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In vitro activity of reconstituted rubisco enzyme from *Gloeobacter violaceus*

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RuBisCO (Ribulose 1,5 bisphosphate carboxylase/oxygenase) by virtue of its dual specificity towards oxygen and carbon dioxide is an important rate-limiting step in photosynthesis and is believed to be the key factor for limited productivity of higher plants and algae. The photoautotrophic growth rate of cyanobacteria is a culmination of several factors including, rates of photosynthetic reactions, stress combating mechanisms and basic biomass generation metabolism in combination with optimal nutrient availability, irradiance, gaseous environment, etc. In case of cyanobacteria, the effect of RuBisCO in affecting the multiplication rate has been observed to show varied response. The current paper presents the RuBisCO activity of an early diverging cyanobacterium, *Gloeobacter violaceus* PCC 7421 and also compares the growth rates and RuBisCO activity of various cyanobacteria. A spectrophotometric estimation in a coupled enzyme assay system of the heterologous expressed *G. violaceus* PCC 7421 RuBisCO in *E. coli*, upon purification, revealed a carboxylation activity of LSu to be 5 nMol of phosphoglycerate min^{-1} mg^{-1} of protein, which is in coherence with the organism's slow growth rate. Further, the *in vitro* complementation of RbcL with RbcS in presence of RbcX of *G. violaceus* facilitated partial reconstitution of the protein and was hence found to cause a four-fold enhancement in its specific activity. The unique characteristics of the primitive cyanobacteria, such as, absence of thylakoids, lack of several photosystem constituting genes, slow carboxylation rate, pose limitation for its rapid multiplication. The RuBisCO carboxylation rate is observed as not the sole but an important parameter for obtaining optimal cell multiplication rates in photo-autotrophically multiplying cyanobacteria.

Keywords. Cyanobacteria; *Gloeobacter violaceus*; growth; RuBisCO

1. Introduction

Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO, EC - 4.1.1.39) is a key enzyme in photosynthesis, catalyzing the carboxylation/oxygenation of Ribulose-1, 5-bisphosphate (RuBP). RuBisCO, despite its prevalent importance in metabolizing the inorganic carbon into organic form for billions of years, suffers from loose specificity for substrate CO_2 and low catalytic rate. In pursuit of overcoming its shortcomings, the enzyme has been subjected to a tradeoff between specificity and catalytic turnover (Tcherkez *et al.* 2006). RuBisCO has been identified to be the limiting factor for photosynthesis and growth in higher plants and algae on basis of several studies based on biochemical constraints (Bassham and Krause 1969; Dietz and Heber 1984), effect on photosynthesis due to differential expression of RuBisCO using antisense

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technology (Stitt *et al.* 1991; Furbank *et al.* 1996), and several other physiological, and genetic studies under saturating irradiance (Bassham and Krause 1969; Farquhar *et al.* 1980; Snir *et al.* 2006). The limiting effect of RuBisCO on photosynthesis and cell growth in cyanobacteria has been found to be variable. The advanced studies on development of plants with higher productivity using cyanobacterial machinery and development of industrially important products from cyanobacteria depend heavily on the basic understanding of limiting factors for their growth.

The doubling time of most of the unicellular cyanobacteria lies in the range of 7 to 12 h, while cyanobacteria like *Synechococcus* PCC 7002 has doubling time of 2.5 h and *Gloeobacter violaceus* takes about a week under best growth conditions to double its number (Rippka *et al.* 1974; Vermass *et al.* 1988; Mori *et al.* 1996; Bernstein *et al.* 2016). Photoautotrophic growth is dependent upon crosstalk between rates of acquisition of light energy, carbon and essential macronutrients, as well as the efficiency with which these resources are directed towards biomass synthesis (Raven 2011). An important aspect of limitation of cyanobacterial productivity is defined by kinetics of light and dark reactions of photosynthesis (Calvin and Benson 1948; Arnon *et al.* 1954). Although, photoinhibition and photorespiration cause drastic effect on photosynthesis and cellular growth, the effect on latter is not very clearly explained (MacKenzie and Campbell 2005; Raven and Larkum 2007; Bailey *et al.* 2008). The cyanobacterial growth has been hypothesized to be limited by several factors like spatial restrictions in the cell that hinder diffusion (Hagemann 2011) and metabolic expenditure involved in partitioning of cellular components (Burnap 2015). The simulation model developed by (Burnap 2015) for distribution of energy and material in cyanobacterial cell, suggests that growth is dependent on partitioning of proteome between machinery involved in providing adaptation to environment and resources involved in cell division. Hence, downregulation of adaptive machinery to basal level would direct major amounts of resources into biomass biosynthesis (Nogales *et al.* 2012).

This paper presents *in vitro* activity of RuBisCO from an early diverging cyanobacteria *Gloeobacter violaceus*. We have also made an attempt to present the effect of various factors on cyanobacterial growth. The available data on cyanobacterial growth and RuBisCO activity has been compared to that of an early diverging cyanobacterium, *Gloeobacter violaceus*. The phylogenetic comparison of the 16S rRNA and other genes from various cyanobacteria suggests that *G. violaceus* diverged at an early stage of evolution of cyanobacterial lineage (Rippka *et al.* 1974; Seo and Yokota 2003; Hoffmann *et al.* 2005; Gupta and Mathews 2010) and is hence an important organism in evolution related studies. *G. violaceus* possesses a simple cell organization without thylakoids and has an unusual structure of photosynthetic apparatus (Rippka *et al.* 1974; Bryant *et al.* 1981; Guglielmi *et al.* 1981). This observation suggests that the photosynthetic electron transfer system and the respiratory systems co-exist in cytoplasmic membrane and possibly also share some of their components (Nakamura *et al.* 2003). The phycobilisome morphology in *G. violaceus* is quite distinct from that found in other cyanobacteria. Further, the fatty acid composition is also different. Sulfoquinovosyl diacylglycerol (SQDG) although considered to play an important role in photosystem stabilization is absent in *G. violaceus* (Rippka *et al.* 1974).

G. violaceus has been used as a model organism for studies based on the evolution of oxygenic photosynthesis (Bernát *et al.* 2012) and is also among the initial set of cyanobacteria whose complete genome was sequenced (Nakamura *et al.* 2003). Various aspects of genus *Gloeobacter* including habitat, distribution, life cycle and identification of variability in the genus remain comparatively unexplored. In the present study RuBisCO from this primitive and evolutionarily interesting cyanobacterium was expressed in *E. coli* and analyzed for its kinetic properties. In accordance with previous reports (Li and Tabita 1997) on the importance of expressing RuBisCO form I as an operon rather than separate subunits, *G. violaceus rbc*L was expressed both in the presence and absence of the chaperone protein RbcX and small subunit RbcS. Further, the effect of the presence of RbcS on the catalysis of RbcL was also analyzed *in vitro*.

2. Materials and methods

2.1 *Construction of expression plasmid, expression in E. coli and purification of target proteins*

The steps followed for expression and purification of *G. violaceus* RuBisCO have been shown in figure 1A. The RuBisCO large subunit (*rbc*L), RuBisCO small subunit (*rbc*S), RuBisCO chaperone (*rbc*X) encoding genes and the RuBisCO operon (*rbc*LSX) were amplified by polymerase chain reaction (figure 1B), ligated into *pCOLDII* vector and transformed into

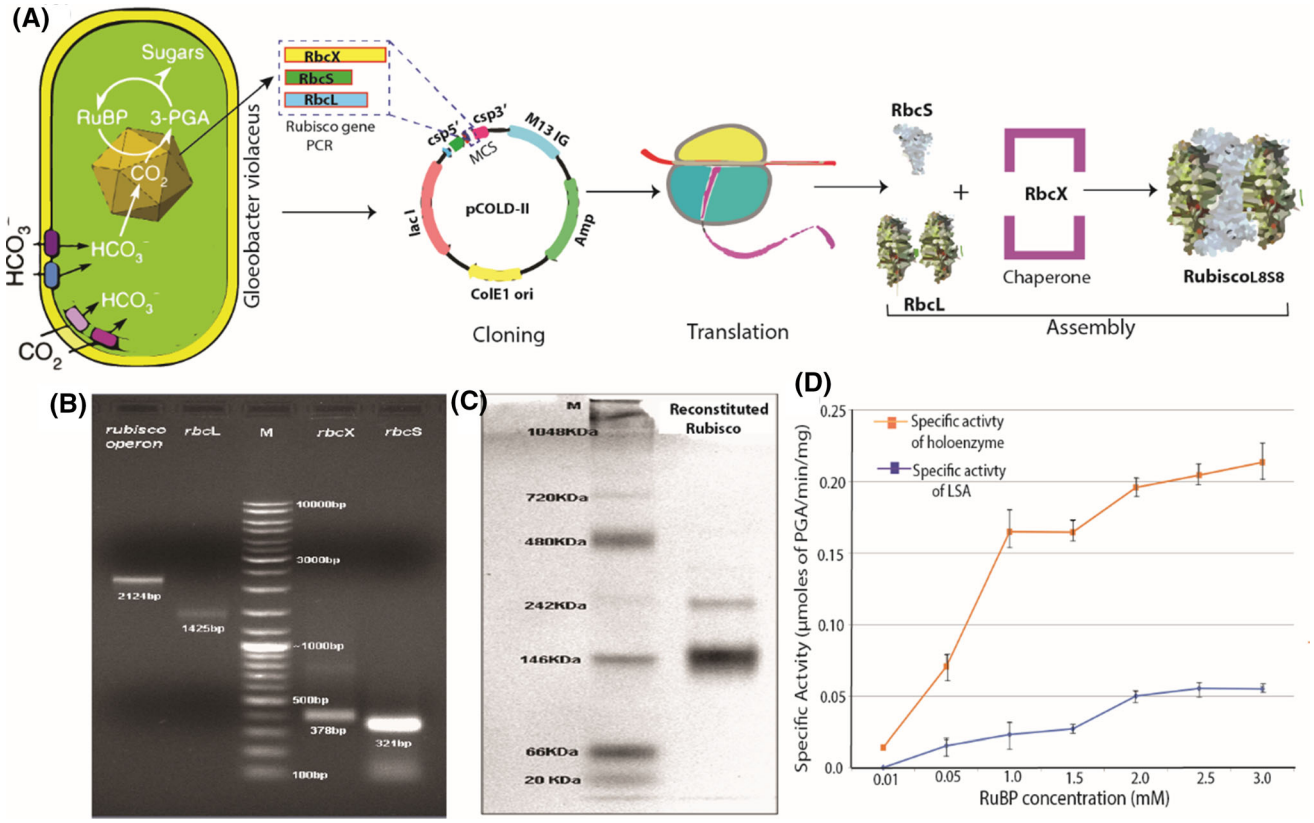

Figure 1. (**A**) Schematic for steps followed for expression and purification of G. violaceus RuBisCO for functional analysis. (**B**) The agarose (1%) gel image of the PCR products of the *G. violaceus* genes under study. lane 1: *rubisco operon*; lane 2: *rbc*L, lane 3: marker SM0331 (Thermoscientific); lane 4: *rbc*X; lane 5: *rbc*S. The estimated sizes of the PCR products obtained are labeled below the respective bands. (**C**) The native PAGE (6%) for *G. violaceus* RbcL after purification by IMAC and renaturation procedures. Lane 1 – Standard native protein marker; Lane 2 – *G. violaceus* RbcL. Standard native protein marker bands (top to bottom in kDa) – 1048, 720, 480, 242, 146, 66 and 20. (**D**) The effect of RuBP concentration on the specific activity of *G. violaceus* RuBisCO large subunit (RbcL blue trendline) and RuBisCO holoenzyme (RbcL + RbcS – red trendline).

E. coli BL21(DE3)pLysS cells for expression of corresponding proteins. The expression of the recombinant proteins was induced by IPTG and cold shock. The proteins expressed in *E. coli* BL21(DE3)pLysS were analyzed for solubility under native and denaturing conditions and subsequently purified by IMAC (figure 1C). The purified proteins viz. RbcL, RbcS and RbcX were subjected to in-gel tryptic digestion for MS/MS analysis. The protein conformation was also analyzed by CD spectroscopy.

2.2 *RuBisCO carboxylase assay*

The RuBisCO assay was performed by NADH oxidation coupled spectrophotometric method (Du *et al.* 1996). The Coupled spectrophotometric assay involving RuBP carboxylation and NADH oxidation is used as a conclusive evidence for RuBisCO activity (Du *et al* 1996). The protein was incubated in presence of activation buffer (HEPES-KOH pH 8.0 – 50 mM, $MgCl_2$ – 25 mM, EDTA – 1 mM, $NaHCO_3$ – 25 mM freshly added) at 25°C for 60 minutes, before the assay was performed. RuBisCO enzyme activity assay was performed at 25°C in a medium containing 100 mM HEPES-KOH pH 8.0, 20 mM $MgCl_2$, 50 mM $NaHCO_3$, 0.2 mM NADH, 5 mM ATP, 20 mM Phosphocreatine, 12.5 U ml^{-1} Phosphocreatine Kinase (PCK), 11.25 U ml^{-1} Phosphoglycerate Kinase (PGK), 10 U ml^{-1} Glyceraldehyde-3-phosphate Dehydrogenase (GAPD) and 0.05 mg Carbonic anhydrase (CA), per mL of reaction. The initial activity was determined by adding 6 µg of the protein to 1ml reaction mixture. The absorbance at 340 nm was measured using Shimadzu spectrophotometer. The Michaelis-Menten constant for RuBP ($K_{M\ RuBP}$) was determined by addition of different amounts of RuBP to bring final concentration from 0.01–3 mM. For complementation

studies, the *G. violaceus* RuBisCO LSu (RbcL) (0.5 μM) was first incubated with RbcX (0.1 μM) at 25°C for 30 min followed by addition of *G. violaceus* RuBisCO SSu (RbcS) (2 μM) and ATP (4 mM) for 30 min in presence of RuBisCO activation buffer and 25 mM $NaHCO_3$ and then analyzed for NADH oxidation.

3. Results

3.1 *Expression of G. violaceus RuBisCO subunits as soluble or insoluble form and purification of G. violaceus RuBisCO*

The *G. violaceus* RbcL recombinant chimeric protein with N-terminal His tag was recovered as a homogenous preparation from the metal chelate column (affinity chromatography using BD Talon Co^{+2} resin). The *G. violaceus* RbcL was purified by IMAC under denaturing conditions, followed by gradual removal of denaturant and elution in native buffer. Similarly, the recombinant RbcS was purified by IMAC and refolded by exchanging into non-denaturing buffer on-column before elution and RbcX purified by IMAC under native conditions. The non-denaturing 6% polyacrylamide gel showed the protein at a position suggesting oligomeric forms with molecular weight ~150 kDa and ~200 kDa (figure 1C). The occurrence of the oligomeric forms of the protein on the native PAGE shows the success of the refolding strategy followed. The purified proteins observed on SDS-PAGE were further investigated by MALDI-TOF analysis and CD spectroscopy. This native PAGE data shows that the proteins (RbcL and RbcS) purified in presence of urea and subsequently subjected to renaturation by gradual removal of denaturant is in near native condition.

3.2 *Catalytic activity of G. violaceus RuBisCO in vitro as analyzed by spectrophotometric RuBisCO assay*

The specific activity of *G. violaceus* PCC 7421 RuBisCO LSu (RbcL) at 2 mM RuBP concentration was found to be 0.005 μmol of PGA min^{-1} mg^{-1} of protein. The activity of the protein increased with an increase in RuBP concentration with the K_M RuBP value of 0.1917 mM and V_{max} value of 0.0002 min^{-1}. The turnover number of the protein was found to be 0.0029 s^{-1} (figure 2). Renatured *G. violaceus* RbcL, when reconstituted with RbcS in presence of RbcX, led to 4-fold enhancement in the activity of the protein (figure 1D). The specific activity observed after complementation was 0.0196 μmol min^{-1} mg^{-1}. The K_M RuBP of the protein for the LSu+SSu (RbcL+RbcS) complex also improved to 0.102 mM and the V_{max} of the protein complex was found to increase up to 0.011 min^{-1}. The turnover number of the protein complex was found to be 0.016 s^{-1}.

4. Discussions

4.1 *Expression and catalysis of RuBisCO*

The *G. violaceus* RbcL and RbcS were found to accumulate in the insoluble fraction. Similar observations were also made in *Anabaena* RuBisCO expression in *E. coli* (Gurevitz *et al.* 1985), where the construct containing the complete *rubisco operon* (*rbc*LXS) led to the expression of *rbc*L but not the other two proteins. Possibly, the intergenic region between *rbc*L and *rbc*X of *G. violaceus* and *Anabaena* is causing transcription termination in *E. coli,* which as such does not occur in the actual organisms.

The type II RuBisCO of *Rhodospirillum rubrum* when expressed in *E. coli* yielded soluble and functionally active protein, as reported in previous studies (Nakano *et al.* 2010). Further, *T. elongatus* BP1 type I RuBisCO (RbcL and RbcS of *T. elongatus* BP1 show 87% and 70% sequence identity to the RbcL and RbcS of *G. violaceus*, respectively) when expressed using *pUC18rbc*LXS in *E. coli* DH5α, has been reported to lead to translation into a soluble protein with specific activity of 1.83 μmol min^{-1} mg^{-1} of protein (Gubernator *et al.* 2008). Similarly, for *Synechococcus sp* (Nägeli) PCC 6301; the RuBisCO operon expressed in *E. coli* has been reported to yield soluble protein in native conformation (Greene *et al.* 2007). The type III RuBisCO from *Archaeoglobus fulgidus* cloned into *pET11a* and expressed in *E. coli* BL21, by IPTG induction was also found to be purified in soluble-native form as determined by gel filtration chromatography and enzyme assay (Kreel and Tabita 2007).

Although a plethora of evidence exists reporting the expression of form I, II and III RuBisCO in *E. coli* in native soluble form, there are also an equally confirmed set of reports showing misfolded or aggregated RuBisCO expression in *E. coli*. The initial attempts to purify natively folded *Anabaena* RuBisCO (form I) from recombinant *E. coli* failed, possibly due to unavailability of adequate folding machinery (Gurevitz *et al.* 1985). In a subsequent research, Li and Tabita (1997) resolved this problem by coexpressing the

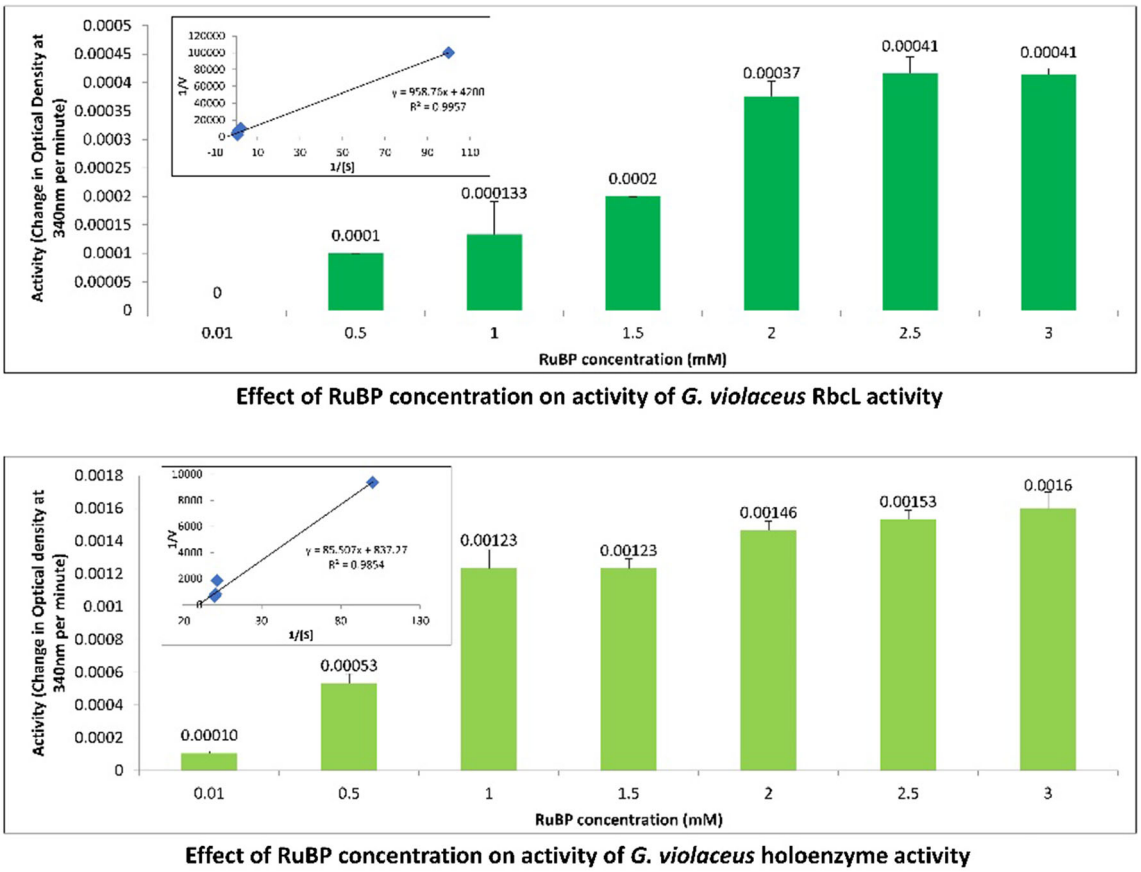

Figure 2. The activity (change in absorbance per minute) of *G. violaceus* RuBisCO RbcL (top panel) and holoenzyme (bottom panel) at different RuBP concentrations.

Anabaena RuBisCO encoding genes with chaperone proteins. Further, the higher plant RuBisCO (form I) from *Nicotiana tabacum* (Whitney and Sharwood 2007), *Zea mays* and *Triticum* (Cloney *et al.* 1993) could not be expressed in *E. coli* in a soluble form. The form I RuBisCO from non-green algae *Galdieria sulphuraria* (Galdieri) Merola and *Phaedactylum tricornutum* (Bohlin) have also been reported to require specific folding proteins for proper assembly of subunits (Whitney *et al.* 2001).

The RbcL and RbcS protein of *G. violaceus* were found to be soluble in denaturing buffer. These proteins were purified by affinity chromatography along with gradual removal of the denaturant. Elian Clark (De Bernardez Clark 1998) reviewed a wealth of literature on methods of refolding denatured recombinant protein by using *in vitro* chaperone activity assisted by additives or slow removal of the denaturants. Colangeli *et al.* (1998) have reported refolding of denatured antigens of *Mycobacterium tuberculosis* by on-column removal of contaminating proteins and gradual exchange into a non-denaturing buffer before elution. The migration of the refolded protein (RbcL) on native PAGE up to standard protein bands of 146 kDa and 242 kDa, shows some degree of multimeric configuration of the protein and hence partial constitution (figure 1C).

The purified RbcL when evaluated for RuBP carboxylation via spectrophotometric assay revealed very low activity for *G. violaceus* RuBisCO, which increased to four times upon reconstitution with small subunit. The carboxylation activity by RbcL in absence of RbcS has been reported by Andrews (1988) to be 100 times lower. Further, Lee and Tabita (1990) also reported reduced carboxylase activity (0.15% of the activity of native L_8S_8 RuBisCO) of the large subunit (in complete absence of small subunit) for *Anacystis nidulans* recombinant RuBisCO. The specific activity, K_M RuBP and K_{cat} of *T. elongatus* BP1 RuBisCO (*rbc*LXS operon in pUC18 vector in *E. coli*) are 1.83 µmol of PGA min^{-1} mg^{-1}, 0.0204 mM, and 2.65 s^{-1}, respectively (Gubernator *et al.* 2008). The K_M RuBP and K_{cat}

reported for *Synechococcus sp* PCC 6301 is 0.044 mM and 3.5 s^{-1}, respectively (Greene *et al.* 2007). The *Anabaena* RbcL when expressed in *E. coli*, in absence of RbcS and the chaperone proteins, was found to be insoluble (Li and Tabita 1997). In the same report, in the presence of RbcS, a soluble RuBisCO was observed with specific activity of 0.2 nMol min^{-1} mg^{-1}. Further, *Anabaena* RuBisCO expressed using the operon *rbc*LXS in *E. coli*, in presence of chaperones GroEl and GroES, yielded a natively folded protein with the specific activity of 13.8 nMol min^{-1} mg^{-1}.

4.2 *Role of chaperones in RuBisCO assembly*

As observed in the present study and in previous reports, the form I RuBisCO from certain cyanobacteria and all reported higher plants is difficult to express in *E. coli* in a correctly folded form. These difficulties have been attributed to the absence of a companion protein in *E. coli* system which can keep the large subunit in solution until it comes in contact with the small subunit of RuBisCO and forms a holoenzyme (Gurevitz *et al.* 1985). Li and Tabita (1997), reported that the *Anabaena* RuBisCO subunits when expressed in absence of each other led to the formation of insoluble aggregates. Their work also demonstrated that the involvement of other chaperone proteins like GroEL and GroES can further increase the solubility of the holoenzyme and hence its activity (Li and Tabita 1997). On the contrary, these chaperones are not necessary for the formation of a biologically active recombinant RuBisCO in *E. coli* from cyanobacteria like *Synechococcus sp.* PCC 6301 and *T. elongatus* BP1 (Greene *et al.* 2007; Gubernator *et al.* 2008). Koay *et al.* (2016) created chimeric RuBisCO LSu by replacing segments of readily expressed and soluble *Synechococcus* LSu (RbcL) with corresponding segments of a difficult to assemble (*in vivo* in *E. coli*) *Chlamydomonas reinhardtii* (P.A. Dangeard) LSu (RbcL) of RuBisCO and expressed them in *E. coli*. The study leads to the identification of regions of *Synechococcus* LSu RuBisCO, possibly responsible for its easy folding and assembly in *E. coli* which includes residues 98–197 and 248–447. The corresponding regions in *G. violaceus* RbcL are 85–90% conserved. As stated by (Bracher *et al.* 2015), the RbcL octamer formation requires chaperonin mediated folding and the L_8S_8 complex assembly is facilitated by RbcX.

4.3 *Congruency in growth rate and RuBisCO activity*

There are competing reports on effect of RuBisCO activity on cyanobacterial growth. As reported by Yamaoka *et al.* (1978), the growth rate is in congruence with the photosynthetic activity of cyanobacteria, i.e., the factors affecting the growth rate have an effect on the photosynthetic efficiency of the organism as well. The RuBisCO overexpression in *Synechocystis* PCC 6803 (1.4–2.1 times) has been found to show enhanced total RuBisCO activity (8.6–52%), greater oxygen evolution (42–54%) as well faster growth when grown with air under 100 μmol/m^2/s irradiance (Liang and Lindblad 2017). Further, Iwaki *et al.*, (2006) found expression of foreign RuBisCO in *Synechococcus* to stimulate photosynthesis while, Daniell *et al.* (1988) reported no effect on photosynthetic rate and cell growth by overexpression of RuBisCO in *Synechococcus*.

In another study using *Synechocystis* PCC 6803 RuBisCO with substituted 1P-site conserved residues, the 6- to 20-fold decrease in RuBisCO K_{cat} was found to be compensated by a 3-fold increase in RuBisCO content which led to very little effect on the photosynthetic rate (Marcus *et al.* 2011). While the ~66% reduction in carboxylation capacity did not affect the photosynthetic rate, any further decrease in K_{cat} led to drastic reduction on rate of photosynthesis, suggesting that the minimal carboxylation capacity required to support growth in *Synechocystis* PCC 6803 under photoautotrophic conditions is ~20% of that of the wild type and hence RuBisCO is not the main rate-limiting factor. Marcus *et al.* (2011) also suggested RuBP generation was a greater limiting factor in comparison to RuBisCO activity for photosynthetic rate and cell growth under saturating Ci and irradiance.

Bernstein *et al.* (2016) analyzed the molecular basis for the high growth rate of *Synechococcus sp* PCC 7002; by monitoring the photo physiological response and transcriptomic measurements. The transcriptomic data reveals faster growth rate is directly linked to higher rates of photosynthetic electron transport, increased synthesis of transcription and translation machinery precursors, elevated abundance of CO_2 transporters and ~300% expansion of cell volume (Bernstein *et al.* 2016). The transcripts found to be inversely proportional to enhanced growth under increased irradiance included the ones for photosystem antenna proteins, regulators of photosynthesis (e.g. thioredoxin responsive regulator), phototaxis signal transduction system and group 2 and 3 RNA

Table 1. Comparison of growth rate and RuBisCO activity of some cyanobacteria

S. no.	Name of organism	Growth rate (doubling time)	Growth conditions	RuBisCO activity
1	*Synechocystis sp PCC 6803*	5.13 h (Zavřel *et al.* 2015)	BG11 medium, 35°C, 220–360 µmol (photons) m^{-2} s^{-1} (Zavřel *et al.* 2015)	K_{cat} 9.08 s^{-1}; K_m rubp 0.14 mM (Marcus *et al.* 2005)
2	*Synechococcus elongatus PCC 6301*	13.6 h (Lepp and Schmidt 1998)	BG11 medium, 37°C, 64.4 mE m^{-2} s^{-1} light intensity	K_{cat} 3.5 s^{-1}; K_m rubp 0.044 mM (Smith and Tabita 2004)
		2 h (Herdman *et al.* 1970)	Medium BG 11 or C Culture grown in 1-l vessels, 38°C, by L/D induction method	
3	*Anabaena 7120*	18.8–1.5 h (Meeks *et al.* 1983)	Basal medium, illumination 7.5 W m^{-2} (Meeks *et al.* 1983)	*Anabaena 7120*: K_m rubp 0.01 mM; K_{cat} 2.5 s^{-1}; specific activity 2.51 µmol min^{-1} mg^{-1} (Larimer and Soper 1993) *Anabaena sp CA strain*: Specific activity 13.8 nmol min^{-1} mg^{-1} (Li and Tabita 1997)
4	*Thermosynechococcus elongatus BP-1*	36 h (Zilliges and Dau 2016)	BG11 medium, 48°C, continuous shaking, illumination 50 µE m^{-2} s^{-1}	K_{cat} 2.65; K_m rubp 0.02 mM; Specific activity 1.83 µmol min^{-1} mg^{-1} (Gubernator *et al.* 2008)
5	*Gloeobacter violaceus PCC 7421*	72 h (Rippka *et al.* 1974)	BG11 medium, 25°C, weak fluorescent illumination ~50 fc (Rippka *et al.* 1974)	K_{cat} 0.016 s^{-1}; K_m rubp 0.102 mM; specific activity 0.0196 µmol min^{-1} mg^{-1}
		> 1 week (Selstam and Campbell 1996)	BG11 medium, 26°C, white fluorescent light illumination of 10 µmol photons m^{-2} s^{-1} (Selstam and Campbell 1996)	

polymerase factors, responsible for transcription of stress induced or adaptive functions (Imamura and Asayama 2009; Bernstein *et al.* 2016). The carboxysome proteins CcmK2 and CcmK4, carbonic anhydrase, RuBisCO subunits and carboxylation transcriptional regulator CcmR, with less abundance in light limiting conditions, were found to show increased expression under light saturated conditions. Further, many genes involved in reductant sink pathways and oxidative stress regulation like cyclic electron transport (psaE) and oxidative phosphorylation (coXAB and sucC), were down regulated under high irradiance, suggesting presence of alternative stress response pathways. The studies conducted by Bernstein *et al.* (2016) suggest that cyanobacterial growth regulation is an intricate crosstalk between multiple pathways, some of which include genes involved in electron transport, carbon fixation, stress response and cell division.

RuBisCO activity, as suggested by various other studies, appears to play an important role in regulating growth rate of cyanobacterial cells (table 1). Organisms like *Synechocystis* PCC 6803 and *Synechococcus elongatus* PCC 6301; with K_{cat} as high as 9.08 s^{-1} and 3.5 s^{-1}, respectively have higher doubling rates in comparison to other cyanobacteria. It is also important to mention that doubling rate of these photoautotrophic unicellular organisms is immensely dependent upon the growth conditions provided. The growth rate of *Synechococcus elongatus* PCC 6301 varies from as high as 2 h to 13.6 h, depending upon the medium, temperature and most importantly irradiance (Asato 2003). The analysis of the factors affecting cell division by Asato (2003) suggest light activated regulatory molecule mediated control of macromolecular biosynthesis and cell cycle. Extensive experimental data suggests that increase in light intensity stimulates transcription of several important photosystem components as well as a shift up in macromolecular metabolism, leading to greater production of high-energy molecules that facilitate improved growth rates (Ihlenfeldt and Gibson 1975; Parrott and Slater 1980; Binder and Chisholm 1990; Li and Golden 1993; Anandan and Golden 1997). However, the maximum growth rate is capped by inherent machinery of the organism, depending heavily on tolerance towards high-energy molecules generated under high irradiance, and the assimilation rate of the energy absorbed. As reported by Bernstein *et al.* (2016), while *Synechococcus* 7002 showed increase in growth rate to up to 2 h doubling time with increase in light intensity to 760 mol photons m^{-2} s^{-1},

the growth rate of *Cyanothece* 51142 declined beyond 430 mol photons m^{-2} s^{-1}.

Further, *Anabaena* 7120 and *Thermosynechoccus elongatus* BP1, with a relatively slow growth rates also have a comparatively smaller turnover number (table 1). *Anabaena* strains from diverse sources have shown to have doubling time ranging from 18 h to 36 h under similar growth conditions (Prasanna *et al.* 2006). The RuBisCO specific activity from *Anabaena* is also found to range between 2.51 µmol/min/mg for *Anabaena* 7120 (Larimer and Soper 1993) and 13.8 nmol/min/mg for *Anabaena* CA strain (Li and Tabita 1997). Even under best growth conditions, the growth rate of *G. violaceus* PCC 7421 does not improve beyond 72 h. The slow growth rate is well congruent with the low RuBisCO activity of the organism. Other factors possibly responsible for the slow growth could be the absence of several genes for proteins for PSI and PSII viz. PsaI, PsaJ, PsaK, PsaX, PsbY, PsbZ and Psb27 (Nakamura *et al.* 2003). Further, as observed by (Yu *et al.* 2015), *Synechococcus elongatus* UTEX 2973 (doubling time 2.3 h) has multi folds faster growth and photosynthetic efficiency as compared to *Synechococcus elongatus* PCC 7942 (8.5 h), the possible reasons for which is the occurrence of a thick layer of thylakoids in former than in the latter. *G. violaceus* being devoid of any thylakoids is incapable of attaining a greater growth rate.

The activity observed for RuBisCO from *G. violaceus*, upon purification from *E. coli*, followed by validation by MS analysis, CD spectroscopy and native PAGE, is very low as compared to the reported activities from other closely related organisms. As expected, in *G. violaceus* form I RuBisCO, the RbcL carboxylation was found to be augmented in the presence of the small subunit RbcS of RuBisCO by up to 4-fold. Further, the study validates the effect of RuBisCO activity on cyanobacterial growth, in amalgamation with the inherent machinery of the organism to support photoautotrophic growth. The understanding of the factors affecting growth of these organisms is essential to utilize their machinery for higher plant productivity enhancement and large-scale production of industrially important products. Although, RuBisCO activity and other components of photosynthetic machinery form an essential aspect for improving growth rates, the external conditions like light intensity and availability of nutrients, oxygen concentration, etc., and the stress tolerance capability are important determinants for attaining optimal cell multiplication rates.

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