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GROWTH PERFORMANCE AND BIOCHEMICAL ANALYSIS OF *LYNGBYA* SP. BDU 90901 UNDER DIFFERENT NITRATE CONCENTRATIONS

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Abstract: The present research work was carried out for assessing the optimum culture conditions for the growth and chemical constituents of *Lyngbya* sp. BDU 90901. The blue green bacteria belonging to Cyanobacteria (*Lyngbya* sp. BDU 90901) was obtained from NFMC, Bharatidasan University, exposed to different nutrient concentrations of nitrogen to claim the biologically effect of proteins, glucose and pigmentation. Growth medium of ASN III was used for vegetative control growth, while experimental growth was performed under different nitrate concentrations. The applied concentrations were 0, ½, 1, 1½ and 2 folds of nitrate concentrations of original ASNIII medium. The metabolites considered for biochemical analysis were pigmentation, protein and glucose content. Optical density was considered for growth parameter and so deliberated from 0^{th} day to 10^{th} day and intervals of 5 days up till 30^{th} day. Under different nitrate concentrations, on 15^{th} day, the rise in chlorophyll-a and carotenes was noticed in 1 and 2 folds respectively, the increment in protein content was 1 fold, the glucose content was high in ½ and 1 fold on the 20^{th} day.

Key words: Marine *Cyanobacteria*, nitrate availability, glucose, proteins and pigment analysis.

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Introduction

Cyanobacteria is the major group of bacteria that occurs throughout the world. They are also known as blue-green algae. These photosynthetic prokaryotes are found in almost aquatic and terrestrial environment (Castenholz & Waterbury 1989).They show specify growth pattern in a specific environment and therefore the distribution, ecology, periodicity, qualitative and quantitative occurrence of *Cyanobacteria* differ widely. They are pioneer oxygen prototroph on earth whose distribution around the world is surpassed only by bacteria. Their diversity ranges from unicellular to multicellular, coccoid to branched filaments, nearly colorless to intensely pigmented, autotrophic to heterotrophic, psychrophilic to thermophilic, acidophilic to alkylophilic, planktonic to barophilic, fresh water to marine including hyper saline (Yoo et al. 1995, Broady 1996, Thajuddin & Subramanian 2005). However their abilities to survive under adverse conditions by forming resting spores, opportunistically colonizing microhabitats and surviving under conditions of high UV-flux through production of UV - absorbing

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pigments, has made them one of the most successful life forms on Earth. It can flourish well either in nutrient rich and warm water or at times in water with apparently low nutrient concentrations, subjected to high temperature and bright light conditions (Bhatnagar 2008). *Cyanobacteria* offer a greatest opportunity as these are considered to be one of the potential organisms useful to mankind in many ways. Blue green bacteria (BGA) have simple metabolic requirement and occupy a wide range of ecological niche. In global programs, the focus has been identifying their antifungal, antiviral, antimitotic, antihelmienthic, anticoagulant, haemagglutinating and toxic metabolites (Kumar et al. 2003, Thajuddin & Subramanian 2005, Ali et al. 2008). The extensively utilized pigments in bioindustry, are the phycobiliproteins, which account for about 20% of total dry weight of many *Cyanobacteria* (Prasanna et al. 2007). Carotenoids are the most common, naturally occurring terpenoid pigments. They carry out important functions in photosynthesis, nutrition and protection against oxidative damage. Most common carotenoids in cyanobacteria are β-carotene, zeaxanthin, ketocarotenoid, echinenone and myxoxanthophyll (Britton et al. 2008). *Cyanobacteria* that produce numerous chemicals including nitrogen containing compounds, polyketides, lipopeptides, cyclic peptides and many others (Shimizu 2003). Several strains of *Cyanobacteria* were found to accumulate polyhydroxyalkanoates, which can be used as a substitute for non biodegradable petrochemical- based plastics. Recent studies showed that oil polluted sites are rich in cyanobacterial consortia capable of degrading oil components (Kumar et al. 2009, Thenmozhi et al. 2011). *Cyanobacteria* are an extremely diverse group of gram negative prokaryotes showing diversity in physiology, morphology, developmental characteristics and habitats (Whitten et al. 2000). *Cyanobacteria* photosynthesis provides oxygen, a key electron acceptor to the pollutant degrading mechanism (Subashchandrabose et al. 2011). *Cyanobacteria* show effective bioremediation of metals (Kumar et al. 2011, Rajeshwari et al. 2011) aromatic compounds (Kumar et al. 2009) organic or recalcitrant pollutants, achieving enhanced rates of degradation and ensuring better survival. Most *Cyanobacteria* are obligate photoautotrophs, but some species can grow as heterotrophs in the dark at the expense of glucose, fructose or sucrose. Under anaerobic conditions, some species can perform lactate fermentation (Oren & Shilo 1979). Nitrogen fixation occurs both in heterocystous *Cyanobacteria* and in some non-heterocystous *Cyanobacteria*. To avoid contact of nitrogenase with oxygen (and then its permanent inactivation) these latter *Cyanobacteria* adopt a temporal separation between the photosynthetic and the nitrogen fixation processes (Bergmann et al. 1997). Increased respiration rates allow to control the oxygen concentration inside the cell, due to diffusion, necessary to carry out cell metabolism. In heterocystous forms, the nitrogen fixation process is spatially separated from the oxygenic photosynthesis. Nitrogen fixation is carried out in specialized cells, the heterocysts (Adams & Duggan 1999). These have many characteristics that allow to reduce diffusion of oxygen, such as a thick cell wall surrounded by a complex external envelope and a reorganization of the photosynthetic apparatus: lack of PS II to avoid internal oxygen production, presence of PS I to obtain ATP through cyclic photophosphorylation. Reducing power is obtained from vegetative cells in the form of sugars. Molecular nitrogen is fixed into ammonia and immediately converted to organic form, usually as glutamine. As nitrogen fixation is a very energy - consuming process,

nitrogenase is produced and heterocysts are differentiated only in the absence of combined nitrogen in the environment surrounding the cell.

Material and methods

The marine cyanobacterial culture (*Lyngbya* sp. BDU 90901) was procured from National Facility of Marine Cyanobacteria (NFMC), Bharatidasan University, Tiruchirapalli, Tamil Nadu. The pure cultures were maintained on ASNIII medium (Table 1) at 24±2°C under a light intensity of 1500 lux and light and dark cycles of 16:8 hrs. Then the cultures were inoculated in ASNIII medium under different nitrate concentrations 0N (0g/L), ½N (0.75g/L), 1N (1.5g/L), 1½N (2.25g/L) and 2N (3.0g/L) medium at 24±2°C under a light intensity of 1500 lux and light and dark cycles.

Table 1 Composition of ASNIII Medium (g/l)

S.No.	Chemical Component	g/L
1	$NaCl_2$	2.5
2	$MgCl_2.6H_2O$	2
3	KCl	0.5
4	**$NaNO_3$** *	-
5	$K_2HPO_4.3H_2O$	0.02
6	$MgSO_4.H_2O$	3.5
7	$CaCl_2.2H_2O$	0.5
8	Citric acid	0.003
9	Ferric ammonium citrate	0.003
10	EDTA(disodium salt)	0.00055
11	$NaNO_3$	0.02
12	Trace metal mix	1ml
13	Distilled water	1000ml
14	pH	7.5
	Trace metal mix (g/l):-	
1	H_3BO_3	2.86
2	$MnCl_2.4H_2O$	1.81
3	$ZnSO_4.7H_2O$	0.222
4	$Na_2MoO4.2H_2O$	0.39
5	$CuSO_4.5H_2O$	0.079
6	$Co(NO_3)_2.6H_2O$	0.0494

Note - * mark indicates **$NaNO_3$** was added in different concentrations in the ASN III medium.

Estimation of Chlorophyll-a and Carotenoids (Sinetova et al. 2012). A volume of 1ml of cyanobacterial culture was taken in centrifuge tubes. Cells were centrifuged at 15000rpm at laboratory temperature for 7 minutes and thoroughly supernatant was discarded. 1ml of methanol was added and the sample was homogenized by vortexing and the samples were incubated at 4°C for 7 minutes. The samples were centrifuged at 15000 rpm for 7 minutes the pellet was bluish purple colour. Triplicates were maintained for calculations of average and standard deviations. The pigment concentration was measured by using methanol as blank by spectrophotometer at 420nm, 665nm and 720nm.

The concentration of chlorophyll-a and carotenoids was calculated by using the formula,

Chl a [µg/ml] = 12.9447 (A_{665} - A_{720})

Carotenoids [µg/ml] = [1000 (A_{470} - A_{720}) - 2.86 (Chl a [µg/ml] / 221

Estimation of glucose content (Plumner 1987). A volume of 1ml of cyanobacterial culture was taken in a test tube, 2ml of distilled water and 1ml of dinitrosalicyclic acid reagent was added. The test tubes were boiled for 5 minutes in hot water bath. The mixture was cooled to room temperature and the level of extinction was measured at 540nm. The total glucose content was calculated using a standard curve established with a glucose solution.

Estimation of total soluble protein content (Lowry et al. 1951; Herbert et al. 1971). Preparation of reagents:

A. 1N sodium hydroxide solution

B. (i) 5% sodium carbonate solution
   (ii) 0.5% copper sulphate solution in 1% sodium potassium tartrate.
   2ml of reagent B (ii) was mixed with 50ml of freshly prepared reagent B (i).

C. 1N Folin - Ceiocalteau reagent

A volume of 0.5 ml of homogenized cyanobacterial suspension was taken in test tubes. 0.5 ml of reagent A was added and boiled for 10minutes and cooled in running tap water. 2.5 ml of reagent B was added in each tubes and incubated at room temperature for 10 minutes. 0.5 ml of regent C was added and the tubes were kept at room temperature for 15 minutes. The intensity of blue colour was read as absorbance at 650nm using appropriate blank. The protein content was estimated using standard calibration curve prepared from bovine serum albumin and expressed in terms of mg/ml.

Results and discussion

The *Lyngbya* sp. BDU90901 is a filamentous nitrogen fixing, marine *Cyanobacteria.* In marine environments living organisms are exposed to nutrient limitation, light intensity and quality, temperature, pH, salinity, draught, pollution etc. Among the nutrients, nitrogen is an essential major element required for the synthesis of various components. Nitrogen is a critical nutrient required for the growth of all organisms. The biological substances such as peptides, proteins, enzymes, chlorophylls, energy transfer molecules (ADP, ATP) and genetic materials such as RNA, DNA contain organic nitrogen (Harisson et al. 1990). Algae play an important role in converting inorganic nitrogen to its organic form through assimilation. Nitrate and nitrite undergoes reduction with the assistance of nitrate reductase and nitrite reductase, respectively. Most *Cyanobacteria* are nitrogen fixers, converting atmospheric nitrogen to ammonia via nitrogenase enzyme (Postgate 1987). The element nitrogen constitutes about 5-10% of the dry weight of a cyanobacterial cell. Nitrogenase reduces nitrogen into ammonia which in turn is metabolized to glutamine in the presence of enzyme glutamine synthetase. Now this glutamine is then transported from heterocyst to the adjacent vegetative cell, where it is converted into glutamate by the enzyme glutamine oxoglutarate aminotransferase. Both glutamine and glutamate, by various transamination reactions from other aminoacids. The fixed nitrogen may be utilized in number of ways like it is assimilated by the *Cyanobacteria* themselves. Soluble

nitrogenous compounds are liberated from healthy cells into the culture medium and after death the nitrogenous compounds are broken down into ammonia, which is eventually converted into nitrate by nitrifying bacteria. During development of heterocyst three DNA excision events take place that allows expression of nitrogen fixing genes. *Cyanobacteria* are versatile tetrapyrrole synthesizers that are able to produce end products representing all major branches of the tetrapyrrole biosynthetic pathway: hemes, chlorophylls, phycobilins and siroheme. *Cyanobacteria* have an elaborated and highly organized system of internal membranes which function in photosynthesis (thylakoids) (Stainer & Cohen – Bazire 1977; Castenholz & Waterbury, 1989). The lipophilic pigments chlorophyll-a (both reaction centers and antenna) and photosynthetic carotenoids are located within the thylakoids, while the hydrophilic antenna pigments are located in the phycobilisomes which are attached to the outside of the thylakoid membranes. Carotenoids comprise the largest class of naturally occurring pigments in organisms. More than 640 carotenoids have been identified to date. Carotenoids in *Cyanobacteria* have two main functions: they serve as light-harvesting pigments in photosynthesis and they protect against photooxidative damage. The reserve carbohydrate is glycogen (Stainer & Cohen – Bazire 1977. Castenholz & Waterbury 1989). *Cyanobacteria* also contain cyanophycin, a nitrogen reserve polymer made of arginine and aspartic acid, polyphosphate granules and carboxysomes, that are a cell reserve of the photosynthesis key enzyme rubisco (ribulose1, 5- phosphate carboxylase). Some *Cyanobacteria* also contain poly beta hydroxyl butyrate granules. Photosynthesis in *Cyanobacteria* uses water as an electron donor and produces oxygen as a byproduct. The marine *Cyanobacteria*, *Lyngbya* sp. BDU 90901 pure cultures were maintained in different nitrate concentrations of ASNIII medium (Table 1), on 0^{th} day (Fig. 1), 5^{th} day (Fig. 2), 10^{th} day (Fig. 3), 15^{th} day (Fig. 4), 20^{th} day (Fig. 5), 25^{th} day (Fig. 6) & 30^{th} day (Fig. 7), revealed that, the increment in protein content was observed in 1 fold on 15^{th} day (Fig. 11), the glucose content was high in ½ and 1 fold on 20^{th} day (Fig.10), the rise in chlorophyll-a (Fig. 8) and carotenes (Fig. 9) was noticed in 1 and 2 folds on 15^{th} day respectively. Nitrate is probably the most abundant source of combined nitrogen for cyanobacterial nutrition. The assimilation of nitrate by *Cyanobacteria* involves nitrate uptake and reduction of intracellular nitrate via nitrite to ammonium, which is the N form incorporated into organic compounds. Nitrite, which can also fulfill the N requirement of *Cyanobacteria*, is taken up into the cell and then reduced to the level of ammonium. Many *Cyanobacteria* are able to grow at the expense of atmospheric nitrogen under aerobic conditions and many more are able to perform nitrogen fixation when anaerobic conditions are provided experimentally. Given the widespread distribution in nature of these microorganisms, it is believed that *Cyanobacteria* contribute significantly to the process of biological nitrogen fixation and thus participate in restoring to the soil combined- nitrogen lost through denitrification.

Conclusions

The *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*. The availability of nitrogen is a key factor in regulating survival of *Cyanobacteria.* The present work concluded that the *Lyngbya* sp. *BDU* 90901grown under different nitrate concentrations,

showed an increment in protein, glucose, chlorophyll-a and carotenes mainly in ½, 1 and 2 folds of nitrate than that of 0 fold. So it is easy to perceive that the growth and rise in biochemical components was observed in the presence of nitrate when compared to that of the absence of nitrate. Thus this study indicates that the nitrogen is a vital element for the growth of *Lyngbya* even it fixes nitrogen.

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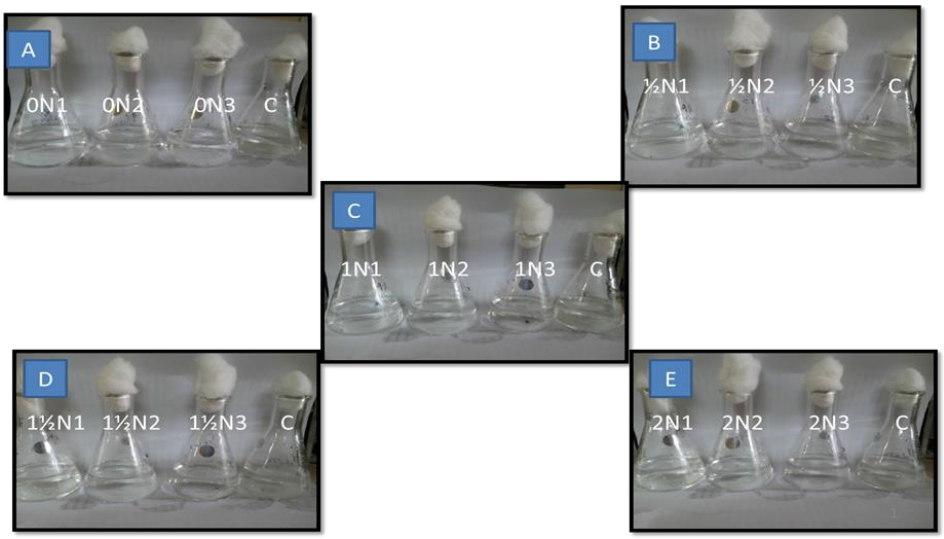

Fig. 1. Growth of *Lyngbya* sp. BDU90901 under different nitrogen concentrations at **0th day culture**. **A**- 0N, **B**-½N, **C**- 1N, **D**-1½N and **E**-2N respectively.

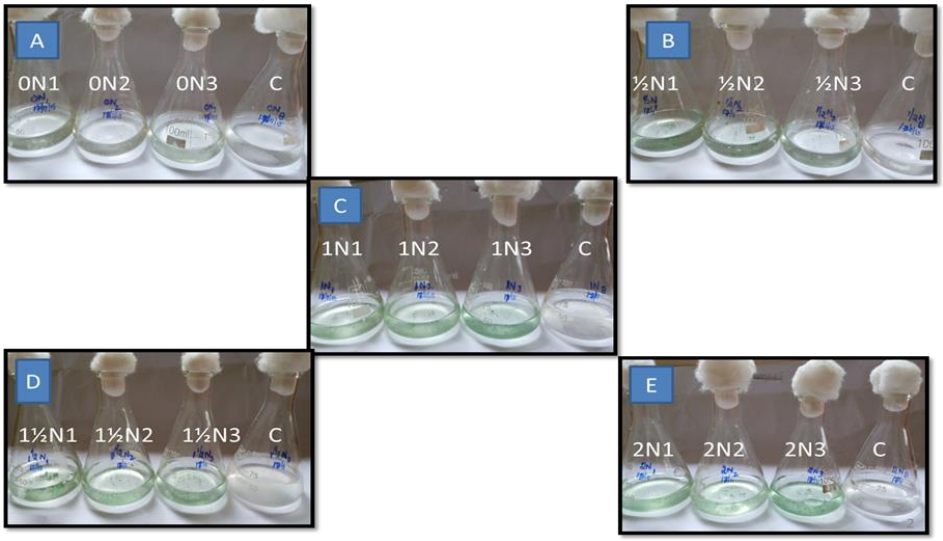

Fig. 2. Growth of *Lyngbya sp.* BDU90901 under different nitrogen concentrations at **5th day culture. A**- 0N, **B**-½N, **C**- 1N, **D**-1½N and **E**-2N respectively.

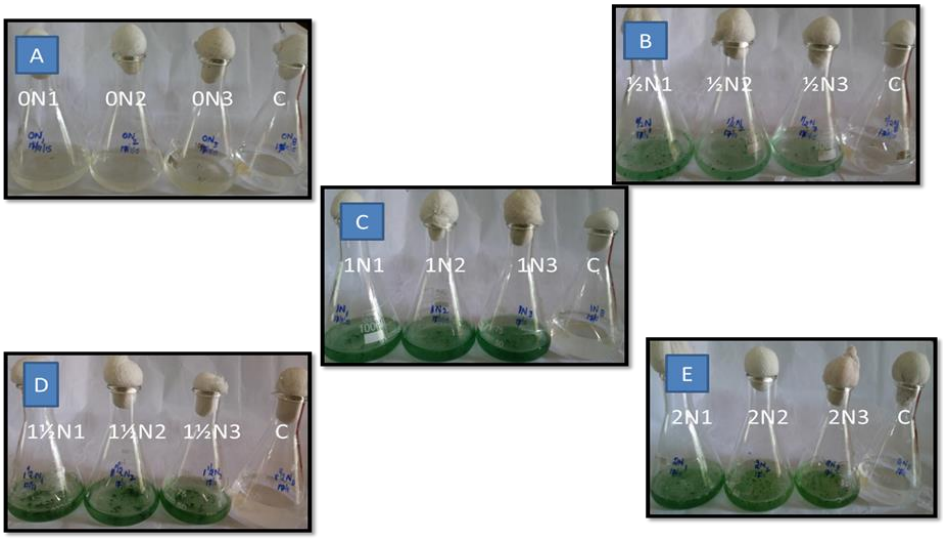

Fig. 3. Growth of *Lyngbya* sp. BDU90901 under different nitrogen concentrations at **10th day culture. A**- 0N, **B**-½N, **C**- 1N, **D**-1½N and **E**-2N respectively.

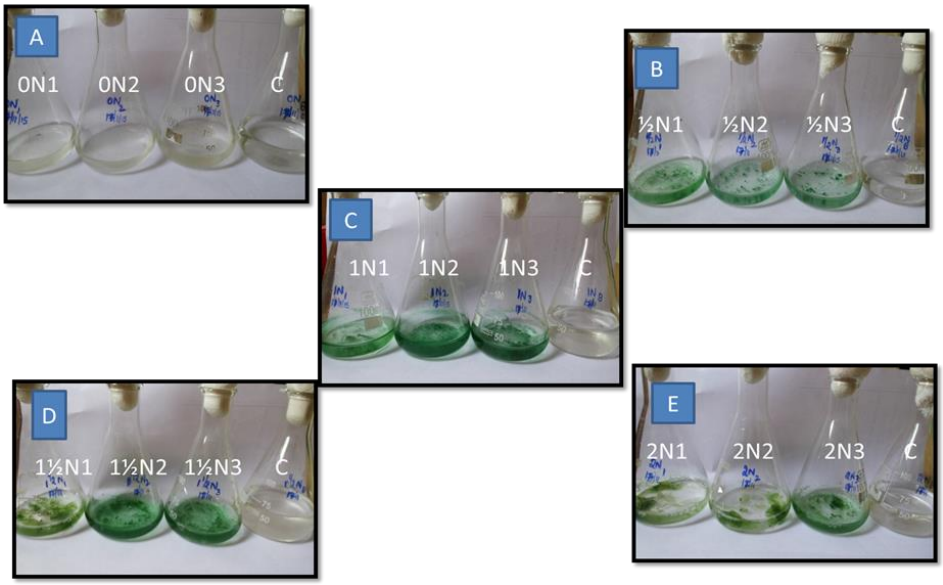

Fig. 4. Growth of *Lyngbya* sp. BDU90901 under different nitrate concentrations at **15th day culture. A**- 0N, **B**-½N, **C**- 1N, **D**-1½N and **E**-2N respectively.

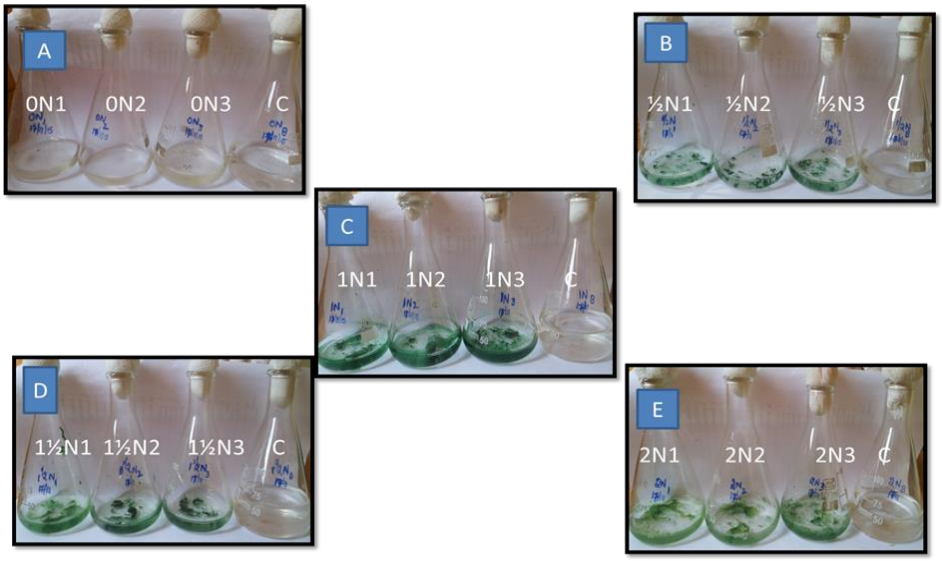

Fig. 5. Growth of *Lyngbya* sp. BDU90901 under different nitrate concentrations at **20th day culture. A**- 0N, **B**-½N, **C**- 1N, **D**-1½N and **E**-2N respectively.

Fig. 6. Growth of *Lyngbya* sp. BDU90901 under different nitrate concentrations at **25th day culture. A**- 0N, **B**-½N, **C**- 1N, **D**-1½N and **E**-2N respectively.

Fig. 7. Growth of *Lyngbya* sp. BDU90901 under different nitrate concentrations at **30th day culture. A**- 0N, **B**-½N, **C**- 1N, **D**-1½N and **E**-2N respectively.

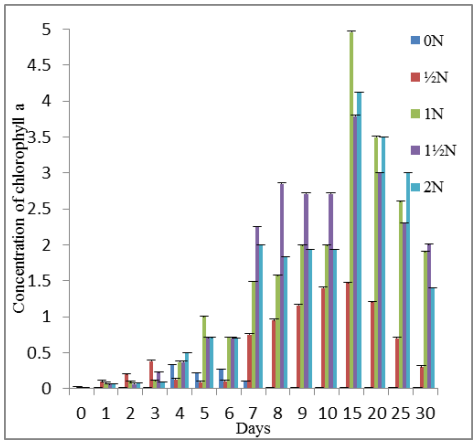

Fig. 8. Estimation of chlorophyll –a from *Lyngbya* sp. BDU 90901 under different nitrate concentrations for 30 days of incubation.

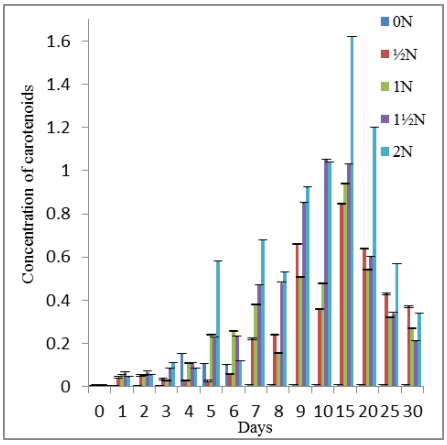

Fig. 9. Estimation of carotenoids from *Lyngbya* sp. BDU 90901 under different nitrate concentrations for 30 days of incubation.

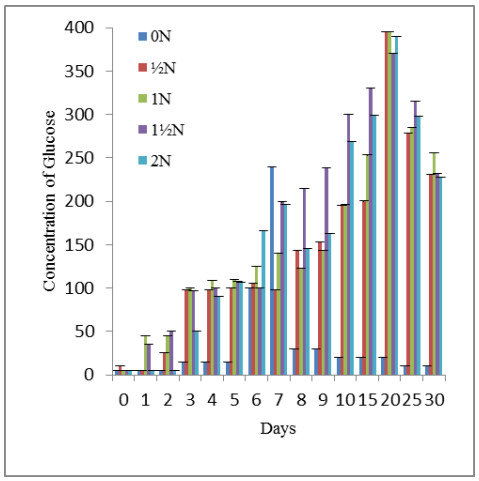

Fig. 10. Estimation of glucose from *Lyngbya* sp. BDU 90901 under different nitrate concentrations for 30 days of incubation.

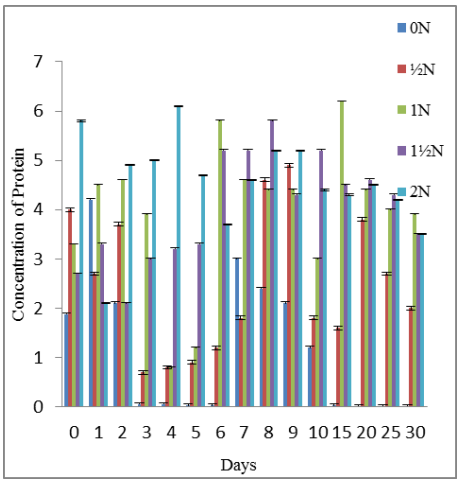

Fig. 11. Estimation of protein from *Lyngbya* sp. BDU 90901 under different nitrate concentrations for 30 days of incubation.