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

G. DINESHBABU*,** V.S. UMA***, M. SHYLAJANACIYAR*, V. RASHMI**, D. PRABAHARAN*, and L. UMA**

National Facility for Marine Cyanobacteria, Department of Marine Biotechnology, School of Marine Sciences, Bharathidasan University, 620 024 Tiruchirappalli, Tamil Nadu, India

Bioprocess Development Laboratory, Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Guwahati, Assam, India

Environmental Safety Division, Indira Gandhi Centre for Atomic Research, Kalpakkam, Tamil Nadu, India

Department of Microbiology, Srimad Andavan College of Arts and Science, Tiruchirappalli, Tamil Nadu, India

Abstract

We have identified an efficient filamentous marine cyanobacterium, *Phormidium valderianum* BDU 20041, which was adaptive to different concentrations of CO₂. In our study, *P. valderianum* BDU 20041 was grown under ambient (0.04%) and elevated (2, 3, 4, 5, 10, and 15%) CO₂ concentration. Its optimal growth occurred at 3% CO₂ supply. *P. valderianum* displayed similar carbon fixation activities through Rubisco, phosphoenolpyruvate 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% CO₂ than under ambient air. This was substantiated by Western blot. The nitrogen-assimilating enzymes shared a similar trend at higher CO₂ concentrations. Our study is one of the few reports that examined the activities of CO₂-fixing and nitrogen-assimilating enzymes together. Due to the active PEPC, this cyanobacterium was found to have active C₃ and C₄ enzymes that promoted higher CO₂ fixation and cellular growth. Thus, it may be an efficient organism for biotechnological exploitation.

**Additional key words:** biomass productivity; elevated CO₂ concentration; nitrate reductase; nitrite reductase.

Introduction

Atmospheric CO₂ 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 CO₂ concentration on the growth, lipid content, and fatty acid profile of microalgae are numerous. The microalgal response to elevated CO₂ concentrations depends extensively on the type of species. There are studies that have recorded increased growth of the cyanobacterial species at elevated CO₂ concentrations; while in contrast, some species were even inhibited by increased CO₂ concentrations (de Morais and Costa 2007, Chiu et al. 2008). Therefore, it is not possible to predict the long-term effects of elevated CO₂ 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 CO₂ are poorly understood. However, there are very few studies on the effect of elevated CO₂ concentration on carbon-fixing enzymes and its implications for fixation (Jouanneau and Tabita 1987, Hayashi et al. 1995).

Variations in CO₂ 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, Burkhart 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 CO₂ concentrations. Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, EC 4.1.1.39) is the primary enzyme in the Calvin-Benson cycle (C₃ cycle), while phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) is a chief enzyme in carbon fixation in the C₄ cycle. Carbonic anhydrase, a zinc metalloenzyme (CA, EC 4.2.1.1), catalyzes the reversible dehydration of HCO₃⁻ to CO₂. As Rubisco is a rate-limiting enzyme at lower carbon concentrations that...
accumulates carbon in carboxylases 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 NADH or NADPH 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 CO2 (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 CO2 concentrations (5 and 10%) (Hayashi et al. 1995).

In addition to carbon fixation, it has been suspected that increased CO2 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 CO2 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 CO2 fixation and nitrogen assimilation of enzymes at different CO2 concentrations. The four major enzymes involved in CO2 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, electrophoretic, and colorimetric assays. The expression of Rubisco at various CO2 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 aeration and illumination with white fluorescent light of a continuous light intensity of 20 μmol(photon) m–2 s–1.

Cyanobacterial growth with continuous CO2: 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 CO2, i.e., 2, 3, 4, 5, 10, and 15% at 0.1vvm flow rate using a 99.9% pure CO2 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% CO2 (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 NaHCO3 with 46.9 kBq of 14C radioactivity and 10 mM MgCl2. 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 14C radioactivity incorporated in the acid-stable end products were measured in a liquid scintillation counter (Ramona Star, Elysia Raytest, Germany). The rate of end product formation was determined from the rate of 14C incorporation and expressed as μmoles of CO2 fixed per mg of protein. From the specific activity of NaH14CO3, the amount of CO2 fixed is determined.

PEPC: The radiometric assay of PEPC activity was performed as defined by Owtrtrim and Colman (1986) with some variations. The standard assay mixture consisted of 50 mM TES buffer at pH 7.5, 5 mM MgCl2, 2.5 mM phosphoenolpyruvate (PEP), 25 mM NaH14CO3, (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 14C incorporation, considering the specific activity of the added NaH14CO3, 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 CO2-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 \( \frac{2 \times (T_0 - T)}{T} \), where \( T \) is time taken with enzyme and \( T_0 \) is without enzyme. Enzyme units is calculated by \( 2 \times (T_0 - T)/(T \times mg \) 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 \( A_{340} \) was recorded for 3–5 min. Enzyme units per mg of protein calculated by \( \Delta A_{340} / [6.22 \times mg(protein) \text{ in ml of reaction mix}] \).

**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 \( \mu g(nitrite \text{ formed}) \) (for NR) or removed (for Nitr) g\(^{-1}\)(dry mass) min\(^{-1}\).

**Glutamine synthetase** activity was estimated in the washed cyanobacterial pellet permeabilized by toluene at 4\(^{\circ}\)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\(^{\circ}\)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 \( \mu g(\gamma\text{-glutamyl hydroxamate formed}) \) 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\%, v/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\(^{\circ}\)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 \( \mu g \) 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. 2A. 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 \( \mu mol(\text{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 \( \mu mol(\text{CO}\(_2\) fixed) \) min\(^{-1}\) mg\(^{-1}\)(protein). The activity at a higher CO\(_2\) concentration was inversely proportional (Fig. 2A).

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.
Western blot of *P. valderianum* BDU 20041 showed that the Rubisco content was higher with increasing CO₂ 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% CO₂-grown cultures revealed a slightly more intense band, indicating higher Rubisco content. The cultures grown at the CO₂ 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 CO₂ above 3%, while the Rubisco content remained the same in all the concentrations.

**Table 1. Growth performance of *Phormidium valderianum* BDU 20041 sparged with different concentrations of continuous CO₂ 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).**

<table>
<thead>
<tr>
<th>CO₂ [%]</th>
<th>Biomass productivity [mg L⁻¹ d⁻¹]</th>
<th>Specific growth rate [μ?/d]</th>
<th>CO₂ fixation rate [mg(C) L⁻¹ d⁻¹]</th>
</tr>
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<tbody>
<tr>
<td>0.04</td>
<td>66.66 ± 3.45&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.183 ± 0.002&lt;sup&gt;c&lt;/sup&gt;</td>
<td>122.21 ± 4.08&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>72.13 ± 3.12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.198 ± 0.003&lt;sup&gt;b&lt;/sup&gt;</td>
<td>126.57 ± 5.17&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>83.33 ± 3.98&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.221 ± 0.007&lt;sup&gt;a&lt;/sup&gt;</td>
<td>152.77 ± 6.74&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>65.00 ± 3.59&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.176 ± 0.004&lt;sup&gt;c&lt;/sup&gt;&lt;sup&gt;d&lt;/sup&gt;</td>
<td>122.24 ± 5.32&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>5</td>
<td>61.33 ± 1.08&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.170 ± 0.005&lt;sup&gt;c&lt;/sup&gt;&lt;sup&gt;d&lt;/sup&gt;</td>
<td>112.43 ± 3.70&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>10</td>
<td>50.33 ± 2.56&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.163 ± 0.004&lt;sup&gt;d&lt;/sup&gt;</td>
<td>92.27 ± 4.24&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>15</td>
<td>51.00 ± 1.87&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.169 ± 0.001&lt;sup&gt;d&lt;/sup&gt;</td>
<td>93.59 ± 3.55&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

**Fig. 2.** Activity of ribulose-1,5-biphosphate carboxylase/oxygenase (Rubisco) (*A*) and phosphoenolpyruvate carboxylase (PEPC) (*B*) of *Phormidium valderianum* BDU 20041 during growth in continuous aeration of ambient air and elevated CO₂, recorded at 12 and 24 h. In order to determine the correlation between the CO₂ assimilation rate and enzyme activities, the CO₂ fixation rate of the organism grown at different CO₂ 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).

**Effect of CO₂ on PEPC activity:** PEPC, the principal C₄ carbon fixation enzyme, showed a pattern similar to the Rubisco activity at the tested CO₂ concentrations. After 12 h, a maximal activity of 0.3 μmol(CO₂ fixed) min⁻¹ mg⁻¹(protein) was observed in the 15% CO₂ cultures, whereas after 24 h, the activity decreased in cultures with CO₂ >3%, similar to the pattern observed with the Rubisco activity. The maximum activity of the enzyme [0.2708 μmol(CO₂ fixed) min⁻¹ mg⁻¹(protein)] after 24 h was observed at the 3% CO₂ concentration (Fig. 2B).

The carbon-fixation rate was reflected in the growth of *P. valderianum* BDU 20041 with different elevated CO₂ 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 CO₂ 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.

Effect of CO₂ on CA and MDH: The CA activity was important factors in the regulation of CO₂ assimilation. Hence, it is worthwhile to infer the relationship between the carbon fixation rate and the recorded activities of the carbon-fixing enzymes. The CO₂-fixation rate stoutly correlated with the activities of Rubisco and PEPC. In P. valderianum BDU 20041, the CO₂-fixation rate increased with CO₂ concentration up to 3% together with the enzyme activities, even after 24 h (Fig. 2).

Nitrogen-assimilating enzymes at elevated CO₂: 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 CO₂ concentrations of 3 and 15%. The activities of all three nitrogen-assimilating enzymes, in samples grown with 3% CO₂, showed higher activities than that under the ambient air and 15% CO₂ after both 12 and 24 h. Irrespective of the cellular demand, the assimilation of nitrogen was very low at 15% CO₂ 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 CO₂ 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 CO₂ concentrations, was found to have optimum CO₂ concentration of 3%. There are many reports on improved cyanobacterial growth at higher CO₂ concentrations (Mo-

Table 2. Activity of nitrogen assimilating enzymes of Phormidium valderianum BDU 20041 grown in ambient air (0.04% CO₂) and elevated CO₂ concentration (3 and 15% CO₂) 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).

<table>
<thead>
<tr>
<th>CO₂ [%]</th>
<th>Nitrate reductase [µg(nitrate formed) g⁻¹(DM) min⁻¹]</th>
<th>Nitrite reductase [µg(nitrate removed) g⁻¹(DM) min⁻¹]</th>
<th>Glutamine synthetase [mg(γ-glutamylhydroxamate formed) g⁻¹(DM) min⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12 h</td>
<td>24 h</td>
<td>12 h</td>
</tr>
<tr>
<td>0.04</td>
<td>3.308 ± 0.123ab</td>
<td>3.787 ± 0.099ab</td>
<td>3.260 ± 0.114ab</td>
</tr>
<tr>
<td>3</td>
<td>3.855 ± 0.275ab</td>
<td>4.151 ± 0.14ac</td>
<td>3.526 ± 0.058ac</td>
</tr>
<tr>
<td>15</td>
<td>2.972 ± 0.236c</td>
<td>1.174 ± 0.294c</td>
<td>2.987 ± 0.154c</td>
</tr>
</tbody>
</table>
roney and Somanchi 1999, Tang et al. 2011). Aphanothece microscopica Nägeli grew without any inhibition at a higher CO₂ 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 CO₂.

Radiometric assay of Rubisco showed higher enzyme activity at 3% which leads to the conclusion that CO₂ 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 CO₂-concentrating mechanism in cyanobacteria. Cultures grown at 15% CO₂ showed a lower activity than the cultures that received ambient CO₂ (Fig. 2A). This result was similar to the pattern observed by Sicher et al. (1994) in tobacco plants grown under elevated CO₂, 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 CO₂ bicarbonate uptake were recorded. In another study, the Rubisco activity of the Chroococcidiopsis sp. strain TS-821 increased at 5% CO₂ but decreased at 10,15, and 20% CO₂, agreed with our finding (Hayashi et al. 1995).

At high concentrations of CO₂ (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 CO₂ concentrations; however, the effect of higher extracellular CO₂ 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 CO₂ 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 CO₂ 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 CO₂ 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 C₄ determinant serine residue at position 923, which is similar to that of the C₄ 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 C₄ PEPC. Hence, this finding suggests the similarity of higher cyanobacterial PEPC to C₄ isomers of higher plants (Shylajanacyiar et al. 2015). CO₂ fixation through PEPC in the selected organism P. valderianum BDU 20041 (Oscillatoriales) is thus justified. PEPC activity was proportional to the CO₂ increase after 12 h, however, the cyanobacterium showed lower PEPC activities at a higher CO₂ concentration at the end of 24-h period (Fig. 2B). Our results are in agreement with the findings of Omoto et al. (2012), who demonstrated that at decreased CO₂ concentrations, the PEPC activity was higher, and at higher CO₂ concentrations, it is speculated to be down-regulated, similarly to Rubisco. The correlation of CO₂ fixation rate with the activities of Rubisco and PEPC is fairly logical. Hudson et al. (1992) also recorded a similar correlation between the CO₂ assimilation rate and the Rubisco activity.

The poor activities of these enzymes at the higher CO₂ concentration could be the reason for stunted cyanobacterial growth at higher CO₂ (~ 10%). In-depth studies to enhance enzyme activities at higher CO₂ could be a major breakthrough for implementation of microalgae carbon capture and storage.

The enzyme CA is involved in the catalysis of the interconversion of HCO₃⁻ and CO₂ in the periplasm space. The CA activity was increasing with CO₂ 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 CO₂-grown cells was observed to be higher until day 4, after which the activity was lower than that of the ambient CO₂-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 CO₂ concentration (2–25%) to ambient CO₂ concentration. The cells grown with ambient air (low C₄ cells) showed higher CA activity in comparison with the cells grown with 2–4% CO₂ (high C₄ cells) (Shiraiwa and Miyachi 1985). Reports are scarce on the effects of transferring cultures from ambient to elevated CO₂ 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 C₄ condition (enhanced CO₂). 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 CO₂ concentrations and the time intervals tested (Fig. 4B).

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 Herrero 1994).

The reduced nitrogen assimilation might be another reason for the reduced growth of the organism at 15% CO₂. 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 CO₂ 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 CO₂ 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 CO₂ 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 CO₂ 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 CO₂ 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 CO₂ concentrations (> 3%) were diminished, thereby causing a reduction in the CO₂ fixation rate and growth at elevated CO₂ concentrations. Further studies need to focus on the reasons for decreased growth at higher CO₂ concentrations.

References


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