S. Meenakshi *et al*, International Journal of Advances in Agricultural Science and Technology,
Vol.5 Issue.1, January- 2018, pg. 42-50 ISSN: 2348-1358
Impact Factor: 6.057
NAAS Rating: 3.77

Proteome Analysis of Nitrogen Starvation Responses in Cyanobacteria

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Abstract:

Carbon dioxide is one of the principle pollutant warming the earth. In the global effort to combat the climate change, several CO_2 capture and storage technologies are deliberated. In the past 150 years, anthropogenic activities have pumped enough carbon dioxide into the atmosphere to raise its levels to 400 ppm, higher than they have been for hundreds of thousands of years. Microalgae can provide solutions to the twin challenges of energy security and environmental pollution. Environmental factors play a significant role and control the availability of CO_2 which affect carbondioxide concentrating mechanism activity. In this study, the effects of nitrate feeding on microalgal growth and related CO_2 fixation were evaluated, as an affinity to increase carbon fixation. On nitrate depletion, the growth of cyanobacterial cells declined. The increasing concentration of nitrate from 5 g/L to 20 g/L rapidly increased the biomass of *Phormidium animale* from (0.51 mg/ml) to (0.80 mg/ml) on the 20th day of growth. Low molecular weight protein bands were suppressed in nitrate stress condition due to decrease in metabolism and photosynthesis in cyanobacterial isolates. High intensity protein bands of molecular weight 29 – 30 kDa was noted in higher nitrate concentration but in low nitrate concentrations have less intense bands in *Anabaena* sp. A number of faint bands was noted in *Nostoc* sp. These results indicated that the protein profile in the carbon concentrating mechanism from growth to protein expression of carbonic anhydrase and RuBisCo.

Keywords*:* Cyanobacteria; Carbonic anhydrase; RuBisCo; Carbondioxide; $NaNO_3$.

1. Introduction:

Microalgae are unicellular or simple multicellular photosynthetic microorganisms that have the capability to fix CO_2 from various sources, industrial exhaust gases, with the environment and soluble carbonate ions. The responses of nutrient shortage are of two main types, one is a stimulation of the uptake and assimilation systems for the limiting nutrient, and secondly, a reduction of growth and metabolism to speed them to the available resources and avoid imbalances (Giordano *et al*., 2000). Higher salinity, which results in the lower CO_2 solubility, increases CCM activity in microalgae (Goyal *et al*., 1992).

A major topic of interest in algal technology is how to divert fixed carbon from cellular biomass to products. Utilization of different nitrogen sources first requires their passage through the permeability barrier of the cytoplasmic membrane into the cyanobacterial cell. At low nitrate concentrations, the endergonic uptake of nitrate in freshwater cyanobacteria takes place through ABC-type transporters that exhibit high affinity for nitrate. Studies reported that algae produced a carboxysomal protein, known as carbonic anhydrase and RuBisCo protein, to diffuse from the nitrate stress condition. Passive diffusion of nitrate takes place in *Synecchococcus* sp. Strain PCC 7942 at nitrate concentrations above 1 mM (Nancy *et al*., 2006).

The production of chlorophyll a, protein, photosynthetic rate and algal biomass can be enhanced under environmental stress factors (González *et al*., 2015). In addition to stress factors, selection and use of microalgal species and strains is also important for enhancing the carbon accumulation. Further optimizing culture conditions, by selecting organisms that can overcome the limitations imposed by ambient conditions, and by selecting strains that produce high protein expression can also lower the unit cost of microalgae. Cyanobacteria are capable of growing on a number of different nitrogen sources. This study investigated the

changes in the proteome by using SDS-PAGE in response to carbon concentrating mechanism by the addition of sodium nitrate ($NaNO_3$).

1. Materials and Methods:

2.1. Determination of growth and biomass productivity:

The selected six cyanobacterial isolates (*Synechococcus* sp., *Anabaena* sp., *Fischerella muscicola*, *Phormidium animale*, *Nostoc* sp. and *Synechococcus elongatus*) were cultivated in different concentrations of nitrate (5, 10, 15, 20 to 25 gm / L) (Ernst *et al*., 2005). Each strains was homogenized well and the optical density of each culture was recorded continuously for 30 days at an interval of ten days by measuring the absorbance at 665nm in a HITACHI U-2001 spectrophotometer. A known volume of the isolate was harvested by centrifugation at 5000 g for 15 minutes and the pellet was washed at least twice with distilled water and freeze dried. The dry weight of the cyanobacterial biomass was determined gravimetrically and the growth was expressed in terms of dry weight grams per litre (Dayananda *et al*., 2005). Proliferation rate and generation time of each cyanobacterial isolate was calculated using Huang *et al*., (2002a).

2.2. Estimation of Chlorophyll a :

Cyanobacterial suspension grown in different concentrations of nitrate was centrifuged at 5000 g for 10 minutes. The pellet was washed twice in distilled water. The pellet was suspended in 4ml of 80% methanol and vortexed. To prevent solvent evaporation, the tube was covered with an aluminium foil. It was incubated in a boiling water bath at 60°C for 1 hour in dark. The tube was cooled and centrifuged at 5000 g for 5 minutes. The supernatant was transferred to another tube and extracted with 2ml of the methanol solvent. The supernatants were pooled and made upto 10ml.The extractions were carried out, in dim light. The absorbance was read at 663nm with methanol as a blank (Myers and Kratz, 1995).

2.3. Estimation of Protein:

Cyanobacterial culture (10 ml) grown in different concentrations of nitrate was homogenized and centrifuged at 2000 g for 15 minutes. The cyanobacterial cells were precipitated with 80% ethanol. The cell precipitate was dissolved with 2 ml of analytical reagent. The solutions were mixed well and incubated at room temperature for 10 minutes. Then, 0.2 ml of Folin Ciocalteau reagent was added to each tube and incubated for 30 minutes along with the blank. The reagent was mixed well and allowed to stand for 10 minutes till the development of a blue colour. The absorbance was measured at 650 nm. Standard graph was plotted using bovine serum albumin (Lowry *et al*., 1951).

2.4. Measurement of photosynthetic rate of algal cells:

Cyanobacterial culture (5 ml) grown in different concentrations of nitrate was homogenized and the magnetic stirrer was placed in the sample chamber. Probe was also inserted into the sample chamber. Oxygen consumption rate was recorded for ten minutes. Respiratory rate was measured by covering the chamber with a black cloth and the readings were recorded. Simultaneously, the estimation of chlorophyll *a* was done for 5ml of cyanobacterial culture extracted in 80% of acetone (Clark *et al*., 1956).

2.5. Isolation of Carboxysome:

Exponential phase of cyanobacterial cells grown in different concentrations of nitrate were resuspended in 50 ml of TEMB buffer before they were ruptured by sonication and centrifuged (12,000 g, 20 minutes). The supernatant was collected and centrifuged at 15,000 g for 1 hr. The resulting pellet was resuspended in 200 µl of lysozyme in TEMB and centrifuged (15,000 g for 1 h 30 minutes). The carboxysome-enriched pellet was resuspended in 2 ml of TEMB, clarified by centrifugation (15,000 g, 1 h 30 minutes) and the brownish pelleted carboxysome was collected and recentrifuged at 15,000 g for 90 minutes. The resulting pellet of carboxysome was resuspended in 1 ml of TEMB before RuBisCo and CA activities were assayed. The isolated carboxysome was visualized in a Scanning Electron Microscope (JOEL-JSM 6390). Purified carboxysomes were disrupted by freezing the pelleted carboxysomes at -20°C for 30 minutes. The pellet was subsequently thawed, resuspended in 100 to 200 µl of TEMB at 4°C, and subjected to centrifugation at 14,000 g for 30 mins. The resulting pellet consisted mainly of carboxysome shell "ghosts," while the supernatant contained most of the RuBisCo and carbonic anhydrase that originally had been packaged in the intact particles (So *et al*., 2004).

2.6. Total carboxylase activity of RuBisCo:

Determination of RuBisCo total carboxylase activity (Activated RuBisCo) of cells cultivated in different concentrations of nitrate was undertaken using the method of Gerard and Driscoll, (1996). Carboxysomal protein was isolated in a pre-cooled RuBisCo extraction solution at pH 7.6. Distilled water was used as the

blank and OD values at 340 nm of the mixture was recorded as the zero-value. The reaction was initiated by adding 0.1 mL of ribulose-1, 5-bisphosphate (RuBP) into the reaction cuvette and OD values at 340 nm was recorded every 1 minute for 5 minutes. Absorbance values were converted to NADH concentrations using an extinction coefficient of 6.22 mM^{-1}. Total carboxylase activity of RuBisCo was calculated using the equation 1 of Wang *et al*., (2011).

2.7. *Assay activity of Carbonic anhydrase:*

Carbonic anhydrase activity was determined in 50 µl of isolated carboxysomal protein cultivated in different concentrations of nitrate. Tris sulfate buffer (1.95 ml) and 1ml of PNPA reagent was added to the carboxysomal protein. An increase at A348 nm was recorded for approximately 5 minutes. The absorbance at 48nm/minute was obtained using the maximum linear rate for both the samples and the blank using the equation 2 of Armstrong *et al*., (1996).

2.8. SDS – PAGE electrophoresis:

Effect of different concentrations of nitrate on carboxysomal protein profile was separated by 12% SDS – PAGE as per the protocol of Laemmli, (1970). A mixture of protein standards was used as a molecular marker.

3. Results and Discussion:

As seen in Table. 1, the effect of different nitrate concentrations on the growth rate was clearly observed. Nitrogen constitute upto 11 % of the dry weight of a cyanobacterial cell. Growth rate varied among unicellular and filamentous forms when subjected to nitrate variation. On nitrate depletion, the growth of cyanobacterial cells declined. In this study, physiological and protein profile were monitored in actively growing six cyanobacterial strains namely, *Synechococcus* sp., *Anabaena* sp., *Fischerella muscicola*, *Phormidium animale*, *Nostoc* sp. and *Synechococcus elongatus*. Beardall and Raven, (2013), stated that the cell growth is a basic feature of algal cell biology, which, together with photosynthesis and nutrition supplements, determines the maximal potential of the algal biomass production. Cell growth probably consists of two phases—earlier cell proliferation and later improvement in cell volume, both of which directly contribute to algal biomass accumulation. The increasing concentration of nitrate from 5 g/L to 20 g/L rapidly enhanced the biomass of *Phormidium animale* from (0.51 mg/ml) to (0.80 mg/ml) on the 20^{th} day of growth. The variation in nitrate concentrations has influenced the growth and metabolism of cyanobacterial species (Lardon *et al*., 2009). Similarly the dry weight of *Synechococcus* sp., *Anabaena* sp., *Fischerella muscicola*, *Nostoc* sp. and *Synechococcus elongatus* were maximum in 20 g/L of nitrate concentration and ranged between 0.26 mg/ml to 0.62 mg/ml. Correspondingly, at nitrate concentration of 20g/L, maximum biomass of 0.80 mg/ml was achieved. Thus, it is inferred from the results that high nitrate concentrations stimulated photosynthetic efficiency. This enabled cyanobacteria to reproduce within a shorter period of time with a greater quantity of biomass.

An increase in nitrate concentration enhanced the photosynthetic activity which was reflected in the correlation coefficient analysis for the selected organisms. The nitrate concentration exhibited a significant positive correlation with dry weight, protein, RuBisCo and CA activity, which in turn emphasized the influence of nitrate on cyanobacterial growth. Chlorophyll a content of the cyanobacterial cells increased with an increase in nitrate concentration but these differences were not statistically significant. The activity of the RuBisCo and CA enzyme which brings about the degradation of chlorophyll pigments, increased during the high nitrate stress. Pigment content enhanced as nitrate concentration increased. Light harvesting and electron transport within the photosynthetic apparatus, CO_2 fixation within the Calvin–Benson cycle, and the enzymatic elimination of reactive oxygen species are all dependent on proteins that are rich in nitrogen (Evans and Vogelmann, 1999 and Burnap *et al*., 2015).

Under nitrogen limitation, there was a decrease in the dry weight, chlorophyll a, protein, CA and RuBisCo activity in the cyanobacterial isolates. Similar results were observed by Berges and Falkowski, (1998) and Hopkinson *et al*., (2014), where the concentration of the photosynthetic carboxylation enzyme RuBisCo and the ratio of RuBisCo activity to electron transport activity declined in nitrate depleted conditions. Saxena *et al*., (2006) observed that under nitrogen starvation/limitation there was a reduction of major and accessory photosynthetic pigments, impairment of photosynthesis due to loss of one major RuBisCo isoenzyme, and suppression of polypeptides. Maximum growth, photosynthetic activity and accumulation of protein was achieved in 20 g/L and with a further increase in nitrate concentration to 25 g/L resulted in the reduction of physiological parameters. Chlorophyll a was enhanced in cyanobacterial species when subjected to high nitrate

concentration. These findings were also reported by Andria *et al*., (1999) and Mangan and Brenner, (2014), where the pigment content was significantly higher in nitrate elevated conditions. In the N-limitation experiment, chlorophyll a was greater during the exponential phase of growth and decreased after the onset of N limitation. Reduction in algal growth was noticed when subjecting the algal isolates under nitrate limitation which in turn affected the cell dry weight, chlorophyll a and photosynthetic activity.

Specific activity was calculated for both RuBisCo and CA enzyme using the assay activity and protein content. Higher specific activity of RuBisCo was observed in *Nostoc* sp. (33.62 mmol CO_2/mg/min) and *Fischerella muscicola* (333 WU/mg) (Table 1) for CA activity. Nitrate availability resulted in an increase of protein content in cyanobacterial species selected for this study. Protein played a major role in cellular metabolism, especially during nitrogen-supplemented growth of *Anabaena* (Banerjee *et al*., 2013). Assimilation of nitrate into organic nitrogen compounds depends on photorespiration (Bloom *et al*., 2014). Andria *et al*., (1999) and Cai *et al*., (2015), reported that the maximum rate of photosynthesis was frequently related to RuBisCo activity.

Compared to other concentrations, 20 g/L nitrate was favourable for the growth of cyanobacterial isolates. The effect of nitrate and incubation period on enzyme activity was statistically optimized using two way ANOVA. *Synechococcus* sp. showed significant differences in RuBisCo activity due to varying nitrate concentrations and incubation period. Turpin, (1991) and Hopkinson *et al*., (2014), also observed that the RuBisCo content per cell decreases under N limitation, resulting in a significant decline in the cellular photosynthetic capacity. Correspondingly, with an increase in RuBisCo content for high nitrate concentration (20 g/L), photosynthetic rate was stimulated (0.67 m mol CO2/mg/min at 20 g/L) in *Synechococcus* sp.

The carbonic anhydrase activity increased in *Synechococcus* sp. on exposure to high nitrate concentration of 20 g/L. An increase in CA activity enhanced the transport of bicarbonate inside the cells through transporters and also increased the RuBisCo activity (Omata *et al*., 1999 and Badger and Price, 2003). *Phormidium animale* and *Synechococcus elongatus* also exhibited significant differences. In RuBisCo and CA activity with respect to nitrate concentration but it was not significantly different with the incubation period. Though there was no significant difference on enzyme activity in *Fischerella muscicola* with regard to nitrate content, it was significantly different during various days of growth.

Effect of environmental modulation and incubation period on RuBisCo and CA activity was statistically optimized using two way ANOVA. CA activity also displayed a distinct correlation with nitrate content of the medium.

The partially purified carboxysomal protein pellets from *Synechococcus* sp. yielded several protein bands of approximately 55 kDa, 29 kDa, 14 kDa on SDSPAGE gel. Several faint bands of high and low molecular weight were observed in the carboxysomal membrane protein. Low molecular weight bands were suppressed in nitrate stress condition due to decrease in metabolism and photosynthesis in cyanobacterial isolates. High intensity protein bands of molecular weight 29 – 30 kDa was noted in higher nitrate concentration from 20 – 25 g/L. Low intensity bands of molecular weight 29 – 30 kDa was noticed in *Anabaena* sp. A number of faint bands were noted in *Nostoc* sp. The assay activity results also indicated a lesser CA and RuBisCo activty in both *Anabaena* sp. and *Nostoc* sp. isolates. For lower concentrations of nitrate, a high molecular weight band of 52 – 55 kDa was suppressed. *Phormidium animale* showed high intensity target bands of RuBisCo (55 kDa) and CA (29 kDa) in various nitrate concentrations.

In *Synechococcus elongatus*, in lower nitrate condition, the band intensity was high and the number of bands also increased but at high nitrate concentration, band intensity was low with the same number of bands. The detailed analysis of carboxysomal protein expression in higher nitrate concentration than low nitrate containing cultures confirmed the identities of the 55 kDa large subunit of RuBisCo, 14.4 kDa small subunit of RuBisCo and 30 kDa carbonic anhydrase proteins were confirmed by SDS-PAGE resolution (Figure 1).

In the correlation coefficient analysis, the nitrate concentration exhibited a significant positive correlation with dry weight, protein, RuBisCo and CA activity, which in turn emphasized the influence of nitrate on cyanobacterial growth.

ISSN: 2348-1358
Impact Factor: 6.057
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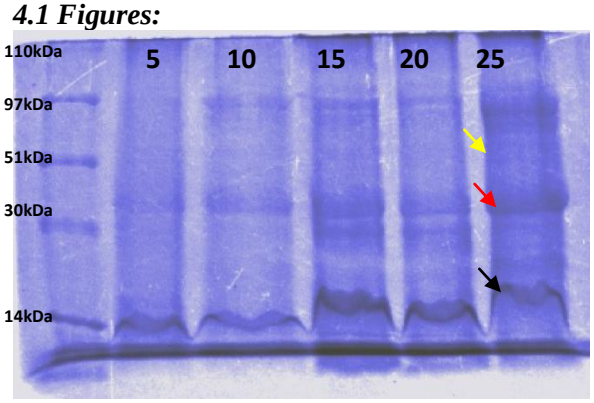
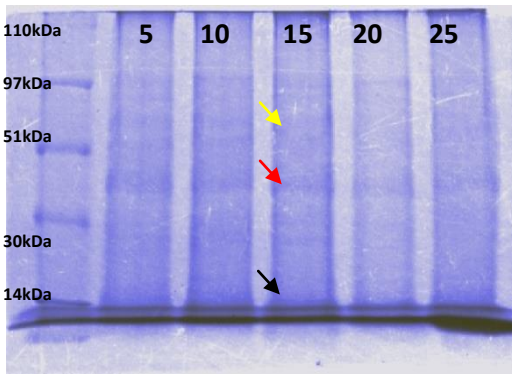
4. Use of Tables, Figures and Equations:

4.1 Figures:



***Synechococcus* sp.**



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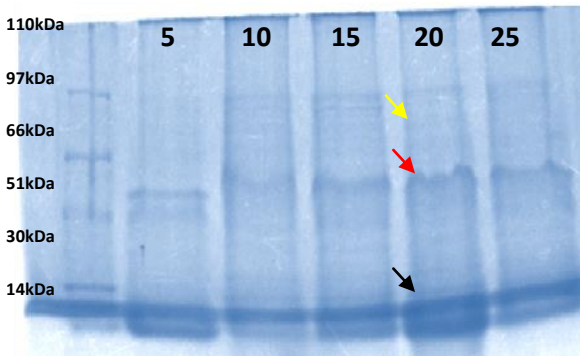
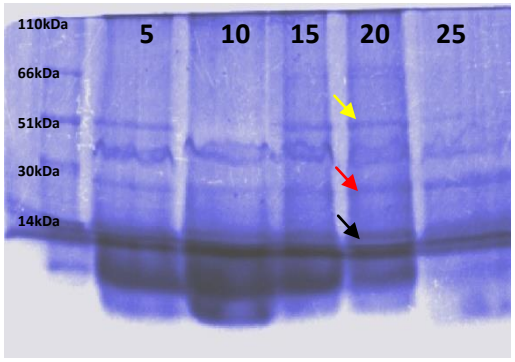
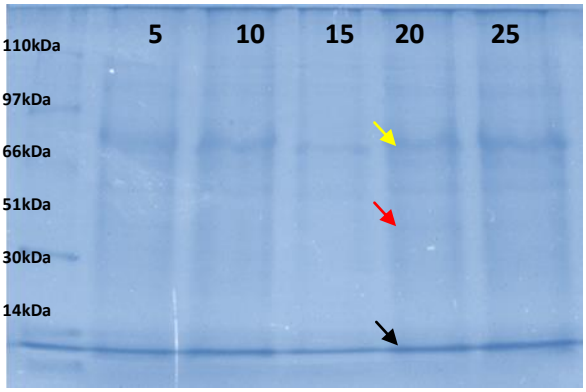


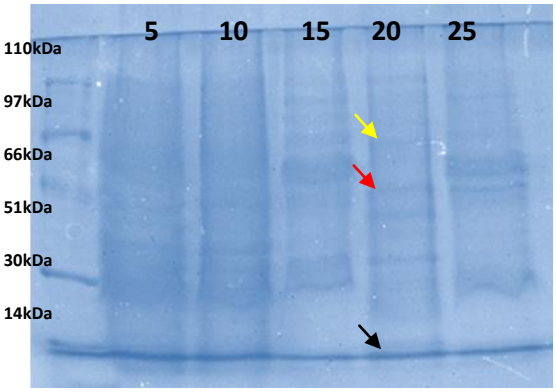
Fischerella muscicola



Phormidium animale



***Nostoc* sp.**



Synechococcus elongatus

Target bands with higher intensity was represented in coloured arrows
Yellow - RuBisCo large subunit (52 - 55kDa)
Red - Carbonic anhydrase (29 - 30kDa)
Black - RuBisCo small subunit (14 – 14.4kDa)

Figure 1: Effect of Nitrate concentrations (g/L) on the protein profile of selected cyanobacteria

ISSN: 2348-1358
Impact Factor: 6.057
NAAS Rating: 3.77

4.2 Tables:

Table 1: Correlation coefficient analysis of the effect of Nitrate on the growth and physiology of cyanobacteria

***Synechococcus* sp.**	**Nitrate**	**Dry weight**	**Chlorophyll a**	**Protein**	**Photosynthetic rate**	**RCA**	**CA**
Nitrate	1						
Dry weight	0.5484	1					
Chlorophyll a	0.0635	0.4923	1				
Protein	-0.3983	-0.0778	-0.3232	1			
Photosynthetic rate	0.7464	0.7115	0.1510	0.2381	1		
RCA	0.2922	0.9346	0.4652	-0.1025	0.4364	1	
CA	0.8906	0.7404	0.4929	-0.3788	0.7919	0.4930	1
***Anabaena* sp.**							
Nitrate	1						
Dry weight	0.5484	1					
Chlorophyll a	0.06352	0.4923	1				
Protein	-0.3983	-0.077	-0.3232	1			
Photosynthetic rate	0.7464	0.7115	0.1510	0.2381	1		
RCA	0.29225	0.9346	0.4652	-0.1025	0.4364	1	
CA	0.89068	0.7404	0.4929	-0.378	0.7919	0.493	1
Fischerella muscicola							
Nitrate	1						
Dry weight	0.2747	1					
Chlorophyll a	0.5321	-0.0555	1				
Protein	0.7550	-0.1529	0.9186	1			
Photosynthetic rate	0.9132	-0.0970	0.7136	0.9223	1		
RCA	0.4593	-0.5208	0.4139	0.6541	0.6540	1	
CA	-0.43079	0.3215	-0.6174	-0.643	-0.6434	-0.117	1
Phormidium animale							
Nitrate	1						
Dry weight	0.7298	1					
Chlorophyll a	0.5910	0.1835	1				
Protein	0.7951	0.6005	0.8833	1			
Photosynthetic rate	0.5384	0.6903	0.4762	0.7809	1		
RCA	0.2706	0.3151	0.7721	0.7919	0.7134	1	
CA	-0.2820	-0.6303	0.5851	0.1906	-0.0892	0.5043	1
***Nostoc* sp.**							

ISSN: 2348-1358
Impact Factor: 6.057
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Nitrate	1						
Dry weight	0.32	1					
Chlorophyll a	0.70	0.4289	1				
Protein	-0.35	0.6548	-0.233	1			
Photosynthetic rate	0.50	0.7193	0.6011	0.4984	1		
RCA	-0.031	0.8993	0.1023	0.9187	0.6790	1	
CA	0.593	0.8065	0.6382	0.4709	0.9814	0.705	1
Synechococcus elongatus							
Nitrate	1						
Dry weight	-0.69	1					
Chlorophyll a	0.082	-0.602	1				
Protein	-0.56	0.2586	0.6069	1			
Photosynthetic rate	0.569	-0.0600	0.0834	0.0052	1		
RCA	0.058	0.3467	0.1408	0.4978	0.8203	1	
CA	0.863	-0.719	0.4786	-0.1508	0.7358	0.345	1

Critical value at 1% level Positive Correlation = 0.8 — **RCA – RuBisCo carboxylase activity**
Critical value at 5% level Positive Correlation = 0.7 — **RAS – RuBisCo activation State**
Negative Correlation — **CA – Carbonic anhydrase**

Table 2: Activity of RuBisCo and Carbonic anhydrase enzyme

Cyanobacteria	RuBisCo activity (mmol CO_2/mg/min)	RuBisCo specific activity (mmol CO_2/mg/min)
	Nitrate concentration (20g/L)	
***Synechococcus* sp.**	5.545	18.48
***Anabaena* sp.**	3.565	17.82
Fischerella muscicola	2.226	11.71
Phormidium animale	4.062	14.50
***Nostoc* sp.**	4.708	33.62
Synechococcus elongatus	7.745	23.46
Cyanobacteria	**Total CA activity (Units)**	**Specific activity of CA (Units/mg)**
***Synechococcus* sp.**	9566	291
***Anabaena* sp.**	5848	167
Fischerella muscicola	6046	333
Phormidium animale	3943	199
***Nostoc* sp.**	587	43
Synechococcus elongatus	6543	293

4.3 Equations:

$$TCA = (10N \times \Delta OD) / (6{:}22 \times 2d\Delta t) \quad (1)$$

TCA: total carboxylase activity of RuBisCo (mmol CO_2 $mL^{-1}min^{-1}$); ΔOD: the margin of the OD value changed in the first minute; 6.22: the light density per mmol NADH at 340 nm; N: the dilution factor; d: the

diameter of the reaction cuvette (cm); Δt: the time (5 minutes). The activation state of RuBisCo or percent activation (Perchorowicz *et al*., 1981) was determined by the ratio of initial to total RuBisCo activities.

$$\text{Units/mg enzyme} = \frac{(\text{A348 nm/ min Test} - \text{A348 nm/ min Blank})\ (1000)}{(5.0)\ (\text{mg enzyme/ml RM})} \quad (2)$$

1000	=	Conversion to micromoles
5	=	Millimolar extinction coefficient of p- nitrophenol at pH 7.6 at 0°C
RM	=	Reaction Mix

5. Conclusion:

The results of the present study showed that the freshwater *Synechococcus* sp. is able to grow in high nitrate concentrations as well as withstand nitrogen limitation up to 30 days. Sequential nitrate limitation for 20th day was the best approach to enhance pigment, photosynthetic rate, RuBisCo and CA in *Synechococcus* sp. The utility of SDS-PAGE profiles of carboxysomal proteins is a good phenotypic tool for distinguishing protein expression under stress condition. The findings therefore have importance not only for understanding of the regulation of carbon accumulation in carboxysome, but also for biochemical strategies to develop microalgae-based integrated carbon sequestration and oxygen production. The results of this analysis will be helpful to apply evaluating the feasibility of anthropogenic CO_2 sequestration in the potential polluted zones.

Acknowledgement:

This work was financially supported, by Women Scientist Scheme (Basic Research Area) from Department of Science and technology (DST), New Delhi.

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 ISSN: 2348-1358
Impact Factor: 6.057
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