



Impact of combined nitrogen sources on growth and synthesis of bioactive compounds in *Synechococcus elongatus* Nägeli, a coccoid cyanobacterium

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The present communication deals with the effect of different combined nitrogen sources viz. urea, ammonium chloride, sodium nitrate and potassium nitrate as a sole nitrogen source on the growth of *Synechococcus elongatus*. The effect of different nitrogen sources were evaluated on the basis of biomass, growth rate, generation time and biological carbon sequestration. Study was conducted on the synthesis of primary, accessory photosynthetic pigments and photo-protective pigment under semi-continuous influence of different sources of combined nitrogen. Sodium nitrate in the nutrient medium could proliferate growth rate and lower generation time of *S. elongatus* and it is also responsible for maximum biological carbon sequestration, synthesis of bioactive compounds like chlorophyll-a, carotenoids and phycobilins. Potassium nitrate as a sole nitrogen source could be responsible for enhanced production of total protein in *S. elongatus* cells. On evaluation of all four different nitrogen sources it could be concluded that sodium nitrate is the best source of nitrogen while urea could be toxic for *S. elongatus*.

Keywords: Bioactive compounds, Combined nitrogen, Cyanobacteria, Generation time, Growth rate

INTRODUCTION

The alteration of solar energy to chemical energy through photosynthesis supports life on Earth. Harvesting of light energy with the help of antenna complex regulates the growth and reproduction of

photosynthetic organisms. Cyanobacteria (Blue-green algae) are a diverse group of photosynthetic prokaryotes distributed worldwide in different biomes including fresh water, marine water, brackish water and waste water; moist soil, tree bark, roof top and building walls (Reynolds &

ARTICLE HISTORY

Submitted: June 27, 2024

Accepted: July 3, 2025

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DOI: 10.21608/ejbo.2025.299856.2900

EDITED BY: Hanaa Amer

Walsby, 1975; Kant et al., 2004a; Tiwari et al., 2009, 2013; Kant et al., 2020; Doli et al., 2023). They are capable to perform 10-25% of global photosynthesis. In recent days, cultivation of cyanobacteria has been practiced worldwide for the production of different human and animal food, food supplements, pigments, pharmaceuticals, biofertilizers and bio-fuels (Kant et al., 2004b, 2006; Chisti, 2007; Kumar et al., 2010; Kant, 2012; Sarma et al., 2020). In addition to light and CO₂, the nitrogen, phosphorus and micronutrients are essential for the growth of cyanobacteria (Yuan et al., 2011). Cultivation modes and nutritional management affect the growth rate and biochemical composition of cyanobacteria (Hsieh & Wu, 2009; Sarma et al., 2023, 2024).

The genus *Synechococcus*, an unicellular gram negative cyanobacterium with highly structured cell wall first described in 1979 (Johnson & Sieburth, 1979; Rippka et al., 1979; Waterbury et al., 1979). The members of genus *Synechococcus* have a circadian rhythm that anticipates the timing of dawn and dusk and regulates the expression of a majority of its genes in a time-dependent manner (Zhang et al., 2024). In nature, nitrogen is highly available but the species of the genus *Synechococcus* are unable to fix atmospheric nitrogen and rely on the availability of a combined nitrogen sources, such as nitrate or ammonia, for growth and intracellular catabolic and anabolic reactions (Esteves-Ferreira et al., 2018).

On the availability of combined nitrogen sources they are capable to harvest light energy and contains chlorophyll *a* as primary photosynthetic pigment (Palsson et al., 1996). They can also produce accessory light harvesting pigment phycobiliprotein (Waterbury et al., 1979) namely c-phycoerythrin, c-allophycocyanin and c-phycoerythrin (Stanier & Cohen-Bazire, 1977). Phycocyanin is a light blue colour accessory pigment absorbing orange and red light. Allophycocyanin is a far red light harvesting protein with high quantum yield and a red protein pigment complex phycoerythrin responsible for photosynthesis (Olson et al., 1988). The genus *Synechococcus* is also considered as a favorable candidate for carotenoid production on a commercial scale (Sarnaik et al., 2019) as it mainly contains β -carotene (Hirschberg & Chamovitz, 1994).

The present study was focused on semi-continuous culturing of cyanobacterium *Synechococcus elongatus* Nägeli under the

influence of different nitrogen sources viz. urea, ammonium chloride, sodium nitrate or potassium nitrate as a sole source of nitrogen to evaluate the effect of different nitrogen sources on synthesis of biomass, evaluate growth rate, generation time and biological carbon sequestration. Study was conducted on the synthesis of primary, accessory photosynthetic pigments and photo-protective pigment carotenoid under semi-continuous influence of different sources of nitrogen content in the culture media. Study was also conducted on the synthesis of total protein content under semi-continuous culture condition.

MATERIALS AND METHODS

Experimental organism

Water samples containing mixotrophic algal growth was collected from Town Kalibari pond, Kailashahar, Unakoti Tripura. The samples were observed under trinocular research microscope (Olympus CH21i) fitted with digital camera and software (Magnus Mag-cam and Mag-Vision). The raw material employed in the experiment is *Synechococcus elongatus* isolated by repeated culturing and sub-culturing in solid and liquid BG-11 media described by Kant et al. (2008) and identified on the basis of monograph (Komárek & Anagnostidis, 1998). The axenic culture of the experimental organism is deposited at the Department of Botany, Chaudhary Charan Singh University, Meerut, Uttar Pradesh, India.

Experimental conditioning of the organism

Experimental conditions were 28±2°C temperature for 14:10 light:dark regime under 140µmol photons m⁻² s⁻¹. Under this condition the experimental organism *S. elongatus* was cultured for 10 days in liquid BG-11 nutrient medium (Stanier et al., 1971) at pH at 7.1 under constant stirring using multi-position magnetic stirrer (LABQUEST-BOROSIL MHPS15P) for uniform growth of the experimental organism.

Culturing media formulation

The present study was conducted using 250ml conical flasks (Borosil) containing 150ml nitrogen free BG-11 medium (Tables 1, 2) supplemented with urea, ammonium chloride, sodium nitrate and Potassium nitrate as nitrogen source. Equivalent value of nitrogen as provided by Sodium nitrate (NaNO₃) 1.5g L⁻¹ is calculated and replaced by Urea (CO(NH₂)₂) 0.53 g L⁻¹, Ammonium chloride (NH₄Cl) 0.944g L⁻¹, Potassium nitrate (KNO₃) 1.784g L⁻¹.

Table 1. Chemical constituents of nitrogen free BG-11 medium

Chemicals	Amount(gL ⁻¹)
K ₂ HPO ₄	0.04
MgSO ₄ .7H ₂ O	0.075
CaCl ₂ .2H ₂ O	0.036
Citric acid	0.006
Ferric Chloride	0.006
EDTA (Disodium salt)	0.001
Na ₂ CO ₃	0.02
Trace Metal Mix	1 mL ⁻¹

Table 2. Chemical Constituents of Trace Metal Mix

Chemicals	Amount(gL ⁻¹)
H ₃ BO ₃	2.86
MnCl ₂ .4 H ₂ O	1.81
ZnSO ₄ .7H ₂ O	0.222
Na ₂ MoO ₄ . 2H ₂ O	0.39
CuSO ₄ . 5H ₂ O	0.079
Co(NO ₃) ₂ .6 H ₂ O	0.0494

Experimental design and application of different nitrogen sources containing media

The experiment was conducted in a complete randomized design in triplicate. Nutrient medium containing sodium nitrate as nitrogen source was used for control while nutrient media supplemented with urea, ammonium chloride and potassium nitrate was used as variants. Exponentially growing *S. elongatus* was semi-continuously cultured for 30 days and harvested on every 10th day and replaced with 50% freshly prepared nutrient medium for the estimation of specific growth rate, generation time, biomass content, CO₂ sequestered, chlorophyll-a, carotenoid, phycobilins and total protein.

Estimation of specific growth rate and generation time

Specific growth rate is usually characterized by the cell volume and doubling time (DT). Specific growth rate is inversely proportional to generation time. The specific growth rate is calculated by using the method described by Mehrara et al. (2007) using the given formula:

$$\text{Specific growth rate (K)} = dV/dT \times 1/V$$

$$\text{Generation time (GT)} = 1/K$$

Estimation of biomass

The biomass of the cyanobacterial culture was expressed in terms of dry weight (Rao et al., 2007).

Estimation of CO₂ sequestered

Microalgae are considered as a prominent biological tool for carbon sequestration by removing CO₂ from the atmosphere and storing it in their cells. The CO₂ sequestered by the *S. elongatus* cells was measured using the method followed by Kumar & Das (2013).

Estimation of Chlorophyll-a

Chlorophyll-a content in the cells was extracted by the method described by Lichtenhaler & Wellburn (1983) using acetone. Chlorophyll-a is completely extractable in polar solvents like acetone /methanol. The whole experiment was carried out in dark to avoid photoreaction and loss of pigments.

Carotenoid estimation

Carotenoids include pigments like carotenes and xanthophylls soluble in polar solvents like acetone, exhibiting a characteristic absorption at 450nm. Extraction of carotenoid was done by the method followed by Jenssen (1978) at 450nm using acetone as blank.

Estimation of Phycobilin

Phycobilin a complex of phycocyanin, allophycocyanin and phycoerythrin measured at 562nm, 615nm and 652nm, respectively by the method followed by Bennett & Bogorad (1973).

Estimation of Total Protein

Total protein content in the cells was estimated by the method developed by Lowry et al. (1951) using Bovine Serum Albumin (BSA) as standard.

Statistical analysis of the data

The data obtained were subjected to statistical analysis of variance (ANOVA) by using completely randomized design in MS Office excel 2007 (Mondal & Mondal, 2016). Standard deviation and standard error were calculated against the values obtained.

RESULTS

The experimental organism used in the present study was isolated from the mixotrophic growth from fresh water pond of Town Kalibari, Kailashahar, Unakoti Tripura (Figure 1A) and

identified as *Synechococcus elongatus* Nägeli on the basis of morphological details of the colonies. Morphologically colonies forming clusters or solitary and the cells are cylindrical straight or slightly curved with blue-green, finely granular homogeneous content with 3-48µm length and 1.2-3µm width in culture condition at 10X and 100X magnification is given in Figure 1(B-D).

Specific growth rate of *S. elongatus* was calculated under the influence of urea, ammonium chloride, sodium nitrate or potassium nitrate as a

sole nitrogen source and it was observed that the specific growth rate under the influence of urea is 0.1615 day⁻¹, ammonium chloride is 0.1937 day⁻¹, sodium nitrate is 0.2674 day⁻¹ and potassium nitrate is 0.1941 day⁻¹. Maximum specific growth rate was observed under the influence of sodium nitrate while minimum was observed under the influence of urea as a nitrogen source. The culture flasks supplemented with ammonium chloride or potassium nitrate showed almost same specific growth rate. Detailed information on specific growth rate of *S. elongatus* is given in Figure 1E.

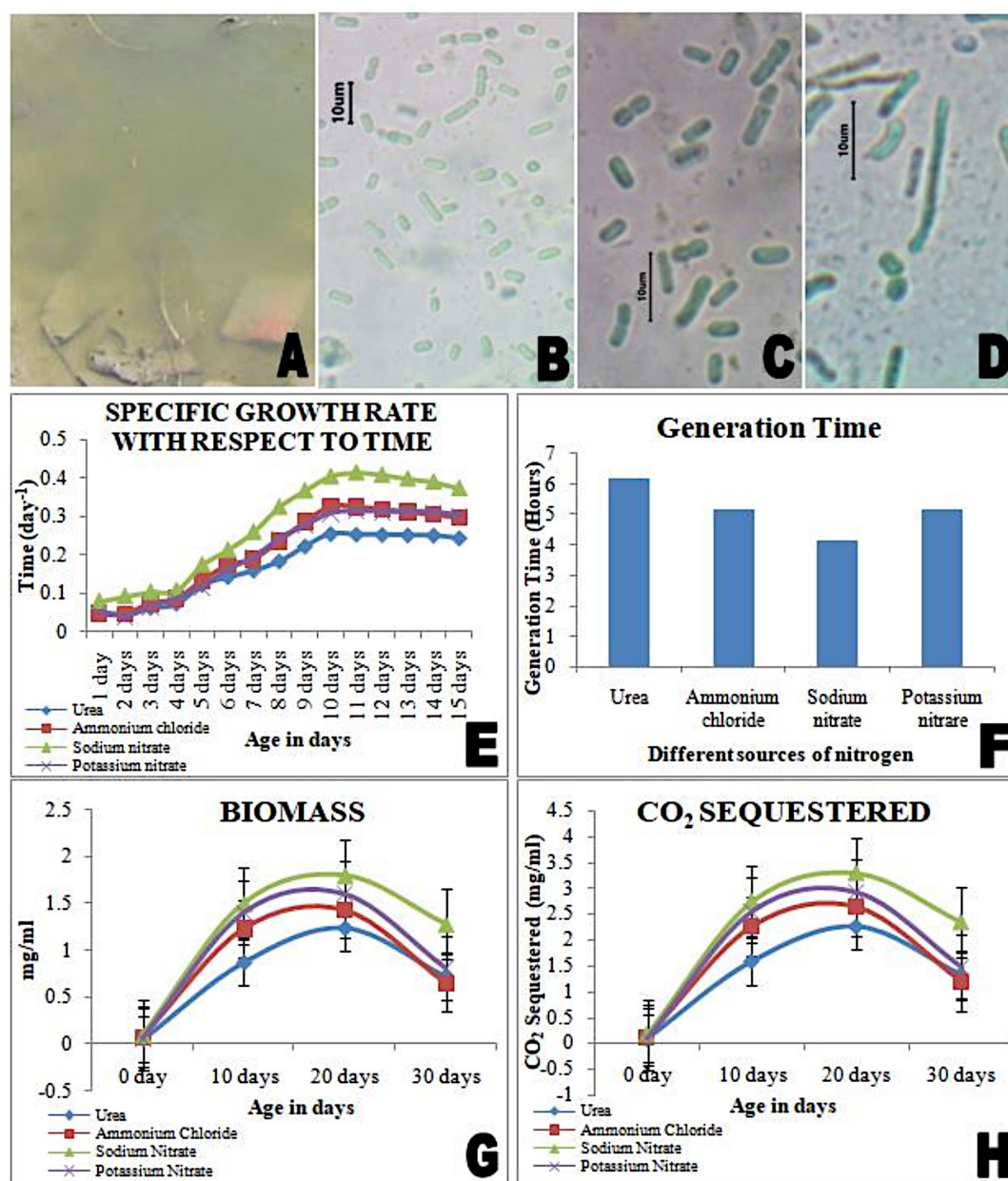


Figure 1(A-G). Description of figures (A) Collection of water sample containing mixotrophic growth; (B) 10 days old *Synechococcus elongatus* under 10X magnification (C-D) 10 days old *S. elongatus* under 100X magnification (E) Growth rate; (F) Generation time (G) Biomass content and (H) CO₂ sequestered under the influence of different nitrogen sources.

On the basis of specific growth rate the generation time was calculated under the influence of four different nitrogen source containing medium. Least generation time of *S. elongatus* was observed 4.13h in sodium nitrate while maximum generation time was observed 6.19h in urea. Generation time under the influence of ammonium chloride and potassium nitrate was observed 5.16h and 5.15h, respectively indicating that both ammonium chloride and potassium nitrate have similar effect on generation time of *S. elongatus*. Detailed results on generation time of *S. elongatus* under different nitrogen sources is given in Figure 1F

A sigmoid curve was observed in all the semi-continuous cultures supplementing every 10th day till 30 days with culture medium containing urea or ammonium chloride or sodium nitrate or potassium nitrate as a sole source of nitrogen for biomass estimation. An increasing trend in biomass content was observed till 20th day supplementing with all the four different medium while after that a gradual decline was observed. Maximum biomass content was observed 1.8mg ml⁻¹ after 20th day of semi-continuous supplementing with nutrient medium containing sodium nitrate as a sole source of nitrogen while minimum biomass content was observed 0.65mg ml⁻¹ in the culture flasks supplemented with ammonium chloride on 30th day. A detailed result on biomass synthesis by *S. elongatus* for a period of 30 days is given in Figure 1G.

A gradual increase in the amount of CO₂ sequestered by *S. elongatus* was observed till 20 days while a decline phase was observed after that under the influence of urea or ammonium chloride or sodium nitrate or potassium nitrate. The graph obtained is sigmoid in nature in all semi-continuously cultured flasks. Maximum amount of CO₂ sequestered was observed 3.294mg ml⁻¹ in the culture flasks supplemented with sodium nitrate for 20 days while minimum amount was observed 1.1185mg ml⁻¹ in ammonium chloride as a sole nitrogen source after a period of 30 days. A detailed result on the amount of CO₂ sequestered by *S. elongatus* for a period of 30 days is given in Figure 1H.

Chlorophyll-a content in *S. elongatus* under the influence of four different nitrogen containing medium A gradual increase in the amount of chlorophyll-a content was observed till 20th day and then declining phase was observed in all the flasks supplemented with four different nitrogen sources containing media. Maximum chlorophyll-a content

was observed 1.425µg ml⁻¹ after 20th day in sodium nitrate while after that a decline in the amount of chlorophyll-a was observed 1.128µg ml⁻¹ after 30 days. Minimum chlorophyll-a content was observed 0.277µg ml⁻¹ after 30th day in ammonium chloride. A detailed result on the synthesis of Chlorophyll-a by *S. elongatus* under the influence of different nitrogen sources is given in Figure 2A.

A gradual inclining and then declining phase were observed in the culture flasks on evaluation of photo-protective pigment carotenoid. Culture flasks supplemented with sodium nitrate as a sole nitrogen source shows a slow and gradual increase till 10th day but after that a stiff peak was observed on 20th day while after that decline in the amount of carotenoid pigment was observed. Maximum carotenoid content after 20 days was observed 0.013µg ml⁻¹ in the culture flasks supplemented with sodium nitrate. Minimum carotenoid content was observed 0.001µg ml⁻¹ when supplemented with urea for 30 days. A detailed result on the synthesis of carotenoid pigment by *S. elongatus* under the influence of urea, ammonium chloride, sodium nitrate or potassium nitrate as a sole source of nitrogen is given in Figure 2B.

A gradual and stiff increase after 10th day with no decline in the amount of c-phycoerythrin content till 30th day was observed in all the culture flasks semi-continuously supplemented with all four different nitrogen sources containing nutrient media. Maximum c-phycoerythrin content was observed 0.075µg ml⁻¹ under the influence of sodium nitrate while minimum content was observed 0.0276µg ml⁻¹ in urea as a sole source of nitrogen after 30 days. A detailed result on the synthesis of c-phycoerythrin pigment by *S. elongatus* under four different nitrogen sources over a period of 30 days is given in Figure 2C.

A steady increase in the amount of c-allophycocyanin till 30th day was observed in all the culture flasks. The culture flasks supplemented with sodium nitrate or potassium nitrate showed similar kind of results while culture flasks supplemented with ammonium nitrate or urea show low concentration of c-allophycocyanin. Maximum c-allophycocyanin content was observed 0.088µg ml⁻¹ in sodium nitrate, while minimum content was observed 0.0252µg ml⁻¹ in urea after 30th day. A detailed result on the synthesis of c-phycoerythrin pigment by *S. elongatus* under the influence of four different nitrogen sources over a period of 30 days is given in Figure 2D.

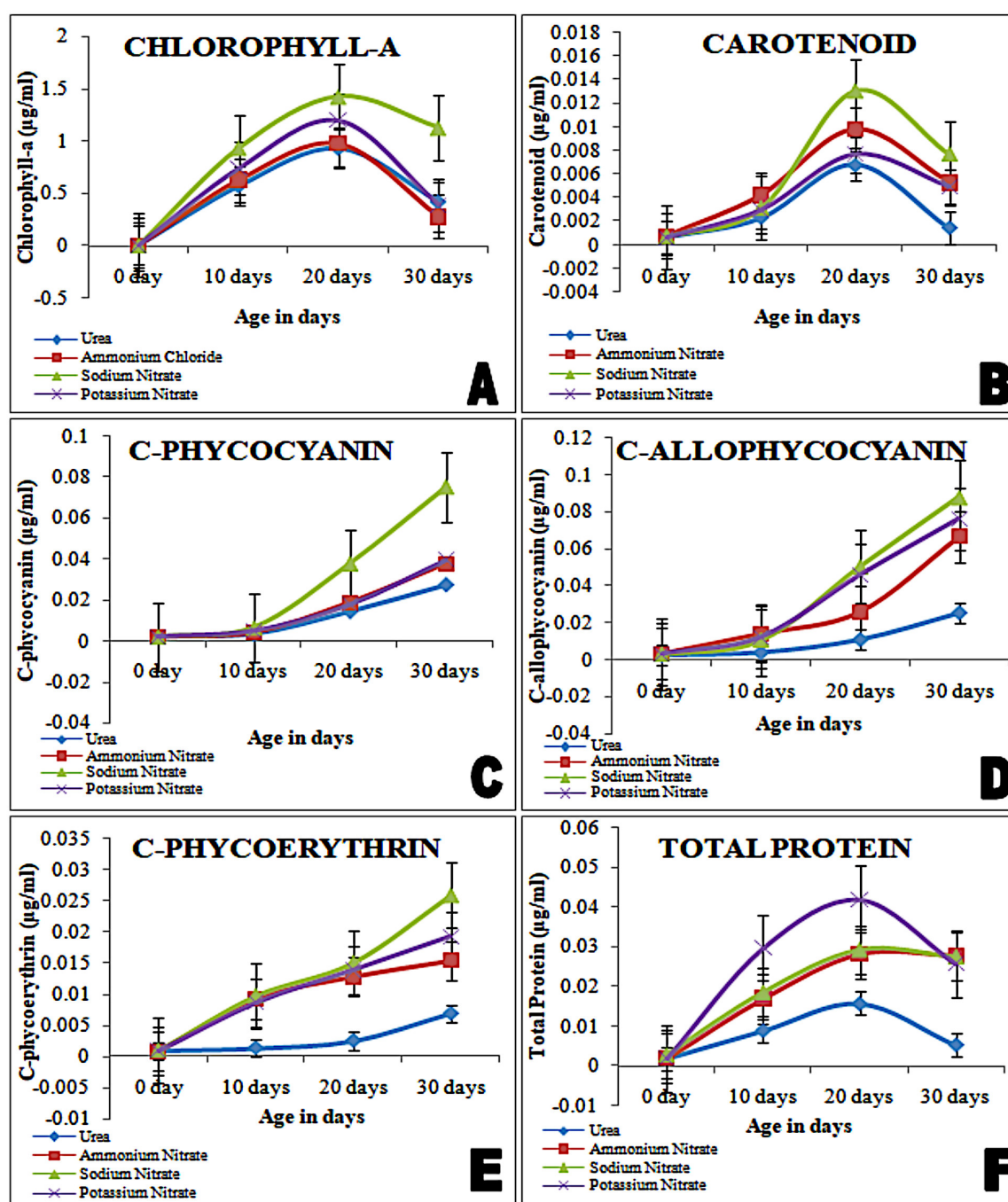


Figure-2(A-G). Figures describing the effect of different nitrogen sources on *S. elongatus* for the synthesis of (A) Chlorophyll-a; (B) Carotenoid; (C) C-phycoerythrin; (D) C-allophycocyanin, (E) C-phycoerythrin and (F) Total Protein.

A steady increase in the amount of c-phycoerythrin content was observed in all the flasks in all the four different nitrogen sources. Culture flasks semi-continuously supplemented with ammonium chloride, sodium nitrate or potassium nitrate show similar results till 20th day. After 2nd harvesting the c-phycoerythrin content in sodium nitrate was more than that of potassium nitrate while the

amount of c-phycoerythrin content in ammonium nitrate was less than that of potassium nitrate. Culture flasks supplemented with urea as a sole nitrogen source showed very low concentration of c-phycoerythrin. Maximum c-phycoerythrin content was observed $0.259\mu\text{g ml}^{-1}$ in sodium nitrate. Minimum content was observed $0.007\mu\text{g ml}^{-1}$ in urea. A detailed result on the synthesis of

c-phycoerythrin under the influence of all the four different nitrogen sources is given in Figure 2E.

A gradual increase in total protein synthesis was observed in all the culture flasks till 20th day while a declining phase was observed in all the flasks after that. Culture flask supplemented with urea showed very low concentration of total protein while high total protein content was observed in the flasks supplemented with potassium nitrate. Culture flasks supplemented with sodium nitrate or potassium nitrate showed similar results indicating that both the nitrogen sources have similar impact on the synthesis of total protein in *S. elongatus* cells. The synthesis of high total protein content under the influence of potassium nitrate indicated that potassium nitrate could boost protein synthesis in *S. elongatus*. Maximum total protein content was observed 0.042 µg ml⁻¹ after 20th day in potassium nitrate. Minimum protein content was observed 0.005 µg ml⁻¹ in urea. A detailed result on total protein content synthesized by *S. elongatus* is given in Figure 2F.

DISCUSSION

For feedstock applications and for biotechnological processes, a strain should be fast-growing, stout, and easy to harvest and have good productivity (Beetul et al., 2016). *S. elongatus*, an euryhaline, unicellular cyanobacterium, an attractive feedstock candidate for biotechnological applications (Pathania & Srivastava, 2021) and is capable of growth under the influence of urea, nitrate, nitrite and ammonium as the primary N source (Pandey et al., 2008; Rabalais et al., 2009; Wawrik et al., 2009; Solomon et al., 2010; Kant et al., 2011; Saito et al., 2014). Kuan et al. (2015) studied growth of *S. elongatus* PCC7942 and revealed that an optimization of components in BG-11 growth medium containing 1.5g L⁻¹ NaNO₃ under an optimal temperature of 33°C and light intensity of 120µmol photons m⁻² s⁻¹ for 12:12h light:dark regime showed a maximum specific growth rate of 0.052 h⁻¹ and a maximum biomass concentration of 0.50 gL⁻¹.

The influence of sodium nitrate, potassium nitrate and ammonium chloride on the growth and phycobilin composition of *Arthrospira platensis*, *Phormidium* sp. and *Pseudoscillatoria* sp. were evaluated in the batch culture period of 12 days (Khazi et al., 2018). *Phormidium* sp. and *Pseudoscillatoria* sp. were able to utilize all tested nitrogen sources; however, ammonium chloride was the best nitrogen source for both strains to achieve maximum growth rate $\mu = 0.284 \pm 0.03$

and $\mu = 0.274 \pm 0.13$ day⁻¹, chlorophyll *a* 16.2 ± 0.5 and 12.2 ± 0.2 mg L⁻¹, and phycobiliprotein contents 19.38 ± 0.09 and $19.99 \pm 0.14\%$ of dry weight, whereas, for *Arthrospira platensis*, the highest growth rate of $\mu = 0.304 \pm 0.0$ day⁻¹, chlorophyll *a* 19.1 ± 0.5 mg L⁻¹, and phycobiliprotein content of $22.27 \pm 0.21\%$ of dry weight were achieved with sodium nitrate.

All nitrogen substrates were suitable for growth; with all species displaying similar growth kinetics when supplied with the same N source (Erratt et al., 2018). However, the highest urea concentration (7mmol-N/L) showed inhibitory properties in two of the species, with lower growth kinetics observed in *Synechococcus* and complete inhibition in *Microcystis aeruginosa*. With the exception of the 7mmol-N/L-urea treatment, NO₃⁻ and urea displayed comparable K values for all species. In contrast, when supplied with NH₄⁺ growth values were halved compared to NO₃⁻ or urea treatments.

Pathania & Srivastava (2021) studied the growth of *S. elongatus* BDU 130192 under three different temp. (30, 34 and 38°C), different NaCl conc., CO₂ in the air and different nitrogen sources viz. NaNO₃, urea, NH₄Cl and found that highest biomass and total carbohydrate accumulation occur at 34°C. From their work they also observed that biomass and total carbohydrate increased with increasing light intensities, while protein, chlorophyll, and carotenoid contents were reduced. They also revealed that *S. elongatus* could grow well until 5.8% NaCl but best growth was observed 1.8% NaCl and 5% CO₂ in the air. Though the cells were able to grow well in urea and NH₄Cl but the best growth was observed under NaNO₃.

The effect of inorganic nitrogen (nitrate and ammonium) and organic nitrogen (urea) on *Microcystis aeruginosa* was studied by Peng et al. (2016) and they observed that chlorophyll-a and phycocyanin were in a trend of rising in the first 12 days with NaNO₃ in the medium while under the influence of urea there was a remarkable decline after a short rise. Chen et al. (2020) studied the effect of urea for biomass production with highest biomass concentration of 8.86g L⁻¹ at 3.81mol L⁻¹ of urea. Chlorophyll degradation was accelerated and primary carotenoid lutein decreased for the first 2 days and then maintained stable in *Chromochloris zofingiensis*.

Cyanobacteria mainly use inorganic compounds like nitrate, ammonium and dinitrogen to fulfill

their N requirements, but urea and other organic sources of N, such as aminoacids, can also be assimilated by some cyanobacteria (Herrero et al., 2001). *Lyngbya* sp. BDU 90901 grown under different nitrate conc., showed an increment in protein, glucose, chlorophyll-a and carotenes mainly in ½, 1 and 2 folds of nitrate than that of 0 fold and it was perceived that the growth and rise in biochemical components were observed in the presence of nitrate when compared to that of the absence of nitrate (Dalavai et al., 2016).

CONCLUSION

From the results obtained it could be concluded that different nitrogen source containing nutrient media could be suitable for growth, biological carbon sequestration and synthesis of natural pigments by the unicellular blue-green alga *S. elongatus*. From the results it could also be concluded that sodium nitrate could be a best source of nitrogen while urea could be toxic. The results also concluded that sodium nitrate in the nutrient medium could proliferate growth rate and lower generation time of *S. elongatus*. Sodium nitrate as a sole nitrogen source in the nutrient media is also responsible for maximum biological carbon sequestration, synthesis of primary photosynthetic pigment chlorophyll-a, photo-protective pigment carotenoids and secondary light harvesting pigment phycobilins. From the results obtained it could also be concluded that potassium nitrate as a sole nitrogen source could be responsible for enhanced production of total protein in *S. elongatus* cells.

Acknowledgement: Authors are thankful to Head, Botany Department, Chaudhary Charan Singh University, Meerut, India for providing necessary facilities. Authors are also thankful to G. L. Tiwari, Retd. Prof. & Head, Botany Department, Allahabad University, Prayagaraj, India for the identification of the experimental organism.

Competing Interests: The authors declare no conflict of interest.

Authors' Contributions: RK developed the idea and elaborated the concept. KS collected the samples and isolated the experimental organism. KS, D, DG and G performed the experiment. KS and RK validated the data. KS wrote the manuscript and RK provided part of the text. RK, MS, NK and NCH edited the manuscript, and finally the manuscript was accepted by all authors and improvement of the manuscript.

Ethical Approval: Not applicable.

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