Research Article

Study of Cellular Constituents and Enzymatic Activity of Cyanobacterial Strains

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Abstract

Five cyanobacterial cultures *viz*. *Anabaena variabilis*, *Nostoc muscorum*, *Westelliopsis prolifica*, *Aulosira fertilissima* and *Tolypothrix tenuis* were analyzed for their physiological activity in terms of soluble proteins, total sugars, nitrogenase activity (ARA), nitrate reductase (NR) activity and glutamine synthetase (GS) activity. These cultures were grown in BG-11medium and their activities were analysed at 7, 14, 21 and 28 days of incubation. Significant differences were observed among the cultures with regard to the activities of enzymes involved in nitrogen metabolism. Comparative analysis indicated that parameters soluble proteins, total sugars and nitrate reductase activity were highest in *Aulosira fertilissima* and the highest values for nitrogenase activity on chlorophyll and GS activity were recorded in *Nostoc muscorum.* Highest nitrogenase activity, nitrate reductase activity, glutamine synthetase activity and soluble proteins were observed at 14th day of incubation in all the cases, followed by a gradual decline whereas total sugars were maximum on 21st day of incubation.

Keywords: Cyanobacteria, Nitrogenase activity, Nitrate reductase (NR) activity, Glutamine synthetase (GS) activity

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Introduction

Blue green algea (BGA) or Cyanobacteria are the photoautotrophic prokaryotes which are known as predominant diazotrophs in wetland rice systems and play a positive role in the maintenance of the nitrogen status of rice fields (De, 1939). Venkataraman (1966) initiated the work on the algalization of the Indian rice fields. It was reported that the supplementation of chemical fertilizer with blue green algae could conserve up to 30% of commercial chemical fertilizers (Venkataraman, 1972, 1981). They are known to fix an average of 27 kg N ha^{-1} in N-free plots in rice ecosystems (Roger and Ladha, 1992; Carreres *et al.*, 1996). Although they preferentially utilize fixed nitrogen and express nitrogenase only in the absence of a suitable source of combined nitrogen, they also utilize a number of assimilatory pathways for assimilating extracellular N-sources to cellular N containing components (Flores and Herrero, 1994). Their ability to regulate the expression of different enzymes (GS and NR) are highly specific. Under the conditions of ammonia limitation, blue green algae use glutamine synthetase and glutamate synthase assimilatory pathway to assimilate ammonia (Miflin and Lea, 1976; Thomas *et al.*, 1975; Wolk *et al.*, 1976). Analysis of enzymes related to nitrogen-assimilation in cyanobacteria may also provide valuable pointers regarding their utility as biofertilizers. Cyanobacteria are also widely used in food industries and in few biotechnological applications (Fatma *et al*., 1994; Thajuddin and Subramanian, 2005). They are known to produce a wealth of high-value bioproducts and have been mass cultivated for centuries as a nutritional supplement (Abed *et al.*, 2009). They store reserve food materials which can be used as the source of proteins, lipids, vitamins, pigments, and certain secondary metabolites (Tan, 2007; Cardozo *et al.*, 2007). Cyanobacterial protein has received worldwide attention for either as food supplement or as an alternative source of food. Some species of *Spirulina*, *Nostoc* and *Anabaena* are consumed as food due to their high protein and fibre content (Anusuya *et al.*, 1981). Cyanobacteria are also known to accumulate starch or glycogen and it was found that its content varies from 10 to 50% of their biomass, depending on the strain and growth conditions. (John *et al.*, 2011). These photosynthetic biomass can be used as a promising resource for the generation of bioethanol, biofuels and other valuable bio products. (Allen, 1984; Ball and Morell, 2003; Ball *et al.*, 2011; Mamo *et al*., 2013; Aikawa *et al.*, 2013). Considering the immence physiological diversity existing in these group of photoautotrophic microorganisms, the present study was undertaken to analyze the nitrogenase activity (ARA), nitrate reductase (NR) activity, glutamine synthetase (GS) activity, soluble proteins and total sugars of the five cyanobacterial species were studied.

Material and Methods

Cultures and growth condition

Five heterocystous blue green algae (BGA) cultures namely *Anabaena variabilis*, *Nostoc muscorum*, *Westelliopsis prolifica*, *Aulosira fertilissima* and *Tolypothrix tenuis* were obtained from Culture Collection of Cyanobacteria at CCUBGA, Indian Agricultural Research Institute, New Delhi, India and maintained and grown in N-free BG-11 medium at 28± 2^{o}C with 16/8 h L/D cycles at 2500–3000 Lux light intensity. The experimental flasks (in triplicated) were inoculated with 2% inoculum of 7 days old actively growing cultures of BGA and their nitrogenase activity (ARA), nitrate reductase (NR) activity, glutamine synthetase (GS) activity, soluble proteins and total sugars of respective cultures was measured periodically at 7 days interval up to 28 days.

Biochemical analysis

Soluble proteins (µg/mg dry weight)

Soluble protein content was estimated by Lowry *et al.* (1951) method. A known volume (0.5 mL) of homogenized algal cell suspension was taken in a test tube. To this, 0.5 mL of reagent (A) was added. The tubes were then heated in a boiling water bath for 10 minutes and cooled in running tap water. Subsequently, 2.5 mL of reagent (B) was added in each and the tubes were incubated at room temperature for 10 minutes. After this, 0.5 mL of reagent (C) was added and tubes were kept at room temperature for 15 minutes. The intensity of blue colour was read as absorbance at 650 nm against appropriate blank. The protein content was estimated using a standard calibration curve prepared from bovine serum albumin.

Total sugars (µg/mg dry weight)

Total sugars content was estimated by method described by Spiro (1966). A known volume (0.25-0.5 mL) of homogenized suspension was taken in tubes and volume was made up to one mL with distilled water. To that, 4 mL of freshly prepared anthrone reagent (100 mg anthrone and 1g thiourea was dissolved in 100 mL of 75% sulphuric acid. The mixture was kept on a water bath at 85°C to dissolve the ingredients completely) was added and tubes were transferred to boiling water bath. After 10 minutes, the tubes were brought to room temperature and absorbance was read at 625 nm. The sugar content was calibrated against the standard curve made with glucose.

Nitrogenase activity (EC 1.18.6.1.)

Nitrogenase activity was determined by acetylene reduction assay (ARA) with the help of Gas Chromatograph (Hardy *et al.,* 1973). Nitrogenase is a versatile enzyme and can reduce variety of substrates with triple bond. Hence, this enzyme can reduce acetylene to ethylene that can be measured to understand the potential of nitrogenase activity. Known volume (5 mL) of suspension was taken in 13 mL rimless tube, stoppered with subaseal and acetylene equal to 10% of air space (1 mL) was injected after removal of equal amount of air. The tubes were incubated for 90 minutes under continuous illumination (52-55 µmol photon m^{-2} s^{-1}) at 28ºC. The reaction was terminated by injecting 0.1 mL of 50% TCA and gas phase was assayed for ethylene. Gas chromatographic quantification of ethylene formed was utilized as an index of nitrogen fixation. Commercially available ethylene was utilized for quantification and vials with an equivalent volume of water served as controls. The ARA values were expressed as nmoles ethylene mg^{-1} chl h^{-1}.

Nitrate reductase (NR) activity (EC 1.6.6.1.)

NR assay was performed according to Herrero and Guerrero (1986). For the assay of enzyme, a known volume of BGA suspension was centrifuged and washed with sterile distilled water and incubated in basal medium containing $NaNO_3$ (10 mM, pH 7.0). Samples (1 mL) were taken out and Sulphanilamide (2 mL) was added and mixed well. This was followed by addition of 2 ml α – (N-1)) napthyl ethylene diamine dihydrochloride (NEDD). The samples tubes were incubated at room temperature and the absorbance was recorded after 30 minutes at 540 nm and the enzyme activity was expressed as µmol NO_2 mg^{-1} protein. A standard curve of nitrite was prepared using known concentrations of sodium nitrite.

Glutamine synthetase (GS) activity (EC 6.3.1.2.)

GS activity was performed according to Shapiro and Stadtman (1970) and Stacey *et al.* (1977). The transferase activity of GS was determined at pH 7.0 by measuring the amount of γ-glutamyl hydroxamate formed using

glutamine as substrate in presence of ADP and arsenate. Whole cell GS activity was analysed after toluene treatment of cells. 0.5 mL of homogenized cell suspension was treated with 0.25 mL toluene and incubated for 10 minutes at 4^0C.The cell suspension was then centrifuged and 0.25 mL of toluene was added and incubated for 10 minutes at 4°C. During incubation, the tubes were repeatedly shaken to ensure complete permeability of cell membranes and release of the enzyme. Further toluene layer was removed and cell pellet was suspended in 0.5 mL imidazole buffer. The reaction was started by addition of 0.5 mL of reaction mixture and incubated at 37^0C for 30 minutes. Reactions were terminated by adding 3.0 mL of stop mixture and absorbance was taken at 540 nm and the activity was directly read from standard curve of y-glutamyl hydroxamate. Transferase activity is expressed as µ mol γglutamyl hydroxamate mg^{-1} protein.

Statistical Analysis

The data was subjected to analysis of variance (ANOVA) using the software. Differences were considered to be significant at the 95% confidence level.

Result

Soluble proteins

Soluble proteins analysed were highest at 14th day of incubation i.e., during the active growth phase of the blue green algal strains. This parameter followed a typical exponential pattern resulting in a slow and gradual decrease towards late log phase. The strains differed significantly with respect to this parameter and *Aulosira fertilissima* showed the maximum soluble protein content (96.61µg mg^{-1} dry weight) followed by *Anabaena variabilis* (87.17µg mg^{-1} dry weight).*Westelliopsis prolifica* showed lowest value (47.42 µg mg^{-1} dry weight) which was *at par* with the *Tolypothrix tenuis* (49.68µg mg^{-1} dry weight).

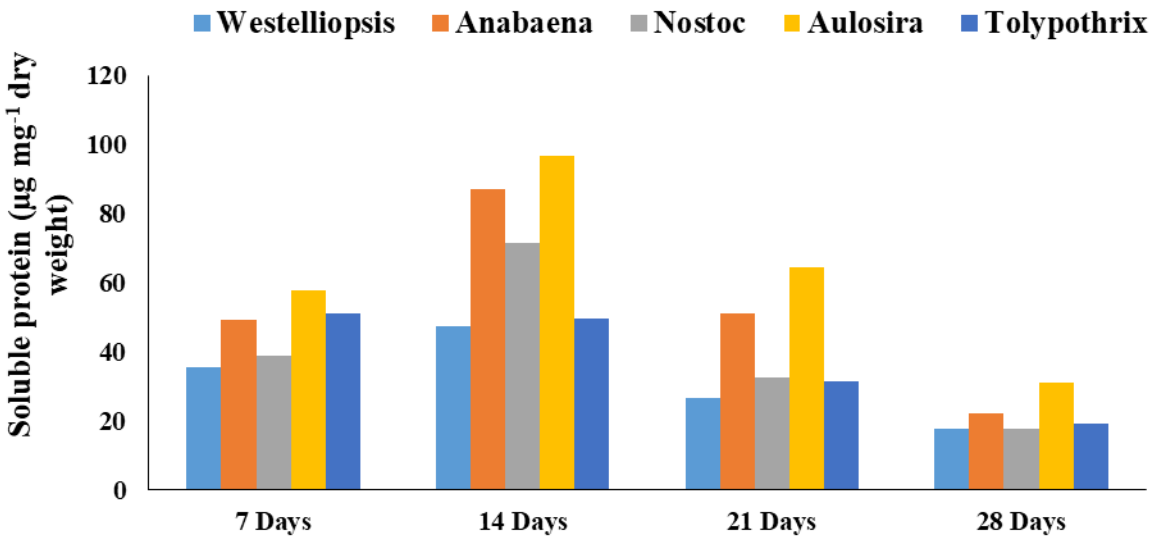

Figure 1 Soluble protein (µg mg^{-1} dry weight) in blue green algal biofertilizers cultures at different days of incubation

Total sugars

The total sugars analysed showed a linear enhancement with incubation time and maximum mean value was exhibited on 21st day of incubation followed by a gradual decline on 28th day of incubation. *Aulosira fertilissima* showed highest mean value of 201.48 µg mg^{-1} dry weight which was *at par* with *Nostoc muscorum* (197.52 µg mg^{-1} dry weight). *Westelliopsis prolifica* showed lowest mean value of 124.61µg mg^{-1} dry weight. *Tolypothrix tenuis* and *Anabaena variabilis* recorded mean value of 178.53 µg mg^{-1} dry weight and 148.24 µg mg^{-1} dry weight respectively.

Nitrogenase activity

Nitrogenase activity was calculated on the basis of per mg of chlorophyll. Highest nitrogenase activity was observed at 14th day of incubation in all the cases, followed by a gradual decline. Thereafter *Nostoc muscorum* exhibited highest ARA value (6841nmoles ethylene mg^{-1} chl h^{-1}), while the lowest activity (3602 nmoles ethylene mg^{-1} chl h^{-1})

was recorded in *Westelliopsis prolifica*. *Anabaena variabilis*, *Aulosira fertilissima* and *Tolypothrix tenuis* were ranked 2nd, 3rd and 4th respectively with respect for this attribute.

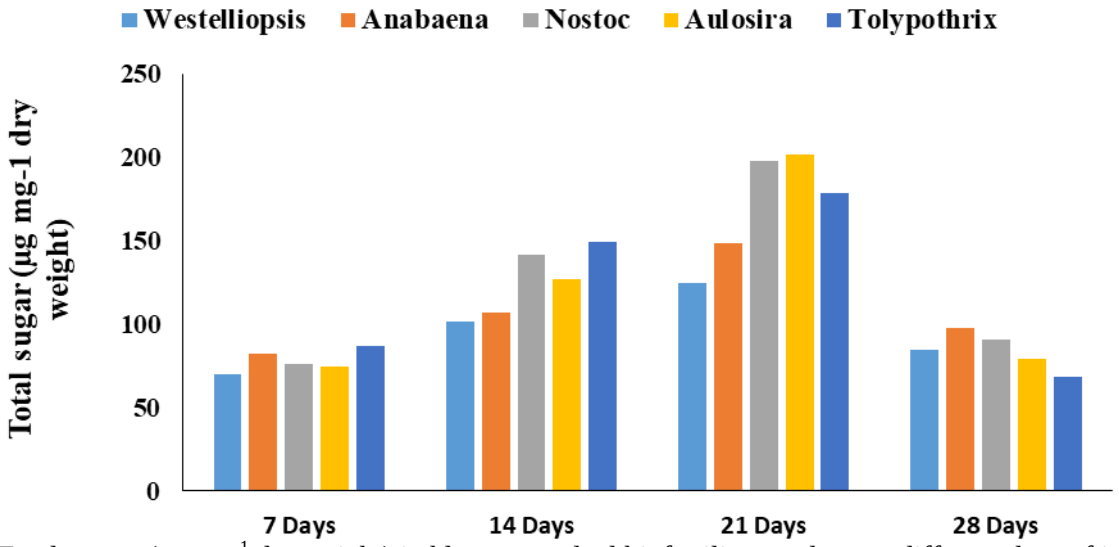

Figure 2 Total sugars (µg mg^{-1} dry weight) in blue green algal biofertilizers culture at different days of incubation

Nitrate reductase (NR) activity

Peak in nitrate reductase activity was also observed at 14th day of incubation followed by slow and gradual decrease in the activity. On further incubation, the cultures differed significantly with respect to this parameter and *Aulosira fertilissima* showed highest NR activity (96.54µmol NO_2^- mg^{-1} protein) whereas *Anabaena variabilis* showed lowest activity (64.07µmol NO_2^- mg^{-1} protein).

Glutamine synthetase (GS) activity

Mean GS activity calculated during incubation time showed that the highest GS activity was observed on 14th day of incubation while decreasing gradually on further incubations. Enzyme activity ranged between 184.12 µ mol γglutamyl hydroxamate mg^{-1} protein for *Nostoc muscorum* to 79.42 µ mol γglutamyl hydroxamate mg^{-1} protein for *Westelliopsis prolifica.* The GS activity of other three strains namely *Tolypothrix tenuis*, *Anabaena variabilis* and *Aulosira fertilissima* was 101.43, 94.72 and 68.42 µ mol γglutamyl hydroxamate mg^{-1} protein respectively.

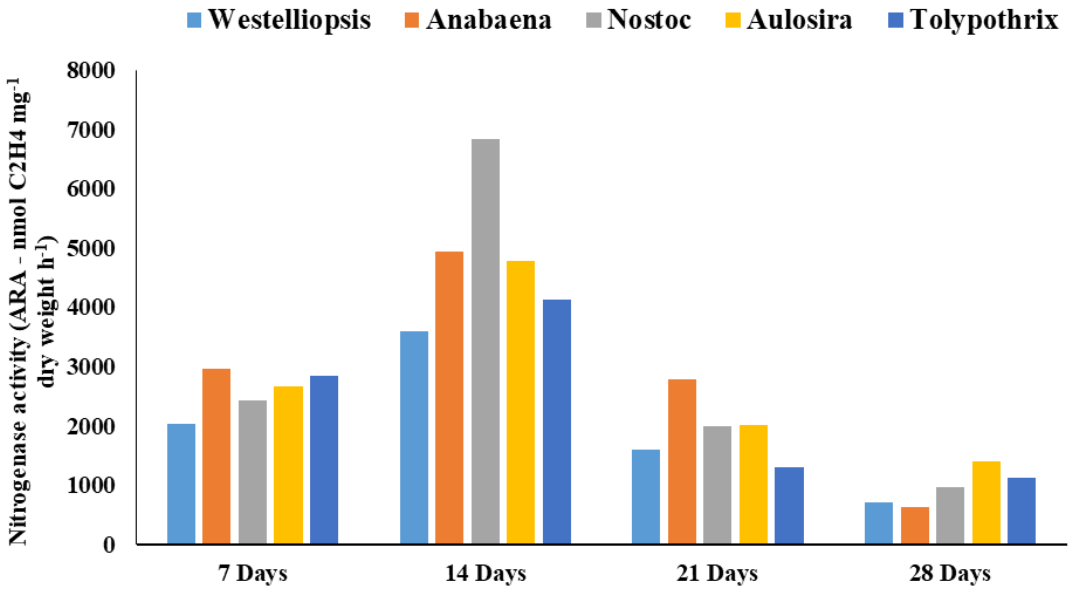

Figure 3 Nitrogenase activity (ARA nmol C_2H_4 mg^{-1} dry weight h^{-1}) in blue green algal biofertilizer cultures at different days of incubation

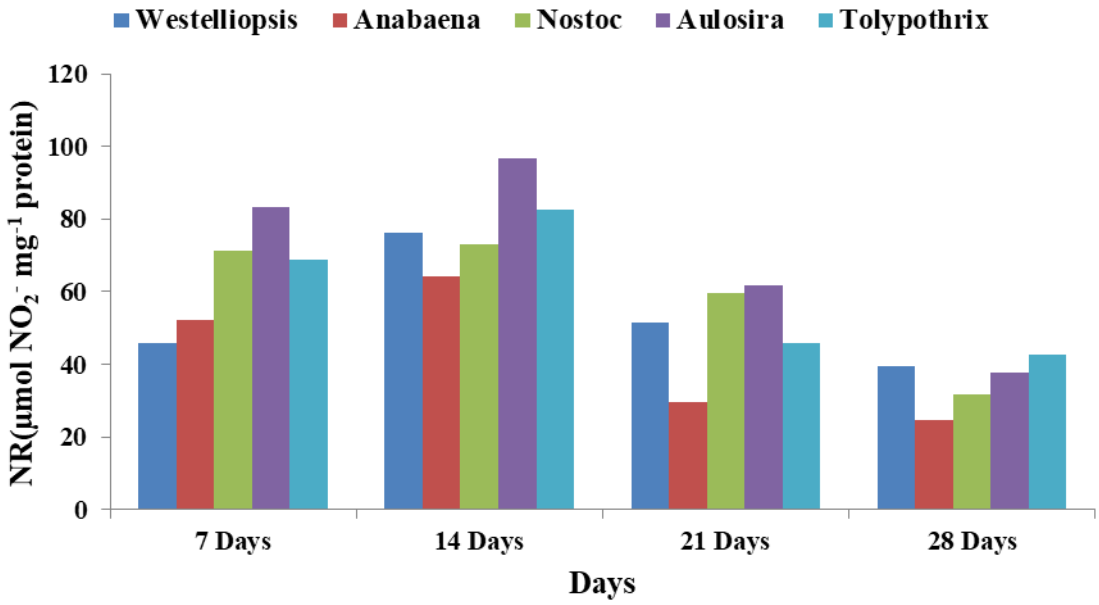

Figure 4 Nitrogen reductases (µmol NO_2^- mg^{-1} protein) in blue green algal biofertilizer cultures at different days of incubation

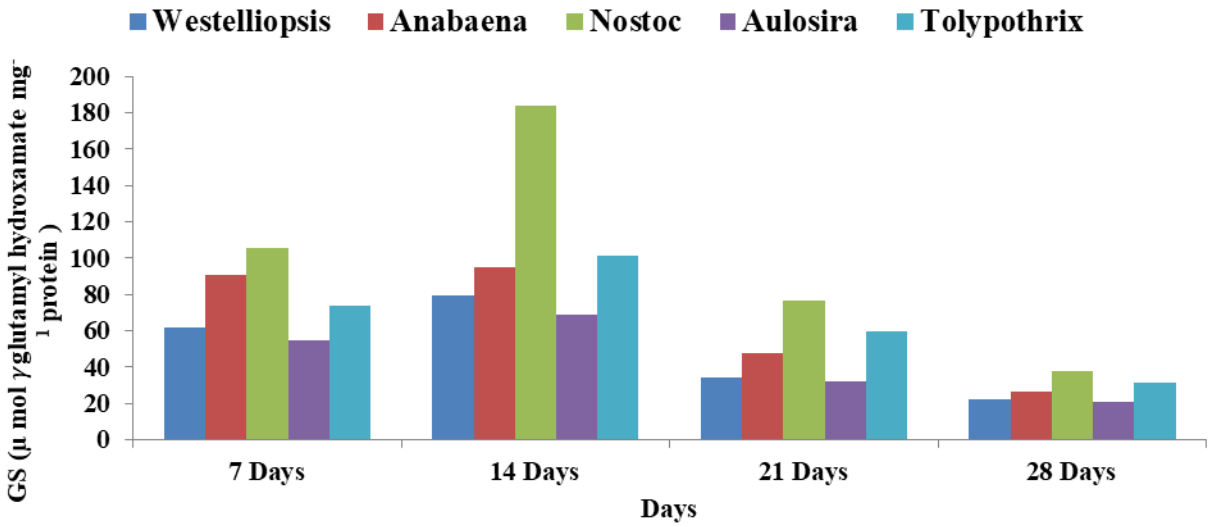

Figure 5 Glutamine synthetase **(**µ mol γglutamyl hydroxamate mg^{-1} protein **)** activityin blue green algal biofertilizer strains at different days of incubation

Discussion

Cyanobacteria have received worldwide attention in their versatility of producing an array of biochemicals. Their protein can be used as food supplement or as an alternative source of food. Some species of cyanobacteria like *Spirulina, Nostoc* and *Anabaena* are commercially grown due to their high protein and fibre content (Anupama, 2000). Prasanna *et al*. (2006) conducted a research on a set of 30 *Anabaena* strains and they found soluble protein ranged from 31.17 $mgmL^{-1}$ to 447.69 mg mL^{-1} in these cyanobacterial strains. Similar results were reported in this study and soluble protein content range varied from 47.42 to 96.61µg mg^{-1} dry weight among the five cyanobacterial strains. Characterization of blue green algal strains has also been carried out for specific parameters in relation to their utilization in value addition (Garcia-Pichel and Castenholz, 1991). Hosmani and Anitha (1998) reported carbohydrate contents of 84.44 mg mL^{-1} in *Microcystis aeruginosa* whereas Hassan *et al*. (2012) found total carbohydrate content of 213.4 and 343.7 mg mg^{-1} dry weight in *Anabaena laxa* and *Nostoc muscorum* respectively. In the present

investigation, the variability observed with respect to total sugar content with range of 124.61 to 201.48µg mg^{-1} dry weight. Nitrogen fixation by cyanobacteria is an economically viable input in rice cultivation and selection and manipulation of high nitrogen fixing strains is an ongoing process in most rice growing countries. Heterocyst differentiation is primarily a response to the N status of the culture (Thomas *et al.*, 1975; Rai *et al.*, 1984). These properties account for their abundance in rice fields (De, 1939) and are considered responsible for the sustained fertility of the rice ecosystem. Under conditions of ammonia limitation cyanobacteria use glutamine synthetase and glutamate synthase pathway which is the major ammonia assimilatory route under nitrogen fixing condition (Wolk *et al.*, 1976; Stacey *et al.*, 1977; Prasanna *et al*., 2006) also conducted research on activities of enzymes related to N-assimilation and reported the value ranged from 602.4 to 22939 nmoles ethylene mg^{-1} chl h^{-1}, 2.83 to 31.83 µmoles NO_2^- $mL^{-1}$and 16.20 to 272.07 µ mol γglutamyl hydroxamate mg^{-1} protein for nitrogenase activity (ARA), nitrate reductase(NR) activity and glutamine synthetase (GS) activity respectively for different *Anabaena* strains. In the present investigation, the variability observed with respect to enzymes related to N-assimilation, were in accordance with results obtained from earlier studies (Dhar *et al.*, 2000; Mishra *et al.*, 2001). Nitrogenase activity was high in most of the strains ranging from a lowest value of 3602 nmoles ethylene mg^{-1} chl $h^{-}1$ for *Westelliopsis prolifica* to highest value of 6841 nmoles ethylene mg^{-1} chl h^{-1} for *Nostoc muscorum*. Nitrate assimilation has been exhaustively reviewed by a number of researchers (Bagchi, 1994). In this study the cyanobacterial strains showed nitrate reductase (NR) activity and glutamine synthetase (GS) activity of 64.07 to 96.54 µmoles NO_2^- mL^{-1} and 68.42 to 184.12 µ mol γglutamyl hydroxamate mg^{-1} protein. Similar reports on Anabaena strains are available in literature (Mekonnen *et al*., 2002).

Conclusion

Variations were observed with respect to cellular constituents soluble proteins and total sugars), nitrogen fixing ability and N assimilation related parameters in the blue green algal cultures during incubation under laboratory conditions. These parameters revealed a typical sigmoid curve showing peak during the exponential active growth phase. Soluble proteins and total sugars were highest at 14^{th} and 21^{st} day of incubation respectively while the N assimilation related parameters namely nitrogenase activity, glutamine synthetase enzyme were higher at 14th day of incubation followed by a slow and gradual decline. Comparative performances of the strains indicated that parameters like, soluble proteins, total sugars and nitrate reductase activity were highest in *Aulosira fertilissima* and *Nostoc* muscorum exhibited peak values for nitrogenase activity and GS activity.

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