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Studies on inorganic carbon uptake and photosynthesis in blue green alga *Nostoc calcicola*

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Abstract

Inorganic carbon uptake and utilization efficiency of a diazotrophic cyanobacterium, *Nostoc calcicola*, an 'usar' land isolate and its bicarbonate resistant mutant was investigated. The wild type strain showed significantly higher rate uptake of inorganic carbon as compared to mutant strain. The rate of photosynthesis and carbohydrate content of wild type strain was higher than mutant strain at ambient atmosphere, while at higher concentrations of inorganic carbon (100-250 mM $NaHCO_3$) mutant strain showed better response. However the photosynthetic rate and carbohydrate content of the mutant strain was higher than wild type at their respective optimal $NaHCO_3$ concentrations (75 and 250mM, respectively). This is indicative of the mutant strain required higher level of bicarbonate in the medium for optimal activity/growth. It may be concluded that the mutant strain is defective in carbon concentrating mechanism (CCM), and may provide a useful tool in understanding of CCM in these organisms, which in turn has a huge potential to act as global CO_2 sink.

Key words

Bicarbonate, Cyanobacteria, Carbon concentrating mechanism, *Nostoc calcicola*, Photosynthesis

Introduction

The earth's climate is the result of complex incompletely understood interactions among the sun, the atmosphere, the oceans, the land and the biosphere. The CO_2 level in the atmosphere was stabilized at about 100,000 years but it has been rising after the industrial revolution to the present level of about 385 ppmv (Solomon *et al.*, 2009). Current predictions are that atmospheric CO_2 is responsible for an estimated 60% of the global warming from the greenhouse gases produced by human activities. Natural photosynthesis and to a lesser extent solution into the ocean sink removes from the atmosphere about half of the CO_2 emitted by anthropogenic activities (Hall *et al.*, 1995). The accumulation of remaining of the CO_2 is of considerable concern. Various solutions have been proposed to mitigate the greenhouse effect of CO_2 emission. One of the options currently explored is the use of cyanobacteria as CO_2 (Badger *et al.*, 2006). Besides acting as CO_2 sink it exhibit close phylogenetic relationship with plant chloroplast (Grotjohann and Fromme, 2005), hence they can be used as model system for understanding the effect of increased CO_2 on the photosynthesis of higher plants

Cyanobacteria are unique photosynthetic microorganisms, which share metabolic activity of eukaryotes and cellular structure of prokaryotes. They are ubiquitous in nature and considered to be next to bacteria as far as their distribution is considered (Riding, 2006). They originated on earth 3.7 b years ago and during the long span of evolution they have acquired remarkable capability to adapt to changes in wide variety of environmental conditions(Riding, 2006). There have been tremendous fluctuations in the concentration of CO_2 in the atmosphere. The concentration of CO_2 prevailing in Precambrian era when cyanobacteria first arose was probably over 100-fold higher than present day condition (Badger *et al.*, 2006). Thereafter with time there was a large decline in CO_2 level and a

simultaneous increase in O_2 concentration. This would have posed significant pressure on cyanobacterial photosynthesis, which caused first evolutionary pressure for the development of carbon concentrating mechanism (CCM) in photosynthetic organisms (Badger *et al.,* 2006; Riding 2006) to concentrate CO_2 at the site of photosynthetic carboxylation (Price *et al.,* 1998). This property has made these organisms an attractive candidate for long term sequestering of atmospheric CO_2 as they raise the internal inorganic carbon (C_i) concentration 1000 times the concentration present in the external environment (Price *et al.,* 1998). Current investigation was undertaken to study the inorganic carbon uptake and utilization efficiency of a diazotrophic cyanobacterium, *Nostoc calcicola*, an 'usar' land isolate and its bicarbonate resistant mutant, capable of tolerating high level of inorganic carbon in the medium.

Materials and Methods

Organism and growth condition : *Nostoc calcicola* Breb, an 'usar' land isolate was cultivated and maintained in combined nitrogen-free Allen Arnon medium with A_5 trace elements and FeEDTA. The cultures were incubated at 25 ± 1°C with photon flux density of 25 $\mu Em^{-2}s^{-1}$ on the surface of culture vessels. The pH of the medium was maintained initially at 7.8 by using 10mM HEPES buffer. The bicarbonate resistant mutant of the cyanobacterium was isolated by chemical mutagenesis using MNNG (Jaiswal and Kashyap, 2002).

Estimation : The photo-pigments Chl *a* and carotenoids were quantified in terms of $\mu g\ mg^{-1}$ protein by following methods of Myers and Kratz (1955) and Allen (1968), respectively. Phycocyanin content of the cyanobacterial samples were analyzed at 610 by freezing and thawing method (Brody and Brody, 1961). The protein was estimated as per Lowry's method as described by Herbert *et al.* (1971).

Exponential phase cells of cyanobacteria were harvested in HEPES buffer (10mM, pH 7.8). O_2-evolution was measured using Clarke type electrode (Jaiswal and Kashyap, 2002). Anthrone reagent was used to determine simple sugars and their polymers by the method described by Herbert *et al.* (1971).

Exponentially growing cells of cyanobacteria were centrifuged, washed and suspended in medium without any inorganic carbon compound for starvation and incubated in dark for 72hr. Thereafter cells were centrifuged, washed and suspended in medium to the required level of cell density (3.36 $\mu g\ ml^{-1}$ Chl *a*). The reaction was initiated by addition of 100 µl $NaH^{14}CO_3$ (specific gravity 5µ Ci; Bhabha Atomic Research Centre, Bombay, India) to 20ml cyanobacterial sample and exposed to light (50 $\mu E\ m^{-2}s^{-1}$). At desired interval, 2 ml of cyanobacterial sample was withdrawn and transferred to scintillation vial containing 0.2 ml acetic acid (50%). The suspension was bubbled to drive out residual radioactivity. Subsequently 10 ml of scintillation cocktail was added and radioactivity was measured in liquid scintillation counter (Beckman, 7000).

Statistical analyses : At least three independent experiments were conducted and statistical analyses (two way ANOVA) were employed according to Scedecor and Cocharan (1967).

Results and Discussion

Photosynthetic O_2 evolution in *N. calcicola* and its HCO_3^{-R} mutant in response to graded concentration of $NaHCO_3$ (Fig 1a) revealed that at ambient atmosphere, O_2 evolution in mutant strain was two folds lower than wild type *N. calcicola.* The increasing level of inorganic carbon enhanced the O_2 evolution in both wild type as well as HCO_3^{-R} mutant, till optimal growth promoting concentrations (75mM for wild type and 250mM for mutant strain), thereafter there was decline in photosynthetic activity. The wild type *N. calcicola* showed maximum O_2 evolution at 75mM $NaHCO_3$ which was 87 % higher than the control. While the mutant strain showed 4.5 folds higher photosynthetic activity at 250mM compared to control. Further, it was 3.3 folds higher than the O_2-evolution recorded in wild type at their respective optimal growth promoting concentration. Analysis of variance (ANOVA) revealed that there were significant differences in O_2 evolution of wild type and mutant strain of *N. calcicola* due to bicarbonate concentrations ($F_{6,6} = 196.09$, $p < 0.001$). The cyanobacterium (wild and mutant strains) responded similarly when CO_2 was used as carbon source (Fig.1b) i.e at ambient CO_2, O_2 – evolution in mutant strain was lower than the wild type, while at higher levels of CO_2 (2-10%), the response of mutant strain was better than wild type. Also mutant strain was able to photosynthesize 2 times higher level of CO_2 as compared to wild type. Statistical analyses revealed that there were significant differences in O_2 evolution of wild type and mutant strain of *N. calcicola* due to CO_2 concentrations ($F_{6,6} = 52.08$, $p < 0.001$).

^{14}C uptake was comparatively higher in wild type strain at all the time of incubation. It was found to be 88% higher than the HCO_3^{-R} mutant at 10 min and 21% at 60 min (Fig. 2a). Experiment was performed to observe the ^{14}C uptake within 20 min of its linear uptake, the results showed a similar variation (Fig. 2b). The uptake rate was 1.6 folds higher in wild type *N. calcicola* as compared to HCO_3^{-R} mutant. The two ways analysis of variance showed that the different level of ^{14}C uptake by the two strains was significantly different at different time interval. ($F_{5,5} = 488.8$, $p < 0.001$).

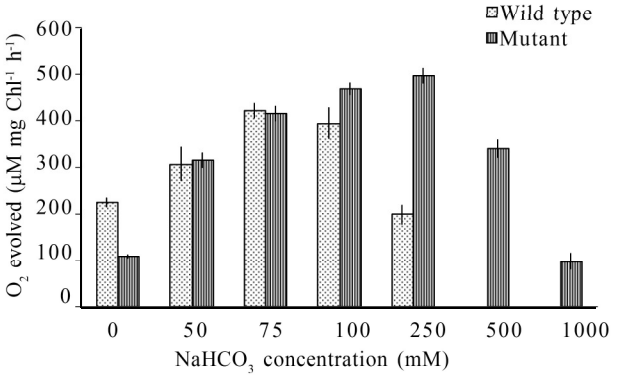


Fig. 1a : Oxygen evolution in *N. calcicola* (wild type and HCO_3^{-R} mutant) grown in varying $NaHCO_3$ concentrations. Cells grown in ambient atmosphere, without any bicarbonate supplementation served as control. Vertical bars on each column represent ± SD

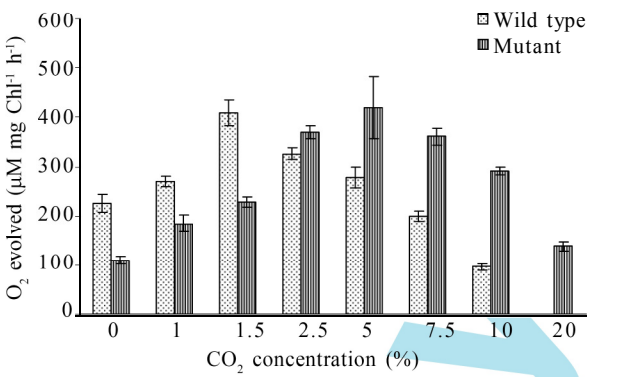
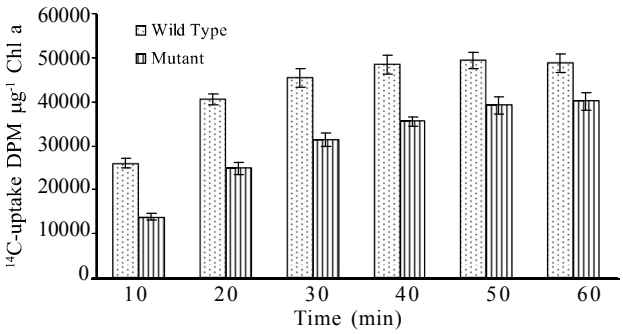


Fig. 1b : Oxygen evolution in *N. calcicola* (wild type and HCO_3^{-R} mutant) grown in varying CO_2 concentrations. Cells grown in ambient atmosphere, without any bicarbonate supplementation served as contron. Vertical bars on each column represent ± SD



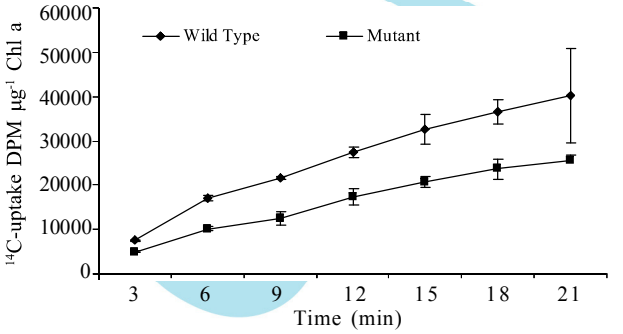


Fig. 2 : ^{14}C-uptake by *N. calcicola* (wild type and HCO_3^{-R} mutant). Vertical bars on each point represent ± SD

It is evident from the results that carbohydrate depletion in the mutant strain was lower as compared to wild type (Fig.3). The decline in carbohydrate level in wild type and mutant strain were 3.6 and 2.8 folds, respectively in comparison to carbohydrate level at 0 hr. In order to compare the depletion in carbohydrate content in wild type and mutant strain statistically in above said conditions two way ANOVA was performed and results revealed that the differences in depletion of carbohydrate of wild type and mutant strain with time were statistically significant ($F_{1,6} = 6.51, p < 0.001$).

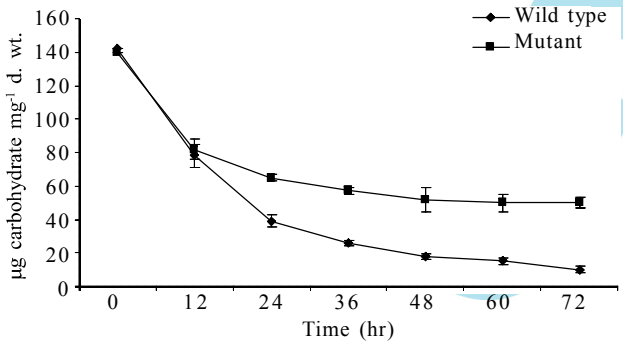


Fig. 3 : Carbohydrate depletion in *N. calcicola* (wild type and HCO_3^{-R} mutant). Vertical bars on each point represent ± SD

Cyanobacterial cells (wild type and mutant strains) grown at ambient atmosphere showed 6 fold increments in the carbohydrate content of the cell within 48hr of transfer (Fig. 4a). At later stage it became stabilized (data not shown). At growth promoting concentrations of CO_2 and $NaHCO_3$, the carbohydrate content of the wild type strain was 67 and 53% higher than control (cells grown in ambient atmosphere). In mutant strain, the carbohydrate content of the cells at optimum $NaHCO_3$ concentration was 2 folds higher than control, while cells grown in optimum level of CO_2 showed 50% higher carbohydrate content as compared to control showed a gradual increase (Fig 4b).

The inorganic carbon compounds available in the environment in the form of CO_2 or HCO_3^- is always limiting for carbon fixation of photosynthetic organisms. However, there is an uncertainty regarding the effect of increased CO_2 on photosynthesis especially if adequate amount of other nutrients are present. Hein and Sand-Jensen (1997) reported 15-19% increment in the primary productivity of ocean in response to elevated CO_2 concentration that will occur over the next 100-200 years. If this occurred the increased growth of producers would remove more CO_2 from the atmosphere and help to slow down global warming. Other studies suggest that the effect varies with different

Table 1: Photopigment ratio in *Nostoc calcicola* and its HCO_3^{-R} mutant, grown in ambient and growth promoting concentration of inorganic carbon (CO_2 / $NaHCO_3$)

$NaHCO_3$(mM)	*Nostoc calcicola (wild*type)		HCO_3^{R} Mutant	
	PC/Chl *a*	Chl *a*/ carotenoid	PC/Chl *a*	Chl *a*/ carotenoid
Ambient level CO_2	5.0	0.55	4.8	0.36
Optimum CO_2	5.52	0.56	5.4	0.37
Optimum $NaHCO_3$	5.80	0.58	5.6	0.36
Maximum CO_2 tolerance limit	6.16	0.57	5.9	0.38
Maximum $NaHCO_3$ tolerance limit	6.5	0.56	5.9	0.37

Cells were grown in ambient and growth promoting concentrations of CO_2 (1.5% and 5%, for wild typr and HCO_3^{-R} mutant , respectively) or $NaHCO_3$ (75 and 250 mM for wild typr and HCO_3^{-R} mutant , respectively). The pigment content were estimated after 10 days of growth. The data presented are mean values of 4 replicates. The growth medium was buffered with HEPES (10mM).

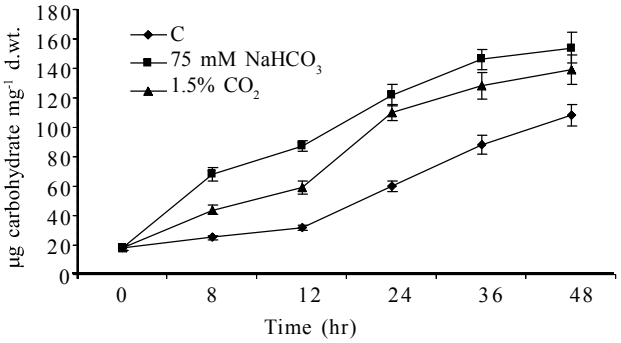


Fig. 4a : Changes in total carbohydrate content of *N. calcicola* (wild Type), in ambient atmosphere and growth promoting concentration of CO_2 (1.5%) and $NaHCO_3$ (75mM). Vertical bars on each point represent ± SD

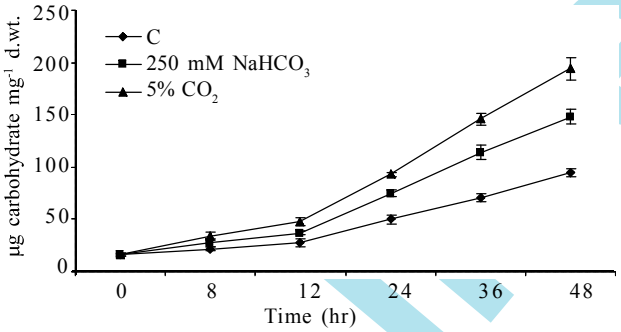


Fig. 4b : Changes in total carbohydrate content of bicarbonate resistant mutant of *N. calcicola*, in ambient atmosphere and growth promoting concentration of CO_2 (5%) and $NaHCO_3$ (250mM). Vertical bars on each point represent ± SD

types of plants in different climate zones (Hall *et al.,* 1995). Increase in inorganic carbon concentration in the medium has been reported to cause an increase in growth and change in morphological characteristics in *N. calcicola* and *Anabaena* sp. (Jaiswal and Kashyap, 2002; Jaiswal *et al.,* 2004). Our observations during the present investigation showed that there was an increase in the photosyhthetic activity of both wild type and mutant strain with inorganic carbon supplementation in the medium, as increase in inorganic carbon concentration in the medium (i.e the ultimate electron acceptor) would lead to an increased photolysis of water, hence increased O_2–evolution. However, the cyanobacterium (both wild type and mutant strain) showed differential response with respect to photosynthetic efficiency in $NaHCO_3$/CO_2 carbon supplemented media. This reflects the preferential utilization of $NaHCO_3$ by the cyanobacterium. This apparent difference could be related to the ecological habitat ('usar' soil) of the cyanobacterium, which is characterized by presence of high concentration of Na^+ and carbonate ion (Singh, 1961; Singh *et al.,* 2008). There was change in pigmentation pattern of both wild type and mutant strain with increasing C_i concentration. The PC/Chl *a* ratio increased at higher level of inorganic carbon, which is in accordance with that of Fu *et al.,* (2007), who reported higher PE, PC, and Chl a contents in cells grown under increased-CO_2 conditions.

Cyanobacteria are known to possess a carbon concentrating mechanism (CCM) that can be adapted to various environmental limitations (Hsin *et al.,* 2007) and they differ in suites of C_i transporters in each genome, the nature of carboxysome structures and the functional role of carbonic anhydrases. (Price *et al.,* 1998; Kaplan *et al.,* 2008). Different CCM activity can be induced within same species depending on availability of C_i-transporters with different affinities and specificities for either CO_2 or HCO_3^- as substrates. In current investigation, the higher photosynthetic efficiency of the cyanobacterium in $NaHCO_3$ can be attributed to active presence of bicarbonate transport system. Five distinct mode of C_i uptake systems has been reported in cyanobacteria (i) an inducible high affinity HCO_3^- transporter (Omata *et al.,* 1999), (ii) an inducible high affinity HCO_3^- transporter (Shibata *et al.,* 2002), (iii) low affinity Na^+ dependent HCO_3 transporter (Price *et al.,* 2004), (iv) a constitutive CO_2-uptake system (Shibata *et al.,* 2001; *Maeda et al.,* 2002; Price *et al.,* 2002) and (v) CO_2 uptake system inducible under C_i limitation (Shibata *et al.,* 2001; *Maeda et al.,* 2002). Growth and photosynthesis of cyanobacterium in CO_2 environment might be correlated to ivth and vth kind of transport system, as the cyanobacterium acclimatize itself

to the existing environment based on availability of C_i by inducing/switching over to CO_2 transport system.

The mutant strain required 3.3 folds higher concentration of Ci in the medium to express its maximum photosynthetic activity (O_2 evolution and carbohydrate content), in comparison to wild type, indicated that the affinity of transport system was altered in mutant strain, this view was further supported by the observation that ^{14}C incorporation rate was low in mutant as compared to wild type. The possibility of reduced rate of C_i transport may also be linked with the requirement of energy derived from photosynthesis as the photosynthesis rate in mutant strain was found to be significantly lower than wild type at lower concentration of inorganic carbon.

The rate of carbohydrate depletion in mutant strain was significantly lower than wild type *N. calcicola* suggesting that the mutant might have slow respiratory activity resulting in lesser break down of carbohydrate than wild type or it may show slower utilization of reserve food material for long term survival in dark (Schmetterer, 1994).

Diazotrophic cyanobacteria play a key role in maintaining the nitrogen and carbon budget. In the current investigation, the ability to photosynthesize at higher level of inorganic carbon and lower rate of C_i uptake by the bicarbonate resistant mutant reflects that the mutant strain is defective in CCM and hence can play an important role as CO_2 sink.

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