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Productivity of the Cyanobacterium *Spirulina platensis* in Cultures using High Bicarbonate and Different Nitrogen Sources

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ABSTRACT

The interactions of bicarbonate (HCO_3^-) and different nitrogen sources in the non-diazotrophic cyanobacterium *Spirulina platensis* in terms of pigments content, photosynthetic O_2 evolution, Ca^{++}-dependent ATPase activity and cytochrome c oxidase activity were examined in the present investigation. The cyanobacterium was acclimated to 300 mol m^{-3} HCO_3^--concentrations in the growth medium containing no nitrogen. The addition of different nitrogen sources in the growth medium resulted in further acclimatization up to 400 mol m^{-3} HCO_3^-. This finding revealed that nitrogen sources incorporated into necessary metabolites, nitrogen storage material and photosynthetic pigments as well. The combined transport of $HCO_3^-$and nitrogen sources ultimately resulted in gradual increase in the examined parameters in order of NO_3^-<NH_4^+<urea. Nitrate and ammonium assimilating enzymes i.e., Nitrate Reductase (NR) and Glutamine Synthetase (GS) were also examined in graded concentration of HCO_3^- and the findings suggested that both the enzymes showed higher activity in the medium containing higher HCO_3^- concentration this suggested that the activity of these enzymes closely linked with carbon fixation. Further, we examined the Ca^{++}-dependent ATPase activity and cytochrome c oxidase activity in the presence of graded concentration of HCO_3^-. The enzyme was more active in the presence of high HCO_3^- concentration; the co-existence of NADPH further enhances the enzyme activities. The use of such cultural practices would help in mass production of biopigments on industrial scale and would paved the way for the development of new technology for CO_2 fixation using the solar light as the energy source.

Key words: Chlorophyll, cyanobacteria, glutamine synthetase, HCO_3^-, nitrate reductase, phycocyanin, phycoerythrin

INTRODUCTION

Cyanobacteria are photoautrophic prokaryotes and are known for their remarkable ability to grow and survive in varying environmental conditions (Bhaya *et al*., 2000). They grow and multiply *via* the autotrophic mode of nutrition. They are able to perform oxygenic photosynthesis even under the condition of low ambient CO_2, because of the active inorganic carbon uptake by PSI-dependent cyclic photophosphorylation (Miyachi *et al*., 1996). The presence of HCO_3^- is known to stimulate membrane potential, indicating the involvement of a primary electrogenic pump for carbon uptake (Kaplan *et al*., 1990). The energy for carbon uptake might be supplied either by ATP or by a trans-membrane electrochemical potential gradient. Reinhold *et al*. (1984) concluded that

the role of Na^+ as component of symport for transport of HCO_3^-. The operation of Na^+ dependent HCO_3^- transport is supported by characterization of a High-CO_2-Requiring (HCR) mutant (IL-2) impaired in HCO_3^- transports (Ronen-Tarazi *et al.*, 1995). Thus interaction between nutrient acquisition and CO_2 fixation mechanism have been studied because both are key points of integration and represent areas where adaptive responses are likely to operate (Mann, 2000).

Diazotrophic cyanobacteria are able to assimilate nitrate, nitrite, ammonium, urea, several amino acids and atmospheric nitrogen as nitrogen source (Herrero *et al.*, 2001). Cyanobacteria have efficient regulatory system to assimilate several nitrogen molecules but when several nitrogen sources are available they prefer the reduced ones (amino acids, urea, ammonium) because of the energy constraint (Garcia-Ferandez and Diez, 2004). The non-diazotrophic cyanobacterial strains also assimilate different nitrogen sources in the form of nitrogen storage material and nitrogen-containing photosynthetic pigments (Kolodny *et al.*, 2006). The nitrogen starvation in non-diazotrophic cyanobacteria leads to- i) degradation of phycocyanin and phycoerythrin, ii) loss of chlorophyll a and iii) cells become almost completely depigmented and reside in dormant stage (Sauer *et al.*, 2001).

Species of *Spirulina* occur in alkaline, brackish and saline water ecosystems of both tropical and semi-tropical regions (Castenholz, 1989). Most of the water bodies in which *Spirulina* grow and survive has high salinity levels (22-60 g L^{-1}), high carbonate/bicarbonate concentration, high pH (8.5-11.0) and temperatures (25-40°C) (Iltis, 1968). *Spirulina platensis* is a non-nitrogen fixing cyanobacterium, grows photoautotrophically with simple expense of light, water and inorganic nutrients. It is ideally marketed as health food because of its high nutritional value, chemical composition and the safety of its biomass (Belay *et al.*, 1993; Vonshak and Tomaselli, 2000). Like other cyanobacteria the cell organization of *Spirulina* is typical of prokaryotes. They are Gram negative prokaryotes in which multi layered cell wall is surrounded by a mucilaginous polysaccharide envelope and thylakoid membrane with phycobilisomes is arranged in parallel bundles. The cell has a number of inclusions namely carboxysomes, ribosomes, DNA fibrils, gas vacuoles, polyglucan granules, polyphosphate granules and large cyanophycin granules.

This study shows the response of *Spirulina platensis* in terms of pigments content, photosynthetic O_2 evolution, Ca^{++}-dependent ATPase activity and cytochrome c oxidase activity under graded concentration of HCO_3^- and in the presence of different nitrogen sources with the HCO_3^-.

MATERIALS AND METHODS

Organism and growth conditions: *Spirulina platensis* used in the present study was obtained from the National Facility for Blue-Green Algae, IARI, New Delhi. The cyanobacterium is a non-diazotrophic, fresh water, filamentous and spiral strain. Zarrouk's synthetic medium (Zarrouk, 1966) was used for routine as well as for experimental purposes. The concentration of different nitrogen sources in this medium being modified according to the experimental design. N_2 grown cultures were washed twice and used as inoculum for incubation in growth medium containing $NaNO_3$ (30 mol m^{-3}), NH_4Cl (10 mol m^{-3}) and urea (10 mol m^{-3}) for 6-days before examining for growth.

Measurement of growth and specific growth rate: Growth was measured as Optical Density (OD) change at 560 nm, which results from variation in Chlorophyll a contents. Specific growth rate constant (k) was calculated by the formula given by Myers and Kratz (1955):

$$k = 2.303(\log N_2 - \log N_1)/(T_2 - T_1)$$

Where:
N_1 = Initial cell density at time T_1
N_2 = The final cell density at time T_2

Determination of pigment content: Chlorophyll a content was estimated according to Grimme and Boardman (1972) and phycocyanin and phycoerthyrin according to Tandeau de Marsac and Houmard (1988).

The Chl a contents is calculated as given below:

$$\text{Chl a (mg mL}^{-1}) = \frac{(\text{Abs 665 nm} \times 16.5 - \text{Abs 650 nm} \times 8.3)}{V_{sample} \times 1000} \times V_{total}$$

Where:
V_{total} = Volume of the reaction mixture
V_{sample} = Volume of the sample

The concentration of phycocyanin and phycoerthyrin was calculated according to the equation given by Bennett and Bogorad (1973) and the extinction coefficients given by Bryant *et al.* (1979).

$$\text{Phycocyanin (mg mL}^{-1}) = \frac{(\text{Abs 620 nm} - 0.70 \times \text{Abs 650 nm})}{7.38}$$

$$\text{Phycoerthyrin (mg mL}^{-1}) = \frac{(\text{Abs 565 nm} - 2.8 \times (\text{PC}) - 13.4 \times (\text{AP})}{12.7}$$

Protein measurement: Protein contents of the cyanobacterial cells were measured according to the method of Lowry *et al.* (1951).

Photosynthetic O_2 evolution: The rate of the photosynthetic O_2 evolution was measured with a Clark type Oxygen Electrode (Hansatech Instrument Ltd. U.K.) at 20°C using a saturating light. The oxygen evolution rate was calculated using software Oxygraph Plus (Version 1.0).

Measurement of Ca^{++}-dependent ATPase activity: Cyanobacterial cells were harvested, washed and resuspended in extraction buffer (30 mol m^{-3} Tris HCl, pH 8.1). The cells were broken by soniprep followed by high speed centrifugation. The cell free supernatant contain crude enzymes was used for measuring Ca^{++} dependent ATPase activities. The enzymatic reaction was activated by adding tripsin to the assay mixture and terminated by tripsin inhibitor (Owers-Narhi *et al.*, 1979).

Determination of cytochrome-c oxidase activity: Cells were harvested, washed and resuspended in Tricine-NaOH buffer (50 mol m^{-3}, pH 7.5) containing 1 mol m^{-3} $MgCl_2$ and were broken by sonication at 4°C. The resultant cell suspension was centrifuged and the pellet thus obtained was resuspended in reaction mixture contained membranes equivalent to 30-50 μg chlorophyll a, 2.5 mol m^{-6} Na-ascorbate, 125 mol m^{-6} Tricine-NaOH buffer (pH 7.5) and

2.5 mol m^{-6} magnesium chloride. Cytochrome [(Horse Heart); 0.01 mol m^{-3}] was added in to the assay mixture. Difference between oxygen uptake with and without cytochrome was taken as the value for cytochrome c oxidation.

Measurement of nitrate reductase (NR) activity: NR activity was measured using dithionite reduced methyl viologen as an artificial electron donor (Manzano *et al*., 1976). Cells were harvested by centrifugation and washed with NR buffer followed by sonication. The supernatant was added with assay mixture. The reaction was terminated by adding 1 M zinc acetate. The resultant mixture was centrifuged and supernatant thus obtained were added with 1% sulphanilamide+0.02% NED. Optical density of the resultant colour was read at 540 nm against a reagent blank. NR activity was calculated in terms of nmol NO_2-produced mg^{-1} protein min^{-1}.

Measurement of glutamine synthetase (transferase) activity: Cyanobacterial cells were harvested by centrifugation and washed with buffer A (Tris-HCl, pH 7.5) and buffer B (buffer A+5 mol m^{-3} $MgCl_2$+10 mol m^{-3} Na glutamate+5 mol m^{-3} 2-mercaptoethanol and 1 mol m^{-3} EDTA, pH 7.5) and the pellet resuspended in buffer B before cell breakage by soniprep. Cell extract thus obtained, was centrifuged at high speed for 30 min and the supernatant used as crude enzyme extract. This enzyme extract (0.5 mL) was mixed with 1 ml reaction mixture (40 mol m^{-3} Tris-HCl, 3 mol m^{-3} $MnCl_2$, 20 mol m^{-3} K-arsenate, 0.4 mol m^{-3} Na ADP, 60 mol m^{-3} hydroxylamine and 30 mol m^{-3} glutamate) and the reaction allowed to proceed in dark for 10 min (30°C). The reaction was terminated after 10 min by adding 2 mL stop mixture (10% $FeCl_3$, 24% TCA, 6N HCl and 6.5 mL DH_2O). The turbid debris in the resultant solution was removed by centrifugation and the intensity of the coffee-colour solution read at 540 nm against the reagent blank, prepared by eliminating glutamine and hydroxylamine. Glutamine synthetase (transferase) activity is expressed as nmol γ-glutamylhydroxamate formed mg^{-1} protein min^{-1} as quantified by a reference to standard curve obtained with γ-glutamyl-hydroxamate in the assay mixture.

RESULTS

The cyanobacterium *Spirulina plantensis* was grown in Zarrouk medium for routine as well as for experimental purposes. Growth (optical density change 560 nm), specific growth rate and pigments content were observed in the cultures grown in Zarrouk medium as well as medium supplemented with graded concentration of HCO_3^- and different nitrogen sources. Growth and specific growth rates were proportionally increased with the increase in HCO_3^- concentration up to 300 mol m^{-3}. Further increase in HCO_3^- concentration has no impact on growth and specific growth rate. Addition of different nitrogen sources in the culture medium further accelerates growth and specific growth rate. The growth under various nitrogen sources occur in the order of NO^-_3<NH^+_4<urea (Fig. 1).

Chlorophyll a and phycobiliproteins (phycocyanin, allophycocyanin and phycoerythrin) are important photosynthetic pigments in *Spirulina*. Therefore, the impact of HCO_3^- and different nitrogen sources on these pigments were examined. The Chlorophyll a contents obtained under graded concentration of HCO_3^- are in agreement with the influence of HCO_3^- on this pigment. Chlorophyll a contents were low in the medium supplemented with 100 mol m^{-3} HCO_3^- further increase in HCO_3^- concentration indicate a positive relationship between the HCO_3^- concentration and chlorophyll a contents. The coexistence of different nitrogen sources in the culture medium triggered further enhancement of chlorophyll a contents. The results in Fig. 2 show that nitrogen

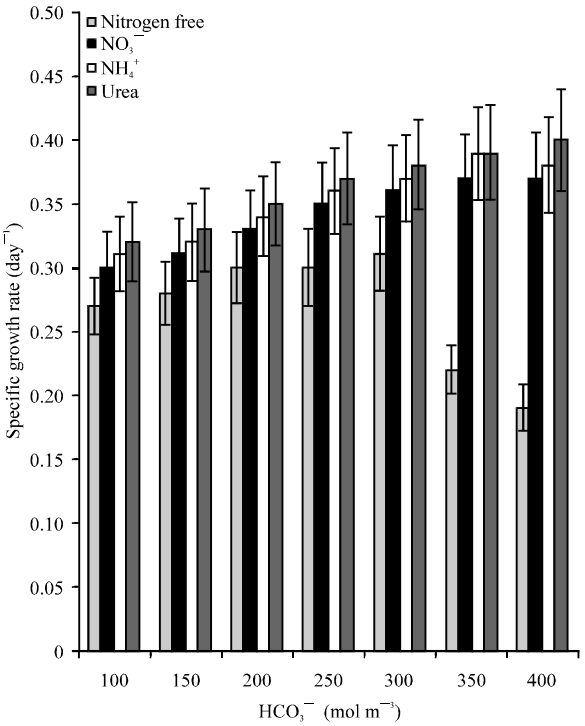

Fig. 1: Specific growth rate of *Spirulina platensis* under graded concentration of HCO_3^- different growth mediums, Values are Mean±SEM of three independent experimental determinations

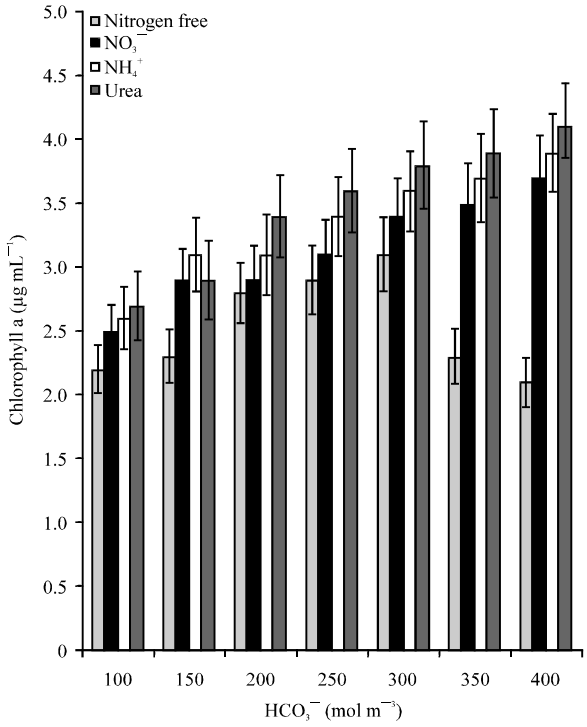

Fig. 2: Effect of bicarbonate concentration on chlorophyll a contents in different growth mediums, Values are Mean±SEM of three independent experimental determinations

supplementation leads to assimilation of high amount of chlorophyll a. Like chlorophyll a, phycocyanin and phycoerythrin contents were higher in the cyanobacterium grown at elevated concentration of HCO_3^-. Addition of different nitrogen sources in the culture increased both phycocyanin and phycoerythrin contents (Fig. 3, 4).

The addition of HCO_3^- at graded concentration showed a concentration dependent enhancement in the rate of photosynthetic O_2 evolution and the photosynthetic rate was saturated at 300 mol m^{-3} HCO_3^-. The PSII activity stimulated when the culture media supplemented with different nitrogen sources (Table 1). The photosynthetic oxygen evolution increased further in the presence of the reductant i.e., NADPH (Table 2).

Ca^{++}-dependent ATPase activity is known to function in photosynthetic generation of ATP molecules. The Ca^{++}-dependent ATPase activity is more pronounced in the culture medium supplemented with graded concentration of HCO_3^-. The addition of various nitrogen sources has a positive relationship for the examined parameter (Table 3). Depending on the results in Table 4, we concluded that Ca^{++}-dependent ATPase activity is comparatively higher in the presence of NADPH this finding suggested that exogenously supplied NADPH provides more electrons for the generation of a transmembrane proton gradient that drives the synthesis of ATP.

Cytochrome c oxidase activity of the *Spirulina platensis* under graded concentration of HCO_3^- and in the absence/presence of NADPH is presented in Table 5 and 6. The activity was enriched in the membranes derived from *Spirulina* cells grown in high HCO_3^- concentration. In comparison,

Table 1: Photosynthetic O_2 evolution (mmol O_2 evolved g^{-1} Chl a) of the *Spirulina platensis* in the presence of different nitrogen sources

Bicarbonate (mol m^{-3})	Zarrouk medium without nitrogen	Zarrouk medium+ 30 mol m^{-3} $NaNO_3$	Zarrouk medium+ 10 mol m^{-3} NH_4NO_3	Zarrouk medium +10 mol m^{-3} urea
100	289±23	301±28	311±28	317±30
150	318±28	332±31	348±32	365±34
200	408±33	432±40	467±44	477±43
250	411±38	456±44	489±45	499±48
300	424±41	478±48	512±49	523±49
350	301±27	502±49	529±51	538±50
400	279±22	512±48	544±52	556±53

N_2 grown cultures were washed twice and used as inoculum for incubation in growth medium containing $NaNO_3$, NH_4NO_3 and urea for 9-days before examining for growth, Values are Mean±SEM of three independent experimental determinations

Table 2: Photosynthetic O_2 evolution (mmol O_2 evolved g^{-1} Chl a) of the *Spirulina platensis* in the presence of different nitrogen sources and in the presence of NADPH

Bicarbonate (mol m^{-3})	Zarrouk medium without nitrogen	Zarrouk medium +30 mol m^{-3} $NaNO_3$	Zarrouk medium +10 mol m^{-3} NH_4NO_3	Zarrouk medium +10 mol m^{-3} urea
100	302±28	319±29	333±31	347±32
150	318±29	337±31	342±33	356±33
200	404±38	421±39	445±41	456±43
250	434±42	488±45	498±49	511±48
300	412±37	536±51	552±52	578±52
350	315±28	578±53	587±55	599±56
400	308±27	599±55	602±58	623±60

N_2 grown cultures were washed twice and used as inoculum for incubation in growth medium containing $NaNO_3$, NH_4NO_3 and urea for 9-days before examining for growth, Values are Mean±SEM of three independent experimental determinations

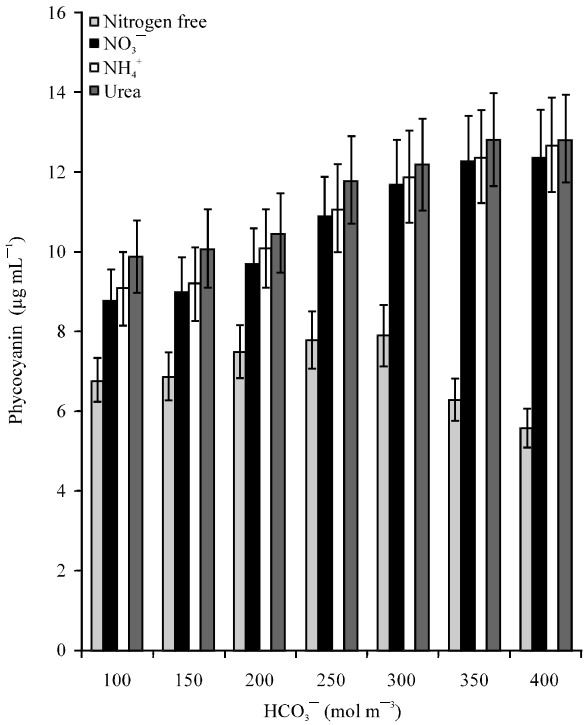

Fig. 3: Effect of bicarbonate concentration on phycocyanin contents in different growth mediums, Values are Mean±SEM of three independent experimental determinations

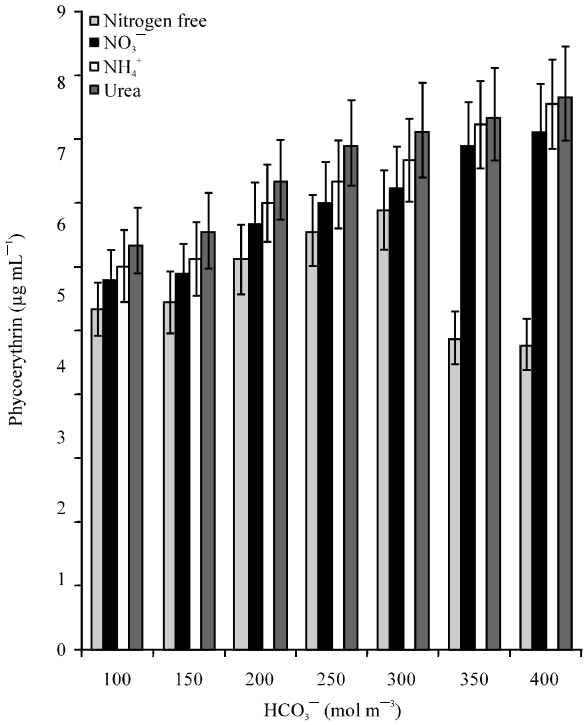

Fig. 4: Effect of bicarbonate concentration on phycoerythrin contents in different growth mediums, Values are Mean±SEM of three independent experimental determinations

Table 3: Ca^{++}-dependent ATPase activity (nmol Pi released min^{-1} mg $protein^{-1}$) of the *Spirulina platensis* in the presence of different nitrogen sources

Bicarbonate (mol m^{-3})	Zarrouk medium without nitrogen	Zarrouk medium +30 mol m^{-3} $NaNO_3$	Zarrouk medium +10 mol m^{-3} NH_4NO_3	Zarrouk medium +10 mol m^{-3} urea
100	22±1.8	24±1.9	25±2.2	27±2.4
150	23±2.1	25±2.2	27±2.5	28±2.6
200	25±2.2	28±2.6	29±2.7	30±2.8
250	28±2.5	30±2.8	31±2.9	33±3.1
300	31±2.8	32±2.9	33±3.1	34±3.3
350	24±2.3	33±3.1	35±3.2	36±3.5
400	21±1.7	33±3.2	36±3.3	37±3.6

N_2 grown cultures were washed twice and used as inoculum for incubation in growth medium containing $NaNO_3$, NH_4NO_3 and urea for 9-days before examining for growth, Values are Mean±SEM of three independent experimental determinations

Table 4: Ca^{++}-dependent ATPase activity (nmol Pi released min^{-1} mg $protein^{-1}$) of the *Spirulina platensis* in the presence of different nitrogen sources and in the presence of NADPH

Bicarbonate (mol m^{-3})	Zarrouk medium without nitrogen	Zarrouk medium +30 mol m^{-3} $NaNO_3$	Zarrouk medium +10 mol m^{-3} NH_4NO_3	Zarrouk medium +10 mol m^{-3} urea
100	23±1.9	25±2.2	27±2.5	29±2.7
150	24±2.2	26±2.3	27±2.5	30±2.7
200	26±2.4	31±2.8	33±3.1	35±3.2
250	29±2.7	33±3.1	34±3.2	37±3.4
300	32±2.9	35±3.3	36±3.3	38±3.6
350	26±2.2	36±3.4	37±3.5	39±3.7
400	23±2.0	36±3.4	38±3.6	39±3.8

N_2 grown cultures were washed twice and used as inoculum for incubation in growth medium containing $NaNO_3$, NH_4NO_3 and urea for 9-days before examining for growth, Values are Mean±SEM of three independent experimental determinations

Table 5: Cytochrome c oxidation (nmol cytochrome c mg $protein^{-1}$ min^{-1}) of the *Spirulina platensis* in the presence of different nitrogen sources

Bicarbonate (mol m^{-3})	Zarrouk medium without nitrogen	Zarrouk medium +30 mol m^{-3} $NaNO_3$	Zarrouk medium +10 mol m^{-3} NH_4NO_3	Zarrouk medium +10 mol m^{-3} urea
100	44±3.7	45±4.1	47±4.3	48±4.4
150	46±3.9	47±4.4	49±4.6	50±4.9
200	52±4.5	55±5.1	59±5.2	63±5.3
250	56±4.8	61±5.8	67±6.1	69±6.4
300	58±5.3	66±5.9	71±6.7	75±6.6
350	52±4.9	69±6.3	74±6.6	79±6.8
400	45±3.9	71±6..6	76±6.9	81±6.9

N_2 grown cultures were washed twice and used as inoculum for incubation in growth medium containing $NaNO_3$, NH_4NO_3 and urea for 9-days before examining for growth, Values are Mean±SEM of three independent experimental determinations

the activity was further increased in the membranes derived from cells grown in different nitrogen sources. The addition of NADPH showed an enhancement in the activity suggested that membrane fractions were active in the NADPH supported reduction of cytochrome.

In cyanobacteria nitrate is reduced by nitrate reductase to nitrite and nitrite is reduced by nitrite reductase to ammonium. The ammonium is assimilated via the GS/GOGAT enzyme system.

Table 6: Cytochrome c oxidation (nmol cytochrome c mg protein^{-1} min^{-1}) of the *Spirulina platensis* in the presence of different nitrogen sources and in the presence of NADPH

Bicarbonate (mol m^{-3})	Zarrouk medium without nitrogen	Zarrouk medium +30 mol m^{-3} $NaNO_3$	Zarrouk medium +10 mol m^{-3} NH_4NO_3	Zarrouk medium +10 mol m^{-3} urea
100	46±4.1	47±4.4	49±4.7	51±4.8
150	48±4.6	49±4.7	50±4.8	52±4.9
200	57±4.9	59±5.2	63±5.7	66±5.9
250	62±5.5	66±5.8	70±6.2	72±6.3
300	67±6.1	71±6.7	76±6.8	79±7.2
350	58±5.2	77±7.1	81±7.3	86±7.8
400	49±4.4	79±7.7	83±7.2	88±7.8

N_2 grown cultures were washed twice and used as inoculum for incubation in growth medium containing $NaNO_3$, NH_4NO_3 and urea for 9-days before examining for growth, Values are Mean±SEM of three independent experimental determinations

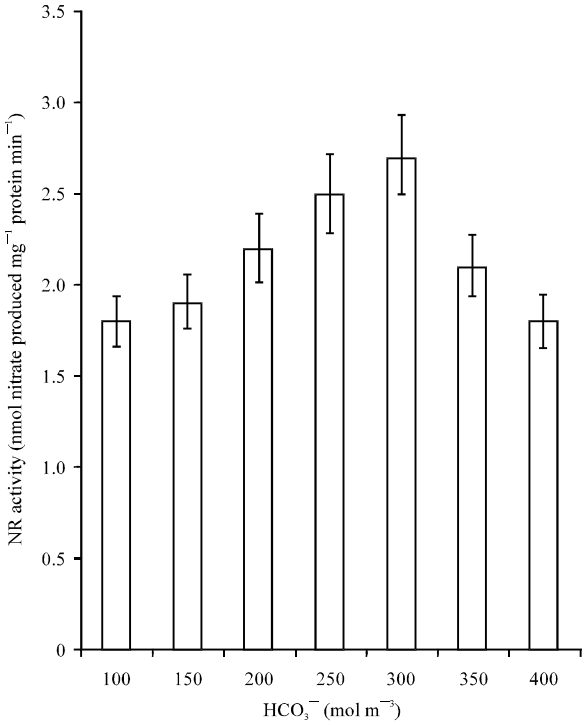

Fig. 5: Effect of bicarbonate concentration on NR activity in the medium containing NO_3^-, Values are Mean±SEM of three independent experimental determinations

Therefore, next series of experiments were planned to examine the effect of different concentration of HCO_3^-on NR and GS activity, because nitrogen metabolism is closely connected with carbon fixation. The cyanobacterial cells were starved for 16 h before the incubating them in growth chamber. Thereafter, cells were supplemented with 30 mol m^{-3} $NaNO_3$ under graded concentration of HCO_3^-. The result as shown in the Fig. 5 showed that rate of NR activity increased up to 300 mol m^{-3} HCO_3^- concentration, thereafter, the rate of NR activity decreased slightly. Likewise, GS activity showed a concentration dependent rising pattern up to 300 mol m^{-3} HCO_3^- followed by declining pattern (Fig. 6).

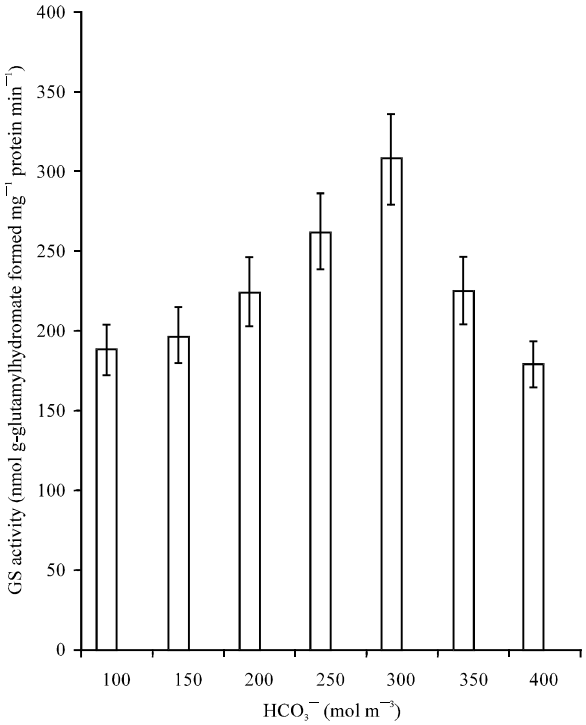

Fig. 6: Effect of bicarbonate concentration on GS activity in the medium containing NO_3^-, Values are Mean±SEM of three independent experimental determinations

DISCUSSION

Species of *Spirulina* display metabolic plasticity in response to environmental stimuli. They are widely distributed in tropical and semi tropical regions. Their ability to grow over a wide range of alkalinity, salinity, light and temperature makes them model photosynthetic prokaryotes for the study of the fundamental processes that participate in regulating their metabolic activities to varying degree of ecological amplitudes. The analysis of growth and photosynthetic pigments under graded concentration of HCO_3^- revealed that growth and pigment concentration was better in the medium containing inorganic nitrogen source. Similar studies of *Spirulina* adoptive responses to varying environmental conditions were conducted by Singh and Kumar (1994), Goksan *et al.* (2007) and Danesi *et al.* (2011). Our findings also agreed with the results found in *Gracilaria lemaneiformis* (Zou and Gao, 2009).

The rate of CO_2 fixation in cyanobacteria depends upon the accumulation of inorganic carbon. The accumulation of inorganic carbon (CO_2 and HCO_3^-) takes place by various transporters such as NDH-I_3, SbtA, BCT1 or CmpR (Price *et al.*, 2008). The present results showed that the photosynthetic rate of *Spirulina platensis* was higher in the medium containing high HCO_3^- concentration this was further enhanced in the presence of different nitrogen sources and in the presence of NADPH. The similar effect of different nitrogen sources on cell growth, cell productivity and chlorophyll content was reported by Danesi *et al.* (2011) on *Spirulina platensis*. The inhibitory effect of nitrogen depletion on photosynthetic electron transport activities was also reported in *Spirulina platensis* (Peter *et al.*, 2010). The uptake and accumulation of inorganic carbon is an energy requiring process. Energy sources for inorganic carbon may include ATP (BCT1 HCO_3^- transporter), NADPH or reduced ferredoxin (CO_2 uptake) or coupling to an electrochemical Na^+

gradient (SbtA or BicA HCO_3^- transporter) (Badger *et al.*, 2002; Badger and Price, 2003). Our result on NADPH dependent effect of photosynthetic O_2 evolution was supported by the above mentioned findings.

In this study we found that graded HCO_3^- concentration alone has caused limited enhancement in the photosynthetic oxygen evolution. The co-existence of various inorganic nitrogen sources in the culture media enhanced photosynthetic oxygen evolution significantly. The similar responses of *Spirulina platensis* under controlled growth conditions were studied by other authors to support our hypothesis (Gitelson *et al.*, 1995; Danesi *et al.*, 2002; Danesi *et al.*, 2011). Some studies on other non-diazotrophic cyanobacteria have been reported to induce PS II activity under varying growth conditions (Richmond *et al.*, 1982; Vonshak *et al.*, 1983; Rangel-Yagui *et al.*, 2004).

The Ca^{++}-dependent ATPase enzyme is responsible for the photosynthetic generation of energy rich ATP molecules. Our findings suggest that the high HCO_3^- concentration alone or in combination with different nitrogen sources have positive effect on Ca^{++}-dependent ATPase activity. Therefore, it is implied that enhanced HCO_3^- concentration alone and in the presence of different nitrogen sources supplied more ATP for various metabolic activities of the examined cyanobacterium. Similar enhancement in Ca^{++}-dependent ATPase activity was observed in *Spirulina platensis* (Hicks and Yocum, 1986; Singh and Singh, 2000).

The cytochrome c oxidase activities were also changed in response to growth conditions. The activity level was low under nitrogen limitation conditions, while in nitrogen repletion condition, the activity was high. The presence of a reductant i.e., NADPH further increased cytochrome c oxidase activity. Similar changes in the activity of cytochrome c oxidase under the different light regime, under Na^+ caused stress and in the presence of various inhibitors have been reported by other authors (Moser *et al.*, 1991; Gabbay-Azaria *et al.*, 1992; Gu *et al.*, 1994; Bagchi *et al.*, 1986; Singh and Singh, 2000). These findings suggested that the energy generated by respiratory activities is consumed to perform various physiological functions. Likewise, results of our study supported that energy generated by respiratory activities may be used to drive carbon and nitrogen uptake and accumulation, because both the processes in cyanobacteria are energy requiring processes.

The non-diazotrophic cyanobacterium *Synechocystis* sp. strain PCC 6308 has been shown to metabolize both inorganic and organic nitrogen sources (Allen and Hutchison, 1980). The fixed nitrogen ultimately incorporated in to necessary metabolites as well as in to nitrogen storage material and nitrogen containing photosynthetic pigments. Under nitrogen limiting conditions the non-diazotrophic cyanobacteria are known to inhibit various metabolic activities (Saha *et al.*, 2003; Kolodny *et al.*, 2006). Our interpretation under varying experimental conditions (i.e., in the presence of high HCO_3^- concentration and different nitrogen sources) is supported by the above findings.

During nitrogen repletion the cyanobacterium *Spirulina platensis* assimilated all nitrogen sources by the enzymes nitrate reductase, nitrite reductase and glutamine synthetase/glutamate synthase (Vonshak, 1997; Jha *et al.*, 2007; Ali *et al.*, 2008). The results as shown in the (Fig. 5, 6) indicates that NR and GS activities were more pronounced in high HCO_3^- concentration this suggested that assimilation of inorganic nitrogen is dependent on CO_2 fixation. Similar effect of inorganic nitrogen availability on algal photosynthesis and carbon metabolism has been reported by Turpin (1991). The studies of Flores and Herrero (1994) also suggested that N-assimilation is coupled to photosynthetic electron transport. This finding is also in agreement with data for other non-diazotrophic strains of cyanobacteria (Herrero *et al.*, 1981; Kolodny *et al.*, 2006).

CONCLUSION

The present study aimed to understand the complete spectrum of *Spirulina* responses to combined impacts of high HCO_3^- and nitrogen sources. On the basis of results obtained the following conclusions are made:

- The HCO_3^- concentration exceeding 300 mol m^{-3} has no significant impact on growth but the presence of different nitrogen sources further increases the affinity for HCO_3^-
- The nitrogen-fixing enzymes viz. NR and GS were found to showed higher activity in the medium containing high HCO_3^- concentration. So, it is concluded that in the examined cyanobacterium both nitrogen and carbon metabolism are linked
- The photo phosphorylation and oxidative phosphorylation i.e., the activity of Ca^{++}-dependent ATPase activity and cytochrome c oxidase were found to enhance in high HCO_3^- concentration. The presence of reductant i.e., NADPH further increases the activity

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REFERENCES

Ali, A., P. Jha, K.S. Sandhu and N. Raghuram, 2008. *Spirulina* nitrate assimilating enzymes (NR, NiR, GS) have higher specific activities and are more stable than those of rice. Physiol. Mol. Biol. Plant., 14: 179-182.

Allen, M.M. and F. Hutchison, 1980. Nitrogen limitation and recovery in the cyanobacterium *Aphanocapsa* 6308. Arch. Microbiol., 128: 1-7.

Badger, M.R. and G.D. Price, 2003. CO_2 concentrating mechanisms in cyanobacteria: Molecular components, their diversity and evolution. J. Exp. Bot., 54: 609-622.

Badger, M.R., D. Hanson and G.D. Price, 2002. Evolution and diversity of CO_2 concentrating mechanisms in cyanobacteria. Fun Plant Biol., 29: 161-173.

Bagchi, S.N., D.T. Singh and H.N. Singh, 1986. Regulation by azide of heterocyst and nitgenase in azide-resistant mutants of the cyanobacterium *Nostoc muscorum*. New Phytol., 102: 51-64.

Belay, A., Y. Ota, K. Miyakawa and H. Shimamatsu, 1993. Current knowledge on potential health benefits of *Spirulina*. J. Applied Phycol., 5: 235-241.

Bennett, A. and L. Bogorad, 1973. Complementary chromatic adaption in a filamentous blue-green alga. J. Cell Biol., 58: 419-435.

Bhaya, D., R. Schwarz and A.R. Grossman, 2000. Molecular Responses to Environmental Stress. In: The Ecology of Cyanobacteria: Their Diversity in Time and Space, Whitton, B.A. and M. Potts (Eds.). Springer, New York, pp: 397-442.

Bryant, D.A., G. Guglielmi, N. Tandeau de Marsac, A.M. Castets and G. Cohen-Bazire, 1979. The structure of phycobilisomes: A model. Arch. Microbiol., 123: 113-127.

Castenholz, R.W., 1989. Subsection III, Order Oscittloriales. In: Bergey's Manual of Systematic Bacteriology, Staley, J.T., M.P. Bryant, N. Pfenning and J.G. Holt (Eds.). Vol. 3. Williams and Wilkins Co., Baltimore, pp: 1771-1780.

Danesi, E.D.G., C.O. Rangel-Yagui, J.C.M. de Carvalho and S. Sato, 2002. An investigation of effect of replacing nitrate by urea in the growth and production of chlorophyll by *Spirulina platensis*. Biomass Bioeng., 23: 261-269.

Danesi, E.D.G., C.O. Rangel-Yagui, S. Sato and J.C.M. de Carvalho, 2011. Growth and content of *Spirulina platensis* biomass chlorophyll cultivated at different values of light intensity and temperature using different nitrogen sources. Braz. J. Microbiol., 42: 362-373.

Flores, E. and A. Herrero, 1994. Assimilatory Nitrogen Metabolism and its Regulation. In: The Molecular Biology of Cyanobacteria, Bryant, D.A (Ed.). Kluwer Academic Publishers, Dardrech, The Netherlands, pp: 487- 517.

Gabbay-Azaria, R., M. Schonfeld, S. Tel-Or, R. Messinger and E. Tel-Or, 1992. Respiratory activity in the marine cyanobacterium *Spirulina subsalsa* and its role in salt tolerance. Arch. Microbiol., 157: 183-190.

Garcia-Ferandez, J.M. and J. Diez, 2004. Adaptive mechanisms of nitrogen and carbon assimilatory pathway in the marine cyanobacteria *Prochlorococcus*. Res. Microbiol., 155: 795-802.

Gitelson, A.A., S. Laorawat, G.P. Keydan and A. Vonshak, 1995. Optical properties de dense algal cultures outdoors and their application to remote estimation of biomass and pigment concentration in *Spirulina platensis* (cyanobacteria). J. Phycol., 31: 828-834.

Goksan, T., A. Zekeriyaoglu and A.K. Ilknur, 2007. The growth of *Spirulina platensis* in different culture systems under green house condition. Turk. J. Biol., 31: 47-52.

Grimme, L.H. and N.K. Boardman, 1972. Photochemical activation of a particle fraction P_1 obtained from the green alga *Chlorella fusca*. Biochem. Biophys. Res. Comm., 49: 1617-1623.

Gu, T.Q., Y. Iwama, A. Murakami, S.P. Adhikary and Y. Fujita, 1994. Changes in the cytochrome c oxidase activity in response to light regime for photosynthesis observed with the cyanophyte *Synechocystis* PCC 6714. Plant Cell Physiol., 35: 1135-1140.

Herrero, A., A.M. Muro-Pastor and E. Flores, 2001. Nitrogen control in cyanobacteria. J. Bacteriol., 183: 411-425.

Herrero, A., E. Flores and M.G. Guerrero, 1981. Regulation of nitrate reductase levels in the cyanobacteria *Anacystis nidulans*, *Anabaena* sps. strain 7119 and *Nostoc* sps. strain 6719. J. Bacteriol., 145: 175-180.

Hicks, D.B. and C.F. Yocum, 1986. Properties of the cyanobacterial coupling factor ATPase from *Spirulina platensis* II. Activity of the purified and membrane bound enzymes. Arch. Biochem. Biophy., 245: 230-237.

Iltis, A., 1968. Salinity tolerance of *Spirulina platensis* (Gom.) geitler (Cyanophyta) in ponds natron Kanem (Chad). Cah ORSTOM Ser Hydrobiol., 2: 119-125.

Jha, P., A. Ali and N. Raghuram, 2007. Nitrate induction of nitrate reductase and its inhibition by nitrite and ammonium ions in *Spirulina platensis*. Physiol. Mol.Biol. Plants, 13: 163-167.

Kaplan, A., R. Schwarz, R. Ariel and L. Reinhold, 1990. The CO_2 Concentrating Mechanism of Cyanobacteria: Physiological Molecular and Theoretical Studies. In: Regulation of Photosynthetic Processes, Kanai, R., R. Katoch, S. Katoch and S. Miyachi (Eds.) Vol. 2, (Special Issue). Botanical Magazine, Tokyo, pp: 53-72.

Kolodny, N.H., D. Bauer, K. Bryce, K. Klucevsek and A. Lane *et al*., 2006. Effect of nitrogen source on cyanophycin synthesis in *Synechocystis* sps. strain PCC 6308. J. Bacteriol., 188: 934-940.

Lowry, O.H., N.J. Rosendrough, A.L. Farr and R.J. Randall, 1951. Protein measurement with folin-phenol reagent. J. Biol. Chem., 193: 265-275.

Mann, N.H., 2000. Detecting the Environment. In: The Ecology of Cyanobacteria: Their Diversity in Time and Space, B.A. Whitton and M. Potts (Eds.). Springer, USA., pp: 233-255.

Manzano, C., P. Candau, C. Gomez-Moreno, A.M. Relimpio and M. Losada, 1976. Ferrodoxin-dependent photosynthetic reduction of nitrate and nitrite by particles of *Anacystis nidulans*. Mol. Cell. Biochem., 10: 161-169.

Miyachi, S., J. Burger, K. Kotzabasis, J. Thielmann and H. Senger, 1996. Photosynthetic characteristics of 3 strains of cyanobacteria grown under low-CO_2 conditions. Z. Naturforsch., 51: 40-46.

Moser, D., P. Nicholls, M. Wastyn and G. Peschek, 1991. Acidic cytochrome C_6 of unicellular cyanobacteria is an indispensable and kinetically competent electron donor to cytochrome oxidase in plasma and thylakoid membranes. Biochem Int., 24: 757-768.

Myers, J. and W.A. Kratz, 1955. A relationship between pigment content and photosynthetic characteristics in a blue green alga. J. Gen. Physiol., 39: 11-22.

Owers-Narhi, L., S.J. Robinson, C.S. De Roo and C.F. Yocum, 1979. Reconstitution of cyanobacterial photophosphorylation by a latent Ca^{2+}-ATPase. Biochem Biophys. Res. Comm., 90: 1025-1031.

Peter, P., A.P. Sarma, K.S. Basha and S.D.S. Murthy, 2010. Nitrogen chlorosis induced alterations in the photosynthetic electron transport activities of the cyanobacterium *Spirulina plantensis*. Afr. J. Basic. Appl. Sci., 2: 60-63.

Price, D.G., M.R. Badger, F.J. Woodger and B.M. Long, 2008. Advances in understanding the cyanobacterial CO_2-concentrating-mechanism. (CCM): Functional components, Ci transporters diversity, genetic regulation and prospects for engineering into plants. J. Exp. Bot., 59: 1441-1461.

Rangel-Yagui, C.O., E.D.G. Danesi, J.C. Monteiro de Carvalho and S. Sato, 2004. Chlorophyll production from *Spirulina platensis*: Cultivation with urea addition by fed batch process. Biores. Tech., 92: 133-141.

Reinhold, L., M. Volokita, D. Zenvirth and A. Kaplan, 1984. Is HCO_3^- transport in *Anabaena* a Na+- symport? Plant Physiol., 76: 1090-1092.

Richmond, A., S. Karg and S. Boussiba, 1982. Effects of bicarbonate and Carbon Dioxide on the competition between *Chlorella vulgaris* and *Spirulina platensis*. Plant Cell. Physiol., 23: 1411-1417.

Ronen-Tarazi, M., R. Schwarz, A. Bouevitch, J. Lieman-Hurwitz, J. Erez and A. Kaplan, 1995. Response of Photosynthetic Microorganisms to Changing Ambient Concentration of CO_2. In: Molecular Ecology of Aquatic Microbes, Joint, I. (Eds.). Vol. G38, NATO ASI Series, Springer-Verlag, Berlin, pp: 323-334.

Saha, S.K., L. Uma and G. Subramanian, 2003. Nitrogen stress induced changes in the marine cyanobacterium *Oscillatoria willei* BDU 1300511. FEMS Microbiol. Ecol., 45: 263-272.

Sauer, J., U. Schreiber, R. Schmid, U. Volker and K. Forchhammer, 2001. Nitrogen starvation-induced chlorosis in *Synechococcus* PCC 7942. Low-level photosynthesis as a mechanism of long-term survival. Plant Physiol., 126: 233-243.

Singh, D.P. and N. Singh, 2000. Calcium and phosphate regulation of nitrogen metabolism in the cyanobacterium *Spirulina platensis* under the high light stress. Curr. Microbiol., 41: 368-373.

Singh, Y. and H.D. Kumar, 1994. Adaptation of a strain of *Spirulina platensis* to grew in cobalt and iodine enriched media. J. Appl. Bacteriol., 76: 149-154.

Tandeau de Marsac, N. and J. Houmard, 1988. Complementary Chromatic Adaptation: Physiological Conditions and Action Spectra. In: Methodology of Enzymology, Packer, L. and A.N. Glazer (Eds.). Vol. 167, Academic Press, USA., pp: 318-328.

Turpin, D.H., 1991. Effects of inorganic N availability on algal photosynthesis and carbon metabolism. J. Phycol., 27: 14-20.

Vonshak, A. and L. Tomaselli, 2000. *Arthrospira* (*Spirulina*): Systematics and Ecophysiology. In: The Ecology of Cyanobacteria, Whitton, A. and M. Potts, (Eds.). Kluwer Academic Publishers, The Netherlands, pp: 505-522.

Vonshak, A., 1997. *Spirulina platensis* (*Arthrospira*): Physiology, Cell-Biology and Biotechnology. Taylor and Francis Publishers, London, Pages: 233.

Vonshak, A., S. Boussiba, A. Abeliovich and A. Richmond, 1983. Production of *Spirulina* biomass: Maintenance of monoalgal culture outdoors. Biotech. Bioeng., 25: 341-349.

Zarrouk, C., 1966. Contribution to the study of Cyanophyceae. Influence of various' physical and chemical factors on growth and photosynthesis of *Spirulina maxima*. Ph.D. Thesis, University of Paris, Paris.

Zou, D. and K. Gao, 2009. Effect of elevated CO_2 on the red algae *Gracilaria lemaneiformis* (Gigartinales, Rhodophyta) grown at different irradiance levels. Phycologia, 48: 510-517.