear responses of PBP accumulation. The quadratic effect of phosphorus (B^2, $p = 0.9817$) was not significant. The low residual variance (9.58) and high R^2 (0.996, Adjusted $R^2 = 0.974$) further confirm the reliability of the model.

Discussion

Effect of nutrients

Nitrogen and phosphorus are key macronutrients regulating pigment biosynthesis, photosynthesis, and overall cellular function in cyanobacteria. The present results clearly demonstrate that variations in nitrate and phosphate concentrations strongly influenced chlorophyll a and different PBP fractions, including PC, APC, and PE, as well as total PBPs across the 14 experimental runs. Nitrogen, mainly as nitrate, is essential for chlorophyll biosynthesis and growth in cyanobacteria, serving as a precursor for nucleic acids, amino acids, and chlorophyll (Farajzadeh and Zamir, 2025). Similarly, phosphate is vital for *Nostoc* sp., forming a key component of nucleic acids, ATP, and phospholipids, crucial for cell division, energy transfer, and overall cellular function, while also enhancing photosynthesis and protein synthesis (Caille et al., 2024). Run 8, under high nitrate and phosphate conditions, showed the highest chlorophyll a and total PBP accumulation. In contrast, runs lacking one or both nutrients, such as Runs 11, 12, and 14 exhibited minimal chlorophyll accumulation. This underscores the essential role of nitrogen and phosphorus in supporting growth and light-harvesting function. These results are consistent with El Shafay et al. (2021), showing that *N. muscorum* cultivated at the highest tested nitrate (2250 mg/L $NaNO_3$) and phosphate (58.5 mg/L $K_2HPO_4.3H_2O$) concentrations exhibited enhanced biomass productivity.

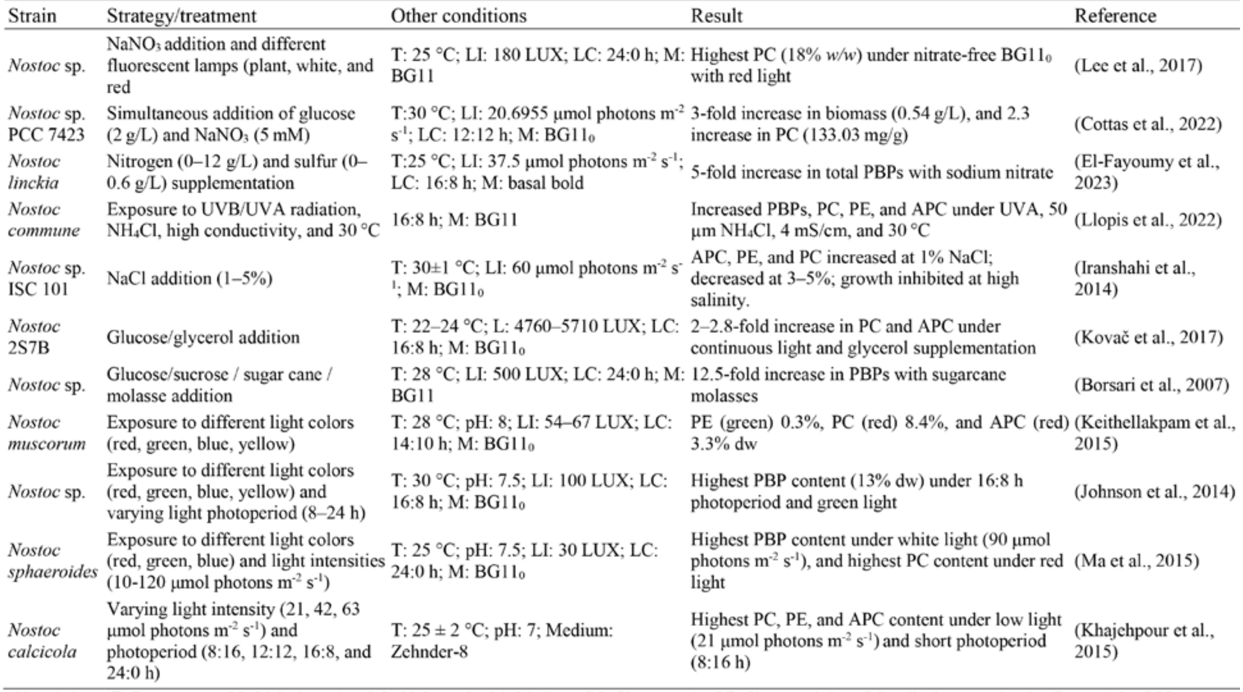
In addition to supporting general cellular growth, nitrate and phosphate have specific regulatory roles in PBP metabolism. These nitrogen-rich pigment–protein complexes function as the primary light-harvesting antenna in cyanobacteria. Nitrogen, primarily as nitrate, is essential for the synthesis of phycobilisome apoproteins and promotes the expression of genes involved in chromophore formation, enabling the assembly of PC, APC, and PE. Phosphate, on the other hand, contributes to cellular energy balance by providing ATP for PBP assembly and stability, and supports nucleotide synthesis and protein turnover, which are crucial for efficient pigment production (Sonani et al., 2016). Consequently, the simultaneous availability of both nutrients, particularly adequate nitrate, promotes not only higher total PBP levels but also a balanced accumulation of individual fractions such as PC, APC, and PE. This trend was evident in nutrient-sufficient runs such as Run 8 and Run 2, where the combination of ample nitrogen and phosphorus supported efficient pigment synthesis and assembly. In contrast, nutrient-limited conditions disrupted this balance, resulting in markedly lower PBP fractions (Runs 12 and 14). In cyanobacteria, severe limitation of key nutrients, such as nitrate or phosphate, not only slows growth but also actively represses PBP synthesis and triggers degradation of light-harvesting complexes. Under nitrogen starvation, phycobilisomes are systematically degraded, leading to reduced PBP content and chlorosis)Richaud et al., 2001). Similarly, phosphate deprivation induces metabolic adjustments that limit investment in pigment-producing structures and prioritize internal phosphorus reallocation, ultimately reducing available resources for pigment biosynthesis (Solovchenko et al., 2020). Statistical analysis also confirmed that nitrate had the most pronounced effect on total PBP production ($p = 0.0099$), while phosphate also significantly influenced PBP accumulation ($p = 0.0154$). These results underscore the synergistic role of nitrogen and phosphorus in promoting efficient PBP accumulation in *Nostoc* sp.

Previous studies have demonstrated that various cultivation strategies and environmental conditions significantly influence PBP production in *Nostoc* species. Table 3 summarizes the effects of factors, such as carbon and nitrogen supplementation, light quality and intensity, photoperiod, salinity, and temperature, on PC, PE, APC, and total PBP accumulation. El-Fayoumy et al. (2021) reported that moderate nitrate supplementation (around 3 g L^{-1}) maximized the production of all major PBP fractions in *Nostoc linckia*, including PC (76.28 ± 4.61 µg/g), APC (37.19 ± 2.44 µg g^{-1}), and PE (146.82 ± 4.11 µ g^{-1}), leading to a total PBP content of 276.29 ± 5.45 µg g^{-1}. Lower nitrate concentrations (0–1.5 g L^{-1}) supported moderate PBP accumulation, whereas higher nitrate levels (6–12 g L^{-1}) caused a dramatic decline in all fractions. In another study, Shanab et al. (2012) reported that *N. muscorum* is highly responsive to nitrogen availability, with increased nitrate concentrations (3–9 g L^{-1}) promoting enhanced PBP production. Similarly, in *Nostoc* sp. PCC 7423, combined supplementation with 2 g/L glucose and 5 mM sodium nitrate increased biomass by ~3-fold (0.54 g L^{-1}) and PC content ~2.3-fold (133.03 mg/g) compared to the basal $BG11_0$ medium (Cottas et al., 2022).

Effect of salinity on growth

Salinity significantly influences cyanobacterial physiology, as variations in external NaCl concentration affect osmotic balance, ion fluxes, and the functioning of pigment-producing pathways. In this study, *N. muscurum* demonstrated a clear, concentration-dependent response to salinity, evident from the substantial variation in chlorophyll a level observed across the experimental runs. Moderate salinity (0.5%) supported higher chlorophyll accumulation, as observed in Runs 8, 4, and 2, with Run 8 exhibiting the highest chlorophyll a level (5.02 ± 0.55 µg mg^{-1} dw). At this level, cells likely maintain osmotic balance more effectively, allowing efficient nutrient uptake and preserving photosynthetic capacity. In contrast, higher salinity (1.5%), as in Runs 3, 6, and 11, resulted in a pronounced reduction in chlorophyll a, indicating that elevated NaCl imposes osmotic and ionic stress. Excess NaCl can impair water uptake and ion homeostasis, disrupt the activity of key metabolic enzymes, as well as the efficiency of the photosynthetic apparatus, ultimately reducing chlorophyll synthesis and growth (Pan et al., 2024). Interestingly, although Runs 8, 4, and 2 shared the same salinity level (0.5%), their chlorophyll contents differed, highlighting that salinity interacts with other environmental factors, particularly nitrate and phosphate availability, to determine the overall photosynthetic output. These results are consistent with Behzadian et al. (2020), demonstrating that increasing

Table 2. Overview of PBP production in *Nostoc* sp. under various cultivation strategies and environmental conditions



Strain	Strategy/treatment	Other conditions	Result	Reference
Nostoc sp.	$NaNO_3$ addition and different fluorescent lamps (plant, white, and red	T: 25 °C; LI: 180 LUX; LC: 24:0 h; M: BG11	Highest PC (18% *w/w*) under nitrate-free $BG11_0$ with red light	(Lee et al., 2017)
Nostoc sp. PCC 7423	Simultaneous addition of glucose (2 g/L) and $NaNO_3$ (5 mM)	T:30 °C; LI: 20.6955 µmol photons m^{-2} s^{-1}; LC: 12:12 h; M: $BG11_0$	3-fold increase in biomass (0.54 g/L), and 2.3 increase in PC (133.03 mg/g)	(Cottas et al., 2022)
Nostoc linckia	Nitrogen (0–12 g/L) and sulfur (0–0.6 g/L) supplementation	T:25 °C; LI: 37.5 µmol photons m^{-2} s^{-1}; LC: 16:8 h; M: basal bold	5-fold increase in total PBPs with sodium nitrate	(El-Fayoumy et al., 2023)
Nostoc commune	Exposure to UVB/UVA radiation, NH_4Cl, high conductivity, and 30 °C	16:8 h; M: BG11	Increased PBPs, PC, PE, and APC under UVA, 50 µm NH_4Cl, 4 mS/cm, and 30 °C	(Llopis et al., 2022)
Nostoc sp. ISC 101	NaCl addition (1–5%)	T: 30±1 °C; LI: 60 µmol photons m^{-2} s^{-1}; M: $BG11_0$	APC, PE, and PC increased at 1% NaCl; decreased at 3–5%; growth inhibited at high salinity.	(Iranshahi et al., 2014)
Nostoc 2S7B	Glucose/glycerol addition	T: 22–24 °C; L: 4760–5710 LUX; LC: 16:8 h; M: $BG11_0$	2–2.8-fold increase in PC and APC under continuous light and glycerol supplementation	(Kovač et al., 2017)
Nostoc sp.	Glucose/sucrose / sugar cane / molasse addition	T: 28 °C; LI: 500 LUX; LC: 24:0 h; M: BG11	12.5-fold increase in PBPs with sugarcane molasses	(Borsari et al., 2007)
Nostoc muscorum	Exposure to different light colors (red, green, blue, yellow)	T: 28 °C; pH: 8; LI: 54–67 LUX; LC: 14:10 h; M: $BG11_0$	PE (green) 0.3%, PC (red) 8.4%, and APC (red) 3.3% dw	(Keithellakpam et al., 2015)
Nostoc sp.	Exposure to different light colors (red, green, blue, yellow) and varying light photoperiod (8–24 h)	T: 30 °C; pH: 7.5; LI: 100 LUX; LC: 16:8 h; M: $BG11_0$	Highest PBP content (13% dw) under 16:8 h photoperiod and green light	(Johnson et al., 2014)
Nostoc sphaeroides	Exposure to different light colors (red, green, blue) and light intensities (10-120 µmol photons m^{-2} s^{-1})	T: 25 °C; pH: 7.5; LI: 30 LUX; LC: 24:0 h; M: $BG11_0$	Highest PBP content under white light (90 µmol photons m^{-2} s^{-1}), and highest PC content under red light	(Ma et al., 2015)
Nostoc calcicola	Varying light intensity (21, 42, 63 µmol photons m^{-2} s^{-1}) and photoperiod (8:16, 12:12, 16:8, and 24:0 h)	T: 25 ± 2 °C; pH: 7; Medium: Zehnder-8	Highest PC, PE, and APC content under low light (21 µmol photons m^{-2} s^{-1}) and short photoperiod (8:16 h)	(Khajehpour et al., 2015)

Abbreviations: T, Temperature; LI, Light intensity; LC, Light cycle; M, Medium; PC, Phycocyanin; PE, Phycoerythrin; APC, Allophycocyanin; dw, Dry weight; PBPs, Phycobiliproteins.

salinity negatively affects *Nostoc* growth. In their study, the maximum specific growth rate was recorded at 1% NaCl, ranging from 0.81% to 3.08%, whereas higher salt concentrations caused a significant decline in growth. Another study reported that in *Nostoc* sp. ISC 101, exposure to low salinity (1% NaCl) resulted in a slight increase in total chlorophyll (1.924 ± 0.074 µg mg^{-1} dw) when compared to the control without salinity (1.860 ± 0.233 µg mg^{-1} dw) (Iranshahi et al., 2014). Higher NaCl levels progressively reduced pigment content and photosynthetic performance. Microscopic analysis indicated structural alterations under salt stress: heterocyst and vegetative cells became narrower, akinetes appeared at 3% NaCl, and significant cellular deterioration was observed at 5%. Although some physiological adjustments occurred, salinity level exceeding 1% imposed substantial stress, compromising cell structure and overall metabolic efficiency.

In addition to chlorophyll, salinity strongly influences PBP synthesis in *Nostoc* sp. by affecting cellular metabolism and the stability of light-harvesting complexes. At low to moderate NaCl levels, mild osmotic stress can stimulate cellular metabolism, boosting the production of essential light-harvesting pigments, such as PC, APC, and PE. In contrast, high salinity causes severe ionic and osmotic stress, disrupting cellular homeostasis, damaging photosynthetic structures, and reducing pigment synthesis. In this circumstances, PBPs degrade, photosynthetic efficiency declines, and resources are diverted from growth and pigment production to survival under stress. In this study, the maximum PBP accumulation occurred at the lowest NaCl concentration (0.5%), with Run 8 reaching 69.59 ± 2.03 µg mg^{-1} dw and Run 2 showing 29.47 ± 10.93 µg mg^{-1} dw by Day 30, whereas higher salinity (1.5%) reduced PBP levels, often below 1 µg mg^{-1} dw (Runs 1, 11, and 14). Statistical analysis also confirmed that salinity significantly affected total PBP production ($p = 0.0105$), supporting the observed trend that moderate NaCl (0.5%) promotes pigment accumulation, while higher levels (≥ 1.5%) strongly inhibit PBP synthesis. Under low salinity, cells likely activate protective mechanisms that boost PBP synthesis, enhancing light capture and energy utilization under mild osmotic stress. This adaptive response supports photosynthetic efficiency and enhances cellular resilience to variable environmental conditions. Severe abiotic stress, such as high salinity, has been shown to impair photosynthetic electron transport and damage light-harvesting complexes in cyanobacteria, leading to reduced pigment synthesis under stressful conditions (Dąbrowski et al., 2021). Iranshahi et al. (2014) observed that low salinity (1% NaCl) slightly enhanced PBP content in *Nostoc* sp. ISC 101 compared to the control. APC, PC, and PE reached their peak levels at 1% NaCl (APC: 1.562 ± 0.097 µg mg-1dw; PC: 0.399 ± 0.042 µg mg^{-1}dw; PE: 0.631 ± 0.039 µg mg^{-1}dw), whereas higher salinity (≥2% NaCl) led to a progressive decline in all PBPs. This was attributed to the disruption of phycobilisome attachment to thylakoid membranes.

Effect of carbon sources

The influence of organic carbon sources such as glucose and acetate on pigment biosynthesis in cyanobacteria is closely tied to their ability to modulate metabolic transitions between autotrophic and mixotrophic modes. Under mixotrophic conditions, cells can simultaneously exploit light energy and externally supplied carbon, allowing for greater metabolic flexibility and, in many cases, higher pigment accumulation and biomass formation (Joun et al., 2023). Glucose is readily assimilated and energizes heterotrophic pathways. It provides ATP and metabolic intermediates that support photosynthetic pigment synthesis when nutrients are sufficient (Noh et al., 2021). Acetate can also serve as a carbon source, but it enters metabolism through different pathways. Its effects depend strongly on concentration and nutrient conditions (Kim et al., 2022).

Chlorophyll a production in *Nostoc* sp. exhibited distinct responses to glucose and acetate. The highest pigment level was observed in Run 8, where a low glucose concentration (0.25%) paired with adequate nitrate and phosphate resulted in 5.02 ± 0.55 µg mg-1 dwchlorophyll a. Notably, under similar nutrient-rich conditions but without glucose supplementation, as in Run 3, chlorophyll a accumulation was minimal (0.14 ± 0.01 µg mg^{-1} dw). This indicates that even modest glucose supplementation can markedly stimulate chlorophyll biosynthesis when nitrogen and phosphorus are not limiting. By comparison, cultures grown without any organic carbon, such as Run 13, still produced a baseline chlorophyll level (~0.99 µg mg^{-1} dw). This reflects that the capability of an organism to maintain photosynthetic machinery under autotrophic conditions, provided that essential nutrients like nitrate are present.

In contrast, cultures provided with high acetate concentrations frequently showed limited chlorophyll accumulation. For example, Run 10 (1% acetate, no glucose) displayed extremely low pigment content (0.17 ± 0.01 µg mg-1dw), demonstrating the inhibitory effect of excess acetate. Acetate metabolism produces acetyl-CoA, which directly enters the tricarboxylic acid (TCA) cycle. However, high concentrations of acetate can acidify the medium, induce metabolic stress, or disrupt cellular redox balance, all of which may suppress chlorophyll biosynthesis (Joun et al., 2023). Nonetheless, even at a relatively high concentration (1%), acetate provided limited metabolic support in nitrogen-deficient cultures. Run 4 (1% acetate, no nitrate) reached 2.28 ± 0.03 µg mg^{-1} dw chlorophyll a, indicating that acetate can sustain some pigment formation under nitrogen limitation. However, its effect is considerably weaker than that of nitrate-rich, glucose-supplemented conditions.

Several studies have highlighted the beneficial effects of exogenous organic carbon on cyanobacterial and algal growth under mixotrophic conditions. Joun et al. (2023) reported that supplementing *Haematococcus pluvialis* cultures with glucose increased biomass by 77%, while acetate led to a 40% improvement over purely autotrophic growth. Glucose primarily fueled the TCA cycle by providing substrates and ATP, although it slightly inhibited photosynthesis, whereas acetate supported both autotrophic and heterotrophic metabolism. Similarly, Borsari et al. (2007) demonstrated that glucose supplementation enhanced *Nostoc* sp.

biomass by 2.5-fold. In another study, Kim et al. (2022) demonstrated that a moderate concentration of sodium acetate (1 g L^{-1}) enhances the growth of *Pseudanabaena mucicola*. However, higher concentrations (5 and 10 g L^{-1}) were found to be inhibitory, and certain sugars, such as galactose and xylose, were toxic.

A similar pattern was observed for total PBPs, with the highest accumulation occurring in Run 8, where low glucose (0.25%) in the absence of acetate led to approximately 70 ± 2.03 µg mg^{-1} dw by day 30. Although glucose did not show a statistically significant effect in the ANOVA ($p > 0.05$), the observed trend indicates that under optimal nutrient conditions, low glucose levels strongly stimulate PBP biosynthetic pathways in *N. muscorum*. Glucose likely enhances ATP generation and provides reducing equivalents necessary for the nitrogen-rich synthesis of PC, APC, and PE, while also supporting the structural integrity of phycobilisomes. However, when glucose is combined with high acetate levels, as observed in Run 2, the accumulation of PBPs decreases (29.47 ± 10.93 µg mg^{-1} dw). This decline is likely due to acetate yields less ATP/NAD(P)H and introducing additional metabolic stress, which restricts pigment production (Gong et al., 2022). In Run 6, which also received 0.5% glucose but lacked phosphate, only 17.93 ± 2.69 µg mg^{-1} dw PBPs was produced, showing that the positive impact of glucose requires balanced nutrient availability. The absence of phosphate limits energy generation and biosynthetic capacity, thereby reducing pigment synthesis despite the presence of organic carbon. Conversely, treatments lacking both glucose and acetate (Runs 11 and 13) accumulated very low PBP levels (<5 µg mg^{-1} dw), suggesting that external carbon sources can play a crucial role in supporting PBP synthesis.

Consistent with these observations, several earlier studies have also highlighted the strong stimulatory effect of organic carbon on pigment accumulation in cyanobacteria. In *Nostoc* sp. PCC 7423, supplementation with 2 g L^{-1} glucose has been reported to increase biomass almost threefold while elevating PC levels by approximately 2.3-fold relative to $BG11_0$ medium (Cottas et al., 2022). Under broader mixotrophic conditions in *Nostoc* sp., Borsari et al. (2007) showed that sugarcane molasses (1.0 g L^{-1}) produced the largest improvement, around a 12.5-fold rise in PBPs, while sucrose (0.5 g L^{-1}) and glucose (1.0 g L^{-1}) resulted in 4.5-fold and 3-fold increases, respectively. Additional evidence from *Synechocystis* sp. further supports the role of glucose in pigment enhancement: in cultures supplemented with 5 mM glucose, substantial increases in chlorophyll a (34.66 mg L^{-1}), PC (84.94 mg/L), APC (34.28 mg L^{-1}), and PE (6.90 mg L^{-1}) were observed after 18 days compared with autotrophic controls (Noh et al., 2021).

Effect of photoperiod

Light is a key environmental factor regulating growth, photosynthesis, and pigment biosynthesis in cyanobacteria. Both photoperiod and spectral quality strongly influence chlorophyll and PBP accumulation. Alternating light/dark cycles provide dark periods that allow the photosynthetic apparatus to recover and repair light-induced damage. This reduces photoinhibition, limits the formation of reactive oxygen species (ROS), and improves energy efficiency, supporting higher pigment synthesis. In contrast, continuous illumination can cause metabolic fatigue and excessive ROS accumulation, ultimately reducing pigment production. Light quality also affects specific pigment production: PC absorbs red light, PE green light, and blue or UV-B light can enhance overall PBP synthesis through photoreceptor activation (Keithellakpam et al., 2015). Light not only drives photosynthesis but also regulates key metabolic processes, including ATP formation, NADP/NADPH balance, and carbon fluxes, all of which affect pigment synthesis and overall cell productivity (Kovač et al., 2017).

The results indicate that the light/dark cycle markedly influences chlorophyll a accumulation in *Nostoc* sp. Cultures under a 16:8 hours photoperiod generally achieved higher chlorophyll content compared to some continuous light (24:0 h) conditions, with Run 8 exhibiting the maximum chlorophyll a. This suggests that intermittent dark periods likely support pigment synthesis by allowing repair of the photosynthetic apparatus, optimizing ATP and NADPH production, and maintaining metabolic rhythms (Pagels et al., 2019). Interestingly, some runs under 24:0 hours, such as Run 4 and Run 2, also showed relatively high chlorophyll under favorable nutrients. In contrast, Run 14, which received continuous light but lacked key nutrients, produced very low chlorophyll (0.45 ± 0.07 μg mg^{-1} dw), suggesting that adequate nutrient supply can partly compensate for the stress of continuous light. This aligns with Khajepour et al. (2015), demonstrating that stronger light and longer photoperiods suppressed chlorophyll a and PBP levels.

Statistical analysis indicated that the photoperiod had a significant effect on chlorophyll a accumulation ($p < 0.05$), whereas its affect on total PBP production was not statistically significant ($p = 0.1879$). Despite the lack of statistical significance for PBPs, the observed trend suggests that a 16:8 h light/dark cycle enhances pigment accumulation compared to continuous illumination. This is consistent with the role of intermittent dark periods in metabolic recovery and efficient photosynthesis. Run 8, grown under 16:8 h, produced the highest PBP level (69.59 ± 2.03 μg mg^{-1} dw), whereas Run 3, which had identical nutrients but was exposed to constant light, reached only 6.93 ± 1.12 μg mg^{-1}dw. Similarly, Run 5 under the 16:8 h cycle accumulated 17.93 ± 1.13 μg mg^{-1} dw of PBPs, in contrast to Run 7, which received continuous light with the same nutrient conditions and produced none. This is consistent with Johnson et al. (2014), demonstrating that a dark period is essential for PBP synthesis in *Nostoc* sp., and a 16:8 h light/dark cycle photoperiod provides optimal conditions. Khajepour et al. (2015) showed that light intensity and photoperiod strongly influence pigment accumulation in *Nostoc calcicola*. Under low light (21 μmol photons m^{-2} s^{-1}) and a short photoperiod (8:16 h light/dark), PC (7.41 ± 0.42 μg mg^{-1}) and PE (5.1 ± 0.1 μg mg^{-1}) reached their highest levels, whereas longer light exposure and higher intensities generally decreased PBP content.

Conclusion

This study demonstrated that pigment production in *N. muscorum* is highly responsive to variations in nutrient availability, salinity, carbon sources, and photoperiod. Results highlight a balance between stress and pigment biosynthesis: mild stress promoted pigment accumulation, whereas severe stress, as observed in Run 14, nutrient limitation, high salinity, and continuous light, inhibited it. Elevated nitrate and phosphate levels consistently supported higher yields of chlorophyll, PC, APC, PE, and total PBPs, while low salinity and minimal acetate further favoured pigment accumulation. Glucose acted as a beneficial supplemental carbon source under nutrient-rich conditions, and a 16:8 h light/dark cycle enhanced chlorophyll synthesis and generally supported improved pigment profiles. Integrating the optimized conditions into controlled photobioreactors, along with dynamic cultivation and co-cultivation strategies, will be key to improving stability, resource efficiency, and waste valorization within circular-economy frameworks. Stress-induced pigment enhancement and targeted metabolic engineering can further boost yields and diversify high-value outputs. Giving the rising demand for natural pigments and bioactive compounds, *N. muscorum* holds significant potential as a sustainable biotechnological platform for applications in food, nutraceutical, pharmaceutical, and cosmetic.

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