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The differential carbon-fixing and nitrogen-assimilating enzyme activities of Oscillatorian marine cyanobacterium *Phormidium valderianum* BDU 20041

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Abstract

We have identified an efficient filamentous marine cyanobacterium, *Phormidium valderianum* BDU 20041, which was adaptive to different concentrations of CO_2. In our study, *P. valderianum* BDU 20041 was grown under ambient (0.04) and elevated (2, 3, 4, 5, 10, and 15%) CO_2 concentration. Its optimal growth occurred at 3% CO_2 supply. *P. valderianum* displayed similar carbon fixation activities through Rubisco, phospho*enol*pyruvate carboxylase (PEPC), carbonic anhydrase, and malate dehydrogenase. The activity of Rubisco, the primary carbon-fixing enzyme, was found to be approximately 80% higher in *P. valderianum* grown under 4% CO_2 than under ambient air. This was substantiated by Western blot. The nitrogen-assimilating enzymes shared a similar trend at higher CO_2 concentrations. Our study is one of the few reports that examined the activities of CO_2-fixing and nitrogen-assimilating enzymes together. Due to the active PEPC, this cyanobacterium was found to have active C_3 and C_4 enzymes that promoted higher CO_2 fixation and cellular growth. Thus, it may be an efficient organism for biotechnological exploitation.

Additional key words: biomass productivity; elevated CO_2 concentration; nitrate reductase; nitrite reductase.

Introduction

Atmospheric CO_2 concentrations were 408.71 ppm in July 2018 and are increasing at a steady rate (NOAA 2018). At this rate, environmental experts believe that the concentrations will reach 500 ppm or higher by the end of the century (Kranz *et al*. 2010). Reports on effects of enhanced CO_2 concentration on the growth, lipid content, and fatty acid profile of microalgae are numerous. The microalgal response to elevated CO_2 concentrations depends extensively on the type of species. There are studies that have recorded increased growth of the cyanobacterial species at elevated CO_2 concentrations; while in contrast, some species were even inhibited by increased CO_2 concentrations (de Morais and Costa 2007, Chiu *et al*. 2008). Therefore, it is not possible to predict the long-term effects of elevated CO_2 on microalgal growth without improving the understanding of underlying mechanisms responsible for photosynthetic acclimation. For example, enzymatic activities that induce photosynthetic adaptation during microalgal growth in elevated CO_2 are poorly understood. However, there are very few studies on the effect of elevated CO_2 concentration on carbon-fixing enzymes and its implications for fixation (Jouanneau and Tabita 1987, Hayashi *et al*. 1995).

Variations in CO_2 concentrations supplied to cultures can affect amendments in cellular carbon acquisition pathways and elemental ratios, which are mostly species-specific (Raven 1997, Tortell *et al*. 2000, Burkhardt *et al*. 2001, Fu *et al*. 2007, Hutchins *et al*. 2007). Most of the studies on this topic have mainly focused on the activity of a single enzyme and its growth at elevated CO_2 concentrations. Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, EC 4.1.1.39) is the primary enzyme in the Calvin-Benson cycle (C_3 cycle), while phospho*enol*pyruvate carboxylase (PEPC, EC 4.1.1.31) is a chief enzyme in carbon fixation in the C_4 cycle. Carbonic anhydrase, a zinc metalloenzyme (CA, EC 4.2.1.1), catalyzes the reversible dehydration of HCO_3^- to CO_2. As Rubisco is a rate-limiting enzyme at lower carbon concentrations that

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Abbreviations: CA – carbonic anhydrase; Chl – chlorophyll; C_i – intracellular CO_2 concentration; GS – glutamine synthetase; MDH – malate dehydrogenase; NIR – nitrite reductase; NR – nitrate reductase; PEPC – phospho*enol*pyruvate carboxylase.
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accumulates carbon in carboxysomes of cyanobacteria, the functioning of carbonic anhydrase is crucial. Malate dehydrogenase (MDH, EC 1.1.1.37) catalyzes the interconversion of malate and oxaloacetate with NAD^+ or NADH as coenzymes, which also play a role in carbon metabolism. In a non-sulfur photosynthetic bacterium, *Rhodobacter sphaeroides*, Rubisco biosynthesis was repressed, and its activities were inhibited at high concentrations of CO_2 (6%) (Jouanneau and Tabita 1987); in contrast, in the thermophilic cyanobacterium, *Chroococcidiopsis* sp. strain TS-821, the enzyme activity was enhanced in response to higher CO_2 concentrations (5 and 10%) (Hayashi *et al*. 1995).

In addition to carbon fixation, it has been suspected that increased CO_2 availability may potentially reduce the energy or nutrients needed for carbon acquisition (Hutchins *et al*. 2007). Reports on *Trichodesmium* sp. have shown that externally supplied CO_2 has a role in determining the nitrogen- and carbon-fixing rates (Barcelos e Ramos *et al*. 2007, Hutchins *et al*. 2007, Levitan *et al*. 2007).

This study aimed to assess the CO_2 fixation and nitrogen assimilation of enzymes at different CO_2 concentrations. The four major enzymes involved in CO_2 fixation, Rubisco, PEPC, CA, MDH, and three nitrogen-assimilating enzymes, nitrate reductase (NR, EC 1.7.99.4), nitrite reductase (NIR, EC 1.7.2.1), and glutamine synthetase (GS, EC 6.3.1.2), were investigated in the Oscillatorian filamentous non-heterocystous cyanobacterium *P. valderianum* BDU 20041. This organism was screened out of 18 selected organisms (data not shown) and was validated through radiometric, electrometric, and colorimetric assays. The expression of Rubisco at various CO_2 concentrations was also confirmed by an immunoblot technique.

Materials and methods

Cyanobacterial strain and culture conditions: The cyanobacterial strain used in this study is a non-heterocystous, filamentous organism, named *Phormidium valderianum* BDU 20041, which was acquired from the germplasm of the National Facility for Marine Cyanobacteria, Bharathidasan University, Tiruchirappalli, India. The organism was grown and conserved in ASN III (artificial sea nutrient) medium (Rippka *et al*. 1979) in a controlled culture room at 25 ± 2°C with continuous aeration and illumination with white fluorescent light of an intensity of 20 µmol(photon) m^{-2} s^{-1} .

Cyanobacterial growth with continuous CO_2: An initial inoculum equivalent to 0.1 g L^{-1}(dry mass) of *P. valderianum* BDU 20041 culture at the mid-log phase was inoculated into ASN III medium. The media containing the cultures were continuously bubbled with different concentrations of CO_2, *i.e.*, 2, 3, 4, 5, 10, and 15% at 0.1 vvm flow rate using a 99.9% pure CO_2 cylinder (*Bhuruka Gases*, Bangalore, India) aided with an aerator for 24 h with a continuous light intensity of 20 µmol(photon) m^{-2} s^{-1}. The temperature was maintained at 25 ± 2°C. The organism in ASN III medium aerated with ~ 0.04% CO_2 (ambient air) served as the control. Unbuffered medium was used for all the experiments. Triplicate values of chlorophyll (Chl) and dry mass were recorded, averaged, and plotted with standard deviations.

Preparation of cell extract: Mid log-grown cultures were harvested after 12 and 24 h by centrifugation at 5,000 × *g*, and the pellets were washed twice with Tris-HCl buffer (pH 7.8). Cold mortar, pestle, and glass powder were used to homogenize the cyanobacterial pellets. The cellular debris and other unwanted components were removed by centrifuging the homogenate at 20,000 × *g* for 30 min. The supernatant was withdrawn and spun again for further purification. The resultant blue-colored supernatant was used as the crude enzyme extract, and the protein concentration was determined according to Lowry *et al*. (1951).

Enzyme assays: carbon-fixing enzymes

Rubisco: The carboxylase activity of the Rubisco enzyme was radiometrically analyzed following the method of Perchorowicz *et al*. (1981) with some modifications. The assay mixture consisted of 2.19 mM ribulose-1,5-bis-phosphate, 0.898 mM $NaHCO_3$ with 46.9 kBq of ^{14}C radioactivity and 10 mM $MgCl_2$. We added 25 µl of assay mixture to 50 µl of the extract, and the mixture was incubated for 5 min. The reaction was terminated by adding 400 µl of 25% trichloroacetic acid (TCA). TCA removes any unbound radiolabelled carbon and the ^{14}C radioactivity incorporated in the acid-stable end products were measured in a liquid scintillation counter (*Ramona Star*, *Elysisa Raytest*, Germany). The rate of end product formation was determined from the rate of ^{14}C incorporation and expressed as µmoles of CO_2 fixed per mg of protein. From the specific activity of $NaH^{14}CO_3$ the amount of CO_2 fixed is determined.

PEPC: The radiometric assay of PEPC activity was performed as defined by Owttrim and Colman (1986) with some variations. The standard assay mixture consisted of 50 mM TES buffer at pH 7.5, 5 mM $MgCl_2$, 2.5 mM phospho*enol*pyruvate (PEP), 25 mM $NaH^{14}CO_3$ (specific activity, 1.8 MBq $mmol^{-1}$) and 25 µl of the crude enzyme. Background radiations were nullified with a blank solution. The assay mixtures without PEP were incubated at 30°C for 10 min. The reaction components were mixed with PEP, and after 10 min, 0.1 ml of 25% trichloroacetic acid was added to stop the reaction. The radioactivity incorporated was measured using a radiospectrophotometer (*Raytest Isotopenmessgeraete*, Germany). The rate of PEPC-mediated oxaloacetate formation per mg of the protein was calculated from the rate of ^{14}C incorporation, considering the specific activity of the added $NaH^{14}CO^3$, and the absolute amount of dissolved inorganic carbon in the assay mixture was determined.

Carbonic anhydrase: The electrometric method of Wilbur and Anderson (1948) was used to assay the activity of carbonic anhydrase. Four ml of ice-cold CO_2-saturated water was added to the reaction mixture of 0.2 M Tris-HCl buffer, pH 8.0, and 0.1 ml of the freshly diluted enzyme.

The time required for the pH to drop from 8.3 to 6.3 was recorded. A unit of enzyme is defined as [2 × (T0 – T)]/T, where T is time taken with enzyme and T0 is without enzyme. Enzyme units is calcualted by 2 × (T0 – T)/(T × mg of protein in reaction mix).

Malate dehydrogenase: The activity was assayed following Baker and Bramhall (1972) and the assay mixture consisted of 6 mM oxaloacetic acid and 3.7 mM of NADH in 0.1 M potassium phosphate buffer at pH 7.4. On addition of the enzyme, decrease in ΔA_{340} was recorded for 3–5 min. Enzyme units per mg of protein calcualted by Δ_{340}/[6.22 × mg(protein) in ml of reaction mix].

Enzyme assays: nitrogen-assimilating enzymes

Nitrate and nitrite reductase: The assay of nitrate reductase and nitrite reductase activity was based on the reduction of nitrate and nitrite, which utilize sodium dithionite-reduced methyl viologen as the electron donor (Herrero and Guerrero 1986). The reaction product, diazotized sulphanilamide, further reacts with N-(1-naphthyl) ethylenediamine dihydrochloride and forms a red dye. The absorbance was read at 540 nm (*Cary 100 bio* UV-VIS spectrophotometer, *Varian*, USA) and the activity was expressed as the µg(nitrite formed) (for NR) or removed (for NIR) g^{-1}(dry mass) min^{-1}.

Glutamine synthetase activity was estimated in the washed cyanobacterial pellet permeabilized by toluene at 4°C. The activity was calculated *in vitro* by incubating the supernatant with 1 ml of reaction mixture for 30 min in the dark at 37°C (Shapiro and Stadtman 1970). At the end of the reaction, the A_{540} was measured (*Cary 100 bio* UV-VIS spectrophotometer, *Varian*, USA), and the activity was expressed as µg(γ-glutamyl hydroxamate formed) g^{-1}(dry mass) min^{-1}.

Western blotting for Rubisco: Proteins separated using 12% SDS-PAGE were transferred onto a nitrocellulose membrane at 12 V for 1 h using a semi-dry Western blotting system (*Bio-Rad*, Hercules, CA, USA). Skimmed milk powder (3%, w/v) in Tris-buffered saline with 0.1% *Tween-20* (TBS-T) was used to block the membrane for an hour. After three washes with TBS-T for 5 min each, the membrane was incubated with polyclonal anti-Rubisco antibody produced in chicken (*Sigma*, St. Louis, MO) at a dilution of 1:1,500 overnight at 4°C. After washing, the membrane was incubated for 1 h with anti-chicken immunoglobulin G (IgG) peroxidase-coated antibody (*Sigma*, St. Louis, MO) at a dilution of 1:10,000. After another wash, the blots in the membrane were developed by the addition of 4-chloro-naphthol (*Sigma*, St. Louis, MO) and 30% H_2O_2 substrate solution. The developed membrane was scanned using *BioRAD Gel Doc EZ* imager and the scanned image was processed with *Image Lab Software 6.0* (*BioRad* Laboratories, USA).

Statistical analysis: Various data obtained in the study were statistically analysed by *Tukey*'s HSD test ($p<0.05$) in *Matlab 16* and plotted in *Microsoft Excel* and *Origin*.

Results

Growth of *P. valderianum* BDU 20041 at different CO_2 concentrations, *i.e.*, 0.04 (ambient air), 2, 3, 4, 5, 10, and 15%, was calculated in terms of Chl *a* (Fig. 1). However, the cultures were capable of tolerating the continuous flow of all the tested CO_2 concentrations; specifically, 3% CO_2 was found to yield increased growth compare to other tested concentrations. This concentration reflected a higher specific growth rate (0.226 ± 0.007 µg d^{-1}) and biomass productivity (83 ± 3.98 mg L^{-1} d^{-1}) at 3% CO_2 (Table 1).

Effect of CO_2 on Rubisco activity and content: The Rubisco activity of *P. valderianum* BDU 20041 harvested after 12 and 24 h at different CO_2 concentrations is shown in Fig. 2*A*. The organism subjected to 3 and 4% CO_2 showed higher Rubisco activity. After 12 h, the enzyme activities of the cultures grown with different CO_2 concentrations increased with an increase in the CO_2 concentration, reaching maximum activity at 15% CO_2. The activity of 15% CO_2-grown cells was 2.26 µmol(fixed CO_2) min^{-1} mg^{-1}(protein), which was more than double of that under ambient air-grown cultures. The enzyme activity after 24 h of growing with 2–5% CO_2 was higher than that under the ambient air by 47.4, 75.5, 80.6, and 54.1%, respectively. The maximum activity recorded at 4% CO_2 was 3.54 µmol(CO_2 fixed) min^{-1} mg^{-1}(protein). The activity at a higher CO_2 concentration was inversely proportional (Fig. 2*A*).

The increased activity of Rubisco after 12 h with the increased CO_2 showed that the activity was CO_2-concentration dependent. To substantiate the above finding, the protein extract of *P. valderianum* BDU 20041 grown in various CO_2 concentrations for 12 h was analysed by immunoblotting for Rubisco. The Western blot of Rubisco corroborated the results of the radiometric assay.

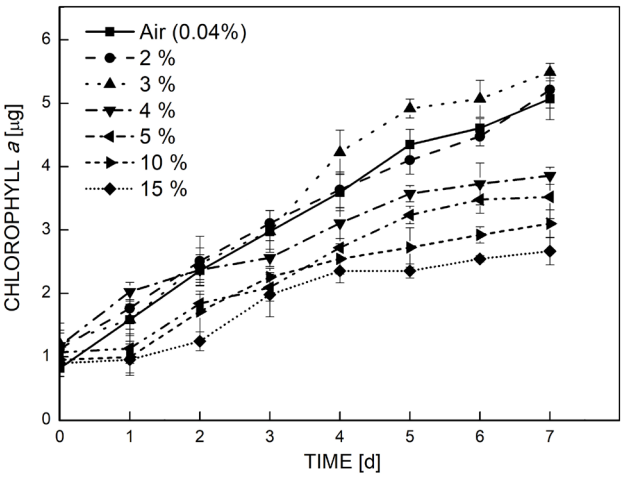

Fig. 1. Effect of the concentration of CO_2 aeration on the growth (expressed as Chl *a* content) of *Phormidium valderianum* BDU 20041. In the cultures, 1.0 g of algal cells were inoculated and cultivated under continuous supply of ambient air, 2, 3, 4, 5, 10, and 15% CO_2. Values are means ± SD (n = 3).

Table 1. Growth performance of *Phormidium valderianum* BDU 20041 sparged with different concentrations of continuous CO_2 for seven days. Values (mean ± SD, $n = 3$) with *the same letters* are not significantly different according to *Tukey*'s HSD test ($p<0.05$).

CO_2 [%]	Biomass productivity [mg L^{-1} d^{-1}]	Specific growth rate [μ?]	CO_2 fixation rate [mg(C) L^{-1} d^{-1}]
0.04	66.66 ± 3.45^{C}	0.183 ± 0.002^{C}	122.21 ± 4.08^{C}
2	72.13 ± 3.12^{B}	0.198 ± 0.003^{B}	126.57 ± 5.17^{B}
3	83.33 ± 3.98^{A}	0.221 ± 0.007^{A}	152.77 ± 6.74^{A}
4	65.00 ± 3.59^{C}	0.176 ± 0.004^{C,D}	122.24 ± 5.32^{C}
5	61.33 ± 1.08^{D}	0.170 ± 0.005^{C,D}	112.43 ± 3.70^{D}
10	50.33 ± 2.56^{E}	0.163 ± 0.004^{D}	92.27 ± 4.24^{E}
15	51.00 ± 1.87^{E}	0.169 ± 0.001^{D}	93.5? ± 3.55^{E}

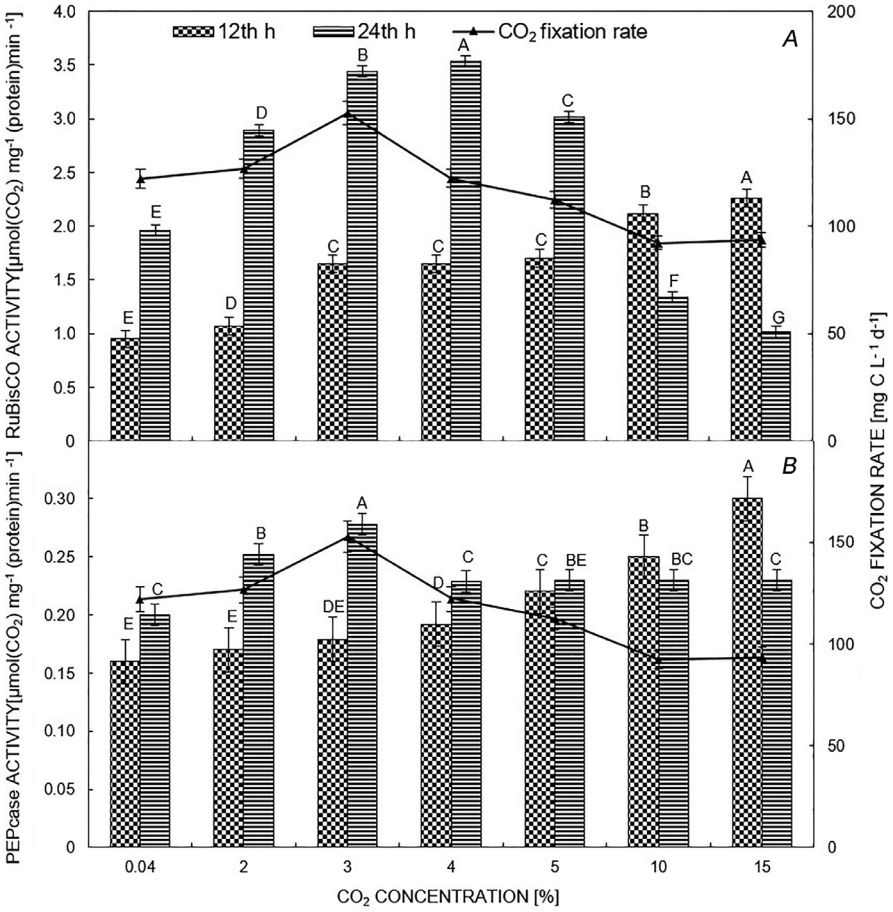

Fig. 2. Activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) (*A*) and phospho*enol*pyruvate carboxylase (PEPC) (*B*) of *Phormidium valderianum* BDU 20041 during growth in continuous aeration of ambient air and elevated CO_2, recorded at 12 and 24 h. In order to determine the correlation between the CO_2 assimilation rate and enzyme activities, the CO_2 fixation rate of the organism grown at different CO_2 concentration is plotted on the secondary *y*-axis. Values are means ± SD ($n = 3$). Different *capital letters* indicate a significant difference by *Tukey*'s test ($p<0.05$).

Western blot of *P. valderianum* BDU 20041 showed that the Rubisco content was higher with increasing CO_2 concentrations (Fig. 3). The Rubisco content was almost four and ten times higher than that of ambient air-grown cultures. The 24-h incubated cultures did not show much deviation in their content, and the 3% CO_2-grown cultures revealed a slightly more intense band, indicating higher Rubisco content. The cultures grown at the CO_2 concentration above 3% (*i.e.*, 4, 5, 10, and 15%) showed the reduced Rubisco content compared to the ambient air-grown cultures (Fig. 3). Interestingly, after 24 h, the activity decreased in cultures grown at CO_2 above 3%, while the Rubisco content remained the same in all the concentrations.

Effect of CO_2 on PEPC activity: PEPC, the principal C_4 carbon fixation enzyme, showed a pattern similar to the Rubisco activity at the tested CO_2 concentrations. After 12 h, a maximal activity of 0.3 μmol(CO_2 fixed) min^{-1} mg^{-1}(protein) was observed in the 15% CO_2 cultures, whereas after 24 h, the activity decreased in cultures with CO_2 >3%,, similar to the pattern observed with the Rubisco activity. The maximum activity of the enzyme [0.2708 μmol(CO_2 fixed) min^{-1} mg^{-1}(protein)] after 24 h was observed at the 3% CO_2 concentration (Fig. 2*B*).

The carbon-fixation rate was reflected in the growth of *P. valderianum* BDU 20041 with different elevated CO_2 concentrations. The amounts and activities of Rubisco and PEPC under given environmental conditions are

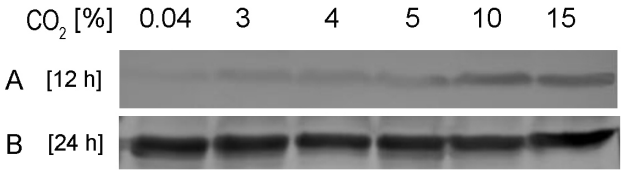

Fig. 3. Western blot analysis for ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) of *Phormidium valderianum* BDU 20041 grown in ambient air and elevated CO_2 concentration for 12 (*A*) and 24 h (*B*). The blots were developed by specific monoclonal antibodies against Rubisco and developed using secondary antibody linked with horse radish peroxidase and 4-chloronaphthol.

important factors in the regulation of CO_2 assimilation. Hence, it is worthwhile to infer the relationship between the carbon fixation rate and the recorded activities of the carbon-fixing enzymes. The CO_2-fixation rate stoutly correlated with the activities of Rubisco and PEPC. In *P. valderianum* BDU 20041, the CO_2-fixation rate increased with CO_2 concentration up to 3% together with the enzyme activities, even after 24 h **(**Fig. 2).

Effect of CO_2 on CA and MDH: The CA activity was reflected in the cultures flushed with different CO_2 concentrations; it showed an increasing activity with enhanced CO_2 concentrations after 12 h. *P. valderianum* BDU 20041 cultures grown with 15% CO_2 showed a maximum activity of 14.11 U mg^{-1}(protein) after 12 h. Nevertheless, unlike Rubisco (C_3) and PEPC (C_4), the CA activity did not show any difference after 24 h. The activity remained almost similar at all the CO_2 concentrations tested (Fig. 4*A*). As in the other three enzymes, MDH activity also increased with the increasing CO_2 concentration after 12 h. Interestingly, the maximum activity of MDH was found at 5% after 24 h (0.25 U mg^{-1}(protein) (Fig. 4*B*).

Nitrogen-assimilating enzymes at elevated CO_2: The nitrogen-assimilating enzymes, NR, NIR, and GS of *P. valderianum* BDU 20041, were assayed for their activity after 12 and 24 h of the exposure to the enhanced CO_2 concentrations of 3 and 15%. The activities of all three nitrogen-assimilating enzymes, in samples grown with 3% CO_2, showed higher activities than that under the ambient air and 15% CO_2 after both 12 and 24 h. Irrespective of the cellular demand, the assimilation of nitrogen was very

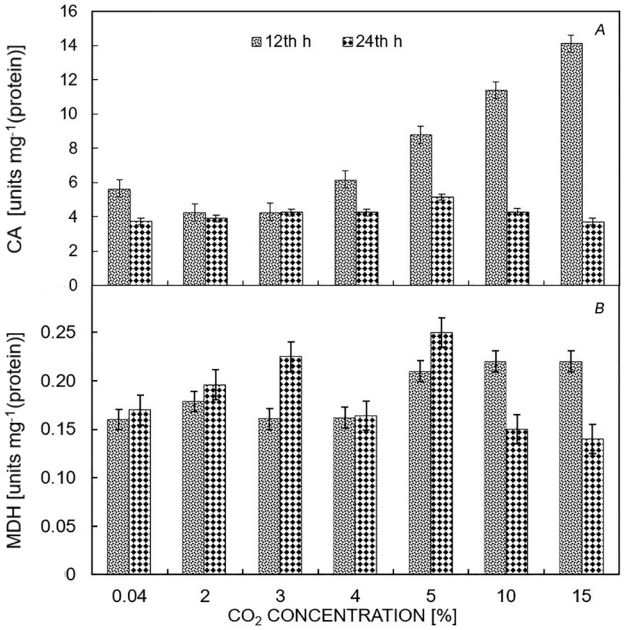

Fig. 4. Carbonic anhydrase (CA) (*A*) and malate dehydrogenase (MDH) (*B*) activity in *Phormidium valderianum* BDU 20041 grown in ambient air and elevated CO_2 concentration (2, 3, 4, 5, 10, and 15% CO_2) for 12 and 24 h. Values are means ± SD (n = 3).

low at 15% CO_2 in *P. valderianum* BDU 20041. This was reflected in the NR and NIR activity, which decreased by 68 and 75% (Table 2) and probably caused reduced growth.

Discussion

Although a wealth of information is available on the structure, function, and activity of the major carbon-fixing enzymes, such as Rubisco and PEPC, the response of these enzymes to higher carbon concentrations is yet to be studied extensively (Sicher *et al*. 1994, Stöckel *et al*. 2013). Our study focused on the effect of different CO_2 concentrations on some important carbon-fixing and nitrogen-metabolizing enzymes at varying time periods of *P. valderianum* BDU 20041 growth.

P. valderianum BDU 20041, when grown at various CO_2 concentrations, was found to have optimum CO_2 concentration of 3%. There are many reports on improved cyanobacterial growth at higher CO_2 concentrations (Mo-

Table 2. Activity of nitrogen assimilating enzymes of *Phormidium valderianum* BDU 20041 grown in ambient air (0.04% CO_2) and elevated CO_2 concentration (3 and 15% CO_2) for 12 and 24 h. Values (mean ± SD, n = 3) with *the same letters* are not significantly different according to *Tukey*'s HSD test (p<0.05).

CO_2 [%]	Nitrate reductase [µg(nitrite formed) g^{-1}(DM) min^{-1}]		Nitrite reductase [µg(nitrite removed) g^{-1}(DM) min^{-1}]		Glutamine synthetase [mg(γ-glutamylhydroxamate formed) g^{-1}(DM) min^{-1}]	
	12 h	24 h	12 h	24 h	12 h	24 h
0.04	3.308 ± 0.123^{B}	3.787 ± 0.099^{B}	3.260 ± 0.114^{B}	3.625 ± 0.213^{B}	3.557 ± 0.113^{B}	4.073 ± 0.217^{A}
3	3.855 ± 0.275^{A}	$4.151 \pm 0.14o^{A}$	3.526 ± 0.058^{A}	4.352 ± 0.281^{A}	4.021 ± 0.226^{A}	4.541 ± 0.183^{A}
15	2.972 ± 0.236^{C}	1.174 ± 0.294^{C}	2.987 ± 0.154^{C}	1.015 ± 0.069^{C}	2.932 ± 0.307^{C}	1.929 ± 0.077^{B}

roney and Somanchi 1999, Tang *et al.* 2011). *Aphanothece microscopica Nägeli* grew without any inhibition at a higher CO_2 concentration of 2–20% (Jacob-Lopes and Franco 2013). However, concentrations of 5% or more harmed the growth of *Chlorella* sp. (Chiu *et al*. 2008). This study aimed to look into the underlying physiological process that promotes growth at elevated CO_2.

Radiometric assay of Rubisco showed higher enzyme activity at 3% which leads to the conclusion that CO_2 concentration up to a certain level enhanced the activity of the primary carbon-fixing enzyme Rubisco in the Oscillatorian cyanobacterial form of *P. valderianum* BDU 20041. Through a variety of approaches (Friedberg *et al*. 1989, Pierce *et al*. 1989, Price and Badger 1989) it has been demonstrated that Rubisco and carbonic anhydrase localization in the carboxysomes is essential for the CO_2-concentrating mechanism in cyanobacteria. Cultures grown at 15% CO_2 showed a lower activity than the cultures that received ambient CO_2 (Fig. 2*A*). This result was similar to the pattern observed by Sicher *et al*. (1994) in tobacco plants grown under elevated CO_2, which showed lower Rubisco activity to ambient air-grown cultures. This result was also concurrent with the finding of Stöckel *et al*. (2013), where lower levels of transcripts associated with CO_2 and bicarbonate uptake were recorded. In another study, the Rubisco activity of the *Chroococcidiopsis* sp. strain TS-821 increased at 5% CO_2 but decreased at 10,15, and 20% CO_2, agreed with our finding (Hayashi *et al.* 1995).

At high concentrations of CO_2 (6%), Rubisco biosynthesis in *Rhodobacter sphaeroides*, a non-sulfur photosynthetic bacterium, was found to be lower (Jouanneau and Tabita 1987). The number of carboxysomes and the Rubisco activity increased in *Synechococcus* PCC7942 cells when transferred to extremely low CO_2 concentrations; however, the effect of higher extracellular CO_2 concentration on the activity of Rubisco has not been examined (Harano *et al*. 2003). Cheng and Fuchigami (2000) have recorded higher Rubisco activity with increasing nitrogen availability. The above finding and the results from this study makes us hypothesize that the increasing CO_2 in the medium causes cells starving for nitrogen and hence it results in reduced Rubisco activity. The Western blot of *P. valderianum* BDU 20041 showed that increasing CO_2 concentration had positive effect on the Rubisco content and it corroborated with our findings in radiometric determination of Rubisco activity. With the support of the radiometric and Western blot assays, our results revealed that both the Rubisco activity and its content were higher at the elevated CO_2 concentration after a 12-h growth period. Thus, it is safe to infer that the variation in activity is probably because of the increased synthesis of Rubisco leading to higher activity.

Our recent *in silico* analysis of the protein sequences of the cyanobacterial PEPC revealed that cyanobacteria from the order Oscillatoriales and Nostocales showed the presence of a C_4 determinant serine residue at position 923, which is similar to that of the C_4 plant, which is replaced by glycine in lower orders. These higher cyanobacterial orders possess a conserved PEPC-activating serine residue at position 39 in the N-terminus that is required for phosphorylation, a very important characteristic feature of the C_4 PEPC. Hence, this finding suggests the similarity of higher cyanobacterial PEPC to C_4 isoforms of higher plants (Shylajanaciyar *et al*. 2015). CO_2 fixation through PEPC in the selected organism *P. valderianum* BDU 20041 (Oscillatoriales) is thus justified. PEPC activity was proportional to the CO_2 increase after 12 h, however, the cyanobacterium showed lower PEPC activities at a higher CO_2 concentration at the end of 24-h period (Fig. 2*B*). Our results are in agreement with the findings of Omoto *et al*. (2012), who demonstrated that at decreased CO_2 concentrations, the PEPC activity was higher, and at higher CO_2 concentrations, it is speculated to be down-regulated, similarly to Rubisco. The correlation of CO_2 fixation rate with the activities of Rubisco and PEPC is fairly logical. Hudson *et al*. (1992) also recorded a similar correlation between the CO_2 assimilation rate and the Rubisco activity.

The poor activities of these enzymes at the higher CO_2 concentration could be the reason for stunted cyanobacterial growth at higher CO_2 (> 10%). In-depth studies to enhance enzyme activities at higher CO_2 could be a major breakthrough for implementation of microalgal carbon capture and storage.

The enzyme CA is involved in the catalysis of the interconversion of HCO_3^- and CO_2 in the periplasm space. The CA activity was increasing with CO_2 concentration till 12 h but after 24 h, the activity of CA decreased and was almost similar at all the concentrations. Our results corroborate the findings of Chen and Gao (2003) in a marine diatom *Skeletonema costatum* and of Sicher *et al*. (1994) in tobacco plants. In tobacco plants, the CA activity of high concentration CO_2-grown cells was observed to be higher until day 4, after which the activity was lower than that of the ambient CO_2-grown cells.

Jaiswal *et al*. (2005) found increasing CA activity in two cyanobacteria, *Anabaena* sp. (ARM 629) and *Nostoc calcicola*, which were transferred from a higher CO_2 concentration (2–25%) to ambient CO_2 concentration. The cells grown with ambient air (low C_i cells) showed higher CA activity in comparison with the cells grown with 2–4% CO_2 (high C_i cells) (Shiraiwa and Miyachi 1985). Reports are scarce on the effects of transferring cultures from ambient to elevated CO_2 concentrations on the CA activity of microalgae. The present investigation indicates that the CA activity decreased when the cells grown in a lower concentration of inorganic carbon were transferred to the high C_i condition (enhanced CO_2). This is because of the continuous addition of carbonates into the medium, which nullifies the necessity for carbonic anhydrase to accumulate carbonates around the carboxysomes. MDH activity did not show a large difference between the different CO_2 concentrations and the time intervals tested (Fig. 4*B*).

For the cyanobacterial growth, ammonia is the preferred source of nitrogen, as the different nitrogen compounds, which serve as nutrients, are first converted to ammonia intracellularly (Johnson *et al*. 2017). In cyanobacteria, nitrate reductases (NR) and nitrite reductases (NIR) are ferredoxin-dependent enzymes, which provide ammonium.

The ammonium is then incorporated into 2-oxoglutarate carbon skeleton, which results in the synthesis of glutamate (Flores and Herroro 1994).

The reduced nitrogen assimilation might be another reason for the reduced growth of the organism at 15% CO_2. Fonseca *et al*. (1997) reported transient increases in the *Nia* transcript in leaves and, especially, in the roots of *Plantago major* during the first 4 d after the transfer to higher CO_2 concentrations. A transient 50% increase in the NR activity in the shoots and a doubling of the NR activity in the roots accompanied this result. In our study, increasing CO_2 concentration to 3% resulted in increased activity of the nitrogen-assimilating enzymes, while an increase to 15% retarded the activity of these enzymes. It is difficult to hypothesize the factor that decides the switch of the mechanism from stimulation to retardation. One possible reason is that, at elevated CO_2 concentrations, the nitrate/nitrogen available for uptake gets reduced for reasons that are poorly understood (Geiger *et al*. 1999), thus lowering the assimilation rates. The changes in nitrogen assimilation at elevated CO_2 have not yet been studied in detail. Knowledge of the regulation of nitrogen metabolism and its interaction with carbon metabolism and growth processes need to be evaluated further through elaborate investigations.

Conclusions: Hence, it can be concluded that the better growth of the marine cyanobacterium *P. valderianum* BDU 20041 at higher CO_2 concentrations of up to 3% is because of the maximum activities of carbon-fixing and nitrogen-assimilating enzymes. The activities of the enzymes at higher CO_2 concentrations (> 3%) were diminished, thereby causing a reduction in the CO_2 fixation rate and growth at elevated CO_2 concentrations. Further studies need to focus on the reasons for decreased growth at higher CO_2 concentrations.

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