Exploring the Oxygenase Function of Form II Rubisco for Production of Glycolate from CO2

Fan Yang  
IMCAS: Institute of Microbiology Chinese Academy of Sciences  https://orcid.org/0000-0001-5590-7951

Junli Zhang  
Institute of Microbiology Chinese Academy of Sciences

Zhen Cai  
Institute of Microbiology Chinese Academy of Sciences

Jie Zhou  
Institute of Microbiology Chinese Academy of Sciences

Yin Li (✉️ yli@im.ac.cn)  
Institute of Microbiology, Chinese Academy of Sciences

Original article

Keywords: Rubisco, oxygenase activity, glycolate production, cyanobacteria, CO2

DOI: https://doi.org/10.21203/rs.3.rs-230143/v1

License: ☺️  This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License
Abstract

The oxygenase activity of Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) converts ribulose-1,5-bisphosphate (RuBP) into 2-phosphoglycolate, which in turn channels into photorespiration, resulting in carbon and energy loss in higher plants. We observed that glycolate can be accumulated extracellularly when two genes encoding the glycolate dehydrogenase of cyanobacteria *Synechocystis* sp. PCC 6803 were inactivated. This inspired us to explore the oxygenase function of Rubisco for production of glycolate, an important industrial chemical, from CO$_2$ by engineered cyanobacteria. Since the oxygenase activity of Rubisco is generally low in CO$_2$-rich carboxysome of cyanobacteria, we introduced Form II Rubisco, which cannot be assembled in carboxysome, into the cytoplasm of cyanobacteria. Heterologous expression of a Form II Rubisco from endosymbiont of tubeworm *Riftia pachyptila* (RPE Rubisco) significantly increased glycolate production. We show that the RPE Rubisco is expressed in the cytoplasm. Glycolate production increased upon addition of NaHCO$_3$ but decreased upon supplying CO$_2$. The titer of glycolate reached 2.8 g/L in 18 days, a 14-fold increase compared with the initial strain with glycolate dehydrogenase inactivated. This is also the highest glycolate titer biotechnologically produced from CO$_2$ ever reported. Photosynthetic production of glycolate demonstrated the oxygenase activity of Form II Rubisco can be explored for production of chemicals from CO$_2$.

Introduction

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) is the key enzyme in photosynthesis (Jensen RG 2000, Erb TJ and Zarzycki J 2018). It is responsible for the primary carbon fixation in Calvin-Benson-Bassham (CBB) cycle, catalyzing the addition of CO$_2$ to ribulose-1,5-bisphosphate (RuBP), leading to the formation of 3-phosphoglycerate (3PGA) (Moroney JV et al. 2013). Despite its pivotal role in the biosphere, Rubisco is notorious for its poor carboxylation activity and specificity (Davidi D et al. 2020). The poor specificity of Rubisco is due to its oxygenase activity, as CO$_2$ and O$_2$ are competitive substrates of Rubisco (Moroney JV, Jungnick N et al. 2013). The oxygenation reaction catalyzed by the oxygenase activity of Rubisco results in the production of 2-phosphoglycolate (2PG) (Eisenhut M et al. 2008). Although 2PG can be metabolized through photorespiration and recycled back into the central carbon metabolism, this process is energy-consuming and leads to carbon loss (Moroney JV, Jungnick N et al. 2013, Fernie AR and Bauwe H 2020).

The oxygenase activity of Rubisco is often considered undesirable but unavoidable (Moroney JV, Jungnick N et al. 2013). A compelling evidence is that active photorespiration is found in nearly all oxygenic photosynthetic organisms to metabolize 2PG, the toxic oxygenation product of Rubisco (Moroney JV, Jungnick N et al. 2013). Engineering Rubisco for a great carboxylation efficiency often comes at a price of decreased CO$_2$:O$_2$ specificity, not to mention the complete removal of its oxygenase activity (Davidi D, Shamshoum M et al. 2020). In fact, there are no CO$_2$ or O$_2$ binding sites found in Rubisco (Moroney JV, Jungnick N et al. 2013). Rubisco binds RuBP and converts it to the 2,3-enediol
form, allowing the subsequent addition of either CO\textsubscript{2} or O\textsubscript{2} (Spreitzer RJ and Salvucci ME 2002). Due to this catalytic mechanism of Rubisco, it is proposed that the oxygenation reaction of Rubisco cannot be eliminated by mutation (Moroney JV, Jungnick N et al. 2013).

Since the oxygenation function of Rubisco cannot be avoided, and the oxygenation product is involved in the overall carbon metabolism, we propose we can take this advantage to employ the oxygenase activity of Rubisco to produce useful chemicals. In *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*), 2PG is subsequently converted to glycolate, a versatile chemical with a wide range of industrial applications in cosmetics, pharmaceuticals and biodegradable polymeric material production (Eisenhut M et al. 2006, Eisenhut M et al. 2008, Zahoor A et al. 2014, Zhan T et al. 2020). Thus, we intended to produce glycolate from CO\textsubscript{2} using the oxygenase activity of Rubisco in *Synechocystis*, providing a unique application avenue of the oxygenase activity in photosynthetic biosynthesis.

**Methods And Material**

**Plasmids and strains construction**

All plasmids constructed in this study were summarized in Supplemental Table 1. *Escherichia coli* DH5a was used as the host for plasmids construction. All plasmids were generated through Gibson Assembly (NEB, China) of amplified inserts and linearized pUC57 plasmid backbones with primers designed using NEBuilder Assembly Tool (http://nebuilder.neb.com/). All *Synechocystis* mutant strains constructed in this study were summarized in Supplemental Table 1. Cyanobacterial strains were generated by transforming cells with certain plasmids which included homologous regions as well as the inserts. Rubiscos were individually overexpressed under the control of the promoter P\textsubscript{cpc560}. The DNA cassette together with a chloromycetin resistance marker was integrated into *pta* site of *Synechocystis* genome. Transformation of *Synechocystis* was performed as previously described (Lindberg P et al. 2010). The colonies were selected on BG-11 plates supplemented with single or combined antibiotics (10 μg/mL chloromycetin, 30 μg/mL erythromycin, 10 μg/mL spectinomycine). Complete segregation and correct gene insertions were checked by PCR and sequencing with primers listed in Supplemental Table 2.

**Culture conditions**

All strains were grown in 50 mL erlenmeyer flask containing 20 mL of BG11 medium at 30\textdegree C under a constant illumination intensity of 100 μmol photons m\textsuperscript{-2}s\textsuperscript{-1}, with atmospheric CO\textsubscript{2} level or supplemented with prescribed concentration of NaHCO\textsubscript{3}. The initial OD\textsubscript{730} was normalized to 0.5. Antibiotics were added to the culture for routine maintenance of mutants when necessary. Growth was monitored by measurements of the optical density at 730 nm (OD\textsubscript{730}) every three days.

**Quantification of extracellular glycolate concentration**
Extracellular glycolate concentrations were determined using the culture supernatants every three days. 10 μL of culture supernatant was analyzed by HPLC equipped with Bio-Rad Aminex® HPX-87H Ion Exclusion Column (300 mm × 7.8 mm) using 8 mM H₂SO₄ as mobile phase, pumped at a flow rate of 0.6 ml/min. The column temperature was maintained at 50°C, peaks were detected using Agilent Technologies 1260 RID (refractive index detector).

**Quantification of intracellular 2PG and glycolate concentration**

The intracellular concentration of 2PG and glycolate were determined after three days of cultivation. To rapidly quench the cell metabolism, 5 mL of cultures were cooled to 0°C within 15 s in a -50°C methanol bath. After centrifugation at 4°C for 5 min at 8,000 g, the cell pellets were washed once with precooled water and resuspended in 2 mL of precooled 80% (vol/vol) methanol solution. After incubation at -20°C for 30 min, the samples were then centrifuged at 4°C for 10 min at 20,000 g. The supernatants were dried by lyophilization and redissolved in 200 μL of water.

The concentration of 2PG and glycolate was determined with AB Sciex Qtrap 6500 LC-MS/MS System. Injection volume was 5 μL. Metabolites were separated with a HyperREZ XP Organic acid column (100 × 7.7 mm, Thermo Fisher Scientific) with H₂O as the solvent. The column was maintained at 40°C with a solvent flow rate of 0.4 mL min⁻¹. The electrospray ionization MS was operated in the negative ion mode. The mass spectra were acquired in multiple-reaction monitoring model for the optimized ion pairs of 2PG and glycolate.

**Enzyme assay**

To prepare the protein samples for SDS PAGE and Native PAGE, *Synechocystis* cells were harvested by centrifugation and resuspended with 1 mL buffer (50 mM Tris-HCl, pH 8.0, 10 mM MgCl₂, 1 mM EDTA) for ultrasonication. After centrifugation, the supernatants were mixed with SDS loading buffer or Native loading buffer at 1:1. The protein samples were detected with SDS PAGE or Native PAGE after the total protein amount was normalized to 7 μg.

**Fluorescence microscopy**

5 μL log-phase cells were spotted onto 1% (w/v in BG11) agarose pads and air-dried before application of a 0.17 mm coverglass. Fluorescence microscopy was performed on a Nikon N-SIM-S Super Resolution Microscope with a 63x/1.4 NA oil-immersion objective using laser lines at 488, and 561 nm.

**Results**
Inactivation of two genes encoding glycolate dehydrogenase in Synechocystis resulted in glycolate production

In *Synechocystis*, glycolate is converted to glyoxylate by two glycolate dehydrogenases (GlcD1 and GlcD2), and subsequently metabolized by three branched routes (Eisenhut M, Kahlon S et al. 2006, Eisenhut M, Ruth W et al. 2008). To completely block the glycolate metabolism, both GlcD1 and GlcD2 encoded by *glcD1* and *glcD2*, respectively, were inactivated (Fig. 1). The resulting mutant was designated as WT-ΔglcD (Table 1). Complete segregation and correct gene insertions at both *glcD1* and *glcD2* sites were verified by PCR and sequencing (Fig. S1).

### Table 1

| Strain          | Genetic background                        | Source of Rubisco                      |
|-----------------|-------------------------------------------|----------------------------------------|
| Wild type       | *Synechocystis* sp. PCC 6803              | -                                      |
| WT-ΔglcD        | WT ΔglcD1::em'; ΔglcD2::spec'             | -                                      |
| RPE-ΔglcD       | ΔglcD Δpta::P_cpc560-4pm-T_rbcS-cm'       | Riftia pachyptila endosymbiont         |
| 4Pm-ΔglcD       | ΔglcD Δpta::P_cpc560-4pm-T_rbcS-cm'       | *Phaeospirillum* molischianum           |
| 5St-ΔglcD       | ΔglcD Δpta::P_cpc560-5st-T_rbcS-cm'       | *Sedimenticola* thiotaurini            |
| 6RBC-ΔglcD      | ΔglcD Δpta::P_cpc560-6rbcL-6rbcST_rbcS-cm' | *Synechocystis* sp. PCC 6803            |

As glycolate metabolism was completely blocked, we next investigated glycolate accumulation in strain WT-ΔglcD. Both the intracellular and extracellular glycolate concentration of WT-ΔglcD were analyzed and compared with that of the WT strain. Samples were taken after three days cultivation supplemented with or without 50 mM NaHCO$_3$. The intracellular glycolate concentration of the WT strain was 0.004 µmol L$^{-1}$OD$_{730}$ and 0.02 µmol L$^{-1}$OD$_{730}$ respectively, when supplemented with or without 50 mM NaHCO$_3$ (Fig. S2). Moreover, the extracellular glycolate concentration was undetectable in the WT strain under both conditions (data not shown). It is evident that glycolate could be rapidly metabolized in the WT strain. On the contrary, strain WT-ΔglcD accumulated glycolate intracellularly and extracellularly under both conditions (Fig. 2 and Fig. S2). The intracellular glycolate concentration of strain WT-ΔglcD was 0.51 µmol L$^{-1}$OD$_{730}$ when supplied with 50 mM NaHCO$_3$, and increased to 1.75 µmol L$^{-1}$OD$_{730}$ without the supply of NaHCO$_3$ (Fig. S2). Furthermore, the glycolate concentration in the medium of strain WT-ΔglcD reached 86.47 µmol L$^{-1}$OD$_{730}$ (mass concentration of 0.02 g/L) and 317.77 µmol L$^{-1}$OD$_{730}$ (mass concentration of 0.06 g/L) after 3 days cultivation respectively, with or without 50 mM NaHCO$_3$ (Fig. 2). Apparently, the majority of glycolate was excreted to the culture by strain WT-ΔglcD, and the intercellular glycolate accumulation could be negligible. We further monitored the glycolate concentration...
in the medium every three days and found that strain WT-ΔglcD produced 0.19 g/L and 0.34 g/L of glycolate after 18 days cultivation respectively with or without the supply of 50 mM NaHCO₃ (Fig. 2). In other words, glycolate can be produced from CO₂ and secreted extracellularly upon inactivation of the two glycolate dehydrogenases in *Synechocystis*. Moreover, strain WT-ΔglcD produces higher concentration of glycolate when no additional NaHCO₃ was supplemented, suggesting ambient level CO₂ is sufficient for glycolate production to occur.

**Overexpression of the native carboxysome-located Rubisco does not contribute to glycolate production**

Given the multiple industrial applications of glycolate, we were encouraged to further increase glycolate production. Glycolate synthetic pathway comprises two reactions (Fig. 1). RuBP reacts with O₂ to generate one molecule of 2PG and one molecule of 3-Phosphoglycerate (3PGA) (Eisenhut M, Ruth W et al. 2008, Fernie AR and Bauwe H 2020). 2PG is then dephosphorylated to glycolate and 3PGA enters the CBB cycle to regenerate RuBP (Eisenhut M, Ruth W et al. 2008, Fernie AR and Bauwe H 2020). In order to identify the bottleneck of glycolate production, the intercellular 2PG concentration in the WT strain and strain WT-ΔglcD were measured. Samples were taken after three days cultivation with or without the supply of 50 mM NaHCO₃. With the intact glycolate metabolism, the intracellular 2PG concentration in the WT strain were below 0.03 µmol L⁻¹OD₇₃₀⁻¹ under both growth conditions (Fig. S2). The intracellular 2PG level in strain WT-ΔglcD was at the same level as compared to the WT strain. However, as mentioned above, the intracellular glycolate concentration in strain WT-ΔglcD became about 100-fold higher than that of the WT strain irrespective of the supply of 50 mM NaHCO₃ (Fig. S2). This indicated that the conversion from 2PG to glycolate in strain WT-ΔglcD was efficient and that the oxygenation of RuBP catalyzed by Rubisco was the rate-limiting step of glycolate production.

Thus, to increase glycolate production, the native Rubisco of *Synechocystis* was overexpressed in strain WT-ΔglcD. The resulting mutant was designated as strain 6RBC-ΔglcD (Table 1) and its capacity for glycolate production was determined with the same growth conditions as mentioned above. After 18 days of cultivation, strain 6RBC-ΔglcD produced 0.16 g/L and 0.35 g/L of glycolate when supplied with or without 50 mM NaHCO₃, respectively. Neither titer is significantly higher than that of strain WT-ΔglcD under the same condition (Fig. 3a and b). In addition, no significant difference was observed in the growth rate of strains 6RBC-ΔglcD and WT-ΔglcD under both conditions (Fig. 3c and 3d). Moreover, the SDS PAGE and native PAGE results suggested that 6RBC was successfully overexpressed and assembled under both conditions (Fig. 3e and f). These results together suggested that overexpression of 6RBC Rubisco did not contribute to increase glycolate production. The reason behind is likely that the native 6RBC Rubisco is encapsulated in a microcompartment found in all cyanobacteria, termed as the carboxysome. It reduces the oxygenase activity of Rubisco by inhibiting the entrance of O₂ and increasing CO₂ concentration around Rubisco (Espie GS and Kimber MS 2011). Thus, to increase
glycolate production, the selected Rubisco is expected to be located outside the carboxysome so as its
oxygenase activity can play a role.

**Overexpression of Form II Rubiscos enhanced glycolate production**

It was previously reported that replacing the native Rubisco of cyanobacteria with Form II Rubisco could
not support the biogenesis of carboxysome, indicating the Form II Rubisco resides outside the
carboxysome (Baker SH et al. 1998, Durao P et al. 2015). If the Rubisco is located in the cytosol, it is
accessible to molecule oxygen and a reduced CO₂ level due to the absence of carbonic anhydrase in the
cytosol (Price GD et al. 2008, Price GD 2011). Thus, we hypothesized that Form II Rubiscos might be
promising candidates to increase glycolate production. To this end, three form II Rubiscos from *Riftia
pachyptila* endosymbiont (RPE Rubisco), *Phaeospirillum molischianum* (4Pm Rubisco) and
*Sedimenticola thiotauroini* (5St Rubisco) were selected and individually overexpressed by using the strong
promoter P<sub>cpc560</sub> in strain WT-ΔglcD (Table 1), resulting in strains RPE-ΔglcD, 4Pm-ΔglcD and 5St-ΔglcD,
respectively (Fig. S1).

Subsequently, glycolate production of these three strains were determined without additional NaHCO₃,
which seemed to be more favorable for strain WT-ΔglcD to produce glycolate. After 18 days of cultivation,
strain 5St-ΔglcD produced 0.3 g/L glycolate, which is not significantly higher than that of strain WT-ΔglcD
(Fig. 3a). Moreover, no significant difference on growth were observed between them (Fig. 3c). This
incapacity for increasing glycolate production could be attributed to the undetectable expression and
assembly of 5St Rubisco (Fig. 3e). In contrast, glycolate production was dramatically enhanced in strains
RPE-ΔglcD and 4Pm-ΔglcD (Fig. 3a). After 18 days cultivation, strain 4Pm-ΔglcD produced 0.66 g/L of
glycolate, about twofold of strain WT-ΔglcD, while strain RPE-ΔglcD produced 0.87 g/L of glycolate, 2.6-
fold of strain WT-ΔglcD (Fig. 3a). However, the growth of strains RPE-ΔglcD and 4Pm-ΔglcD were
significantly impaired (Fig. 3c). The expression and assembly of RPE Rubisco and 4Pm Rubisco were
also detected (Fig. 3e). RPE Rubisco was copiously overexpressed and well assembled. By contrast, 4Pm
Rubisco was successfully overexpressed but not assembled well. This explained their different capacity
on enhancement of glycolate production. Taken together, these results showed that overexpression of
Form II Rubisco indeed increased glycolate production.

**Supply of NaHCO₃ increased glycolate production by strains RPE-ΔglcD and 4Pm-ΔglcD**

As mentioned above, glycolate production by strain WT-ΔglcD decreased when supplied with 50 mM
NaHCO₃ (Fig. 2). Thus, we further investigated whether glycolate production of strains RPE-ΔglcD and
4Pm-ΔglcD would also be repressed when supplied with 50 mM NaHCO₃.
Surprisingly, glycolate production by strains RPE-ΔglcD and 4Pm-ΔglcD was not decreased, but instead sharply when NaHCO$_3$ was available (Fig. 3b). Strain 4Pm-ΔglcD produced 1.46 g/L of glycolate in 18 days when supplemented with 50 mM NaHCO$_3$, which is about 7.7-fold of the titer of strain WT-ΔglcD under the same condition (Fig. 3b). This is also more than twofold of the titer produced by Strain 4Pm-ΔglcD without additional NaHCO$_3$. Additionally, 4Pm Rubisco assembled better in strain 4Pm-ΔglcD upon addition of 50 mM NaHCO$_3$, which could contribute to the increased glycolate production (Fig. 3f).

Among these three strains, strain RPE-ΔglcD was inarguably the best glycolate producer, generating 2.82 g/L after 18 days of cultivation, about 15-fold of the titer of strain WT-ΔglcD under the same growth condition (Fig. 3b). Moreover, the expression and assembly of RPE did not differ upon addition of NaHCO$_3$ (Fig. 3f), suggesting that the increased glycolate production was not related to the assembly of RPE Rubisco. However, the growth of strains RPE-ΔglcD and 4Pm-ΔglcD were also significantly impaired under this condition (Fig. 3d).

Thus, we further investigated glycolate production of strain RPE-ΔglcD when supplied with different concentration of NaHCO$_3$. Glycolate production of strain RPE-ΔglcD increased along with increasing the concentration of NaHCO$_3$, and approached a plateau of 2.84 g/L when supplied with 30 mM NaHCO$_3$ (Fig. 4a). Notably, the growth of strain RPE-ΔglcD gradually reduced along with the increased glycolate production (Fig. 4b). The intracellular glycolate concentration in RPE-ΔglcD was also increased, from 5.6 μmol L$^{-1}$OD$_{730}$$^{-1}$ in the absence of NaHCO$_3$, to 10.4 μmol L$^{-1}$OD$_{730}$$^{-1}$ when adding 50 mM NaHCO$_3$ (Fig. S2). It was previously reported that intracellular accumulation of glycolate is toxic to the cell (Eisenhut M, Ruth W et al. 2008). The retarded growth of strain RPC-ΔglcD upon adding increased concentration of NaHCO$_3$ was probably related to the elevated intracellular glycolate concentration in strain RPE-ΔglcD.

### Supply of CO$_2$ decreased glycolate production by strain RPE-ΔglcD.

Cyanobacteria can use both HCO$_3^-$ and CO$_2$ as external inorganic carbon source (Price GD, Badger MR et al. 2008, Price GD 2011). As supply of HCO$_3^-$ increased glycolate production of strains RPE-ΔglcD and 4Pm-ΔglcD, we then wondered what would be the effect if supplying CO$_2$. Since strain RPE-ΔglcD produces much higher glycolate concentration than that of strain 4Pm-ΔglcD, we chose strain RPE-ΔglcD to study the effect of CO$_2$.

To this end, the external organic carbon supplied was changed from NaHCO$_3$ to CO$_2$. The glycolate production and growth of strain RPE-ΔglcD were evaluated under 1% or 3% CO$_2$ (Fig. 4c and 4d). After 12 days of cultivation, strain RPE-ΔglcD produced 0.87 g/L glycolate under 1% CO$_2$, and the glycolate titer decreased to 0.47 g/L under 3% CO$_2$ (Fig. 4c). Additionally, the growth of strain RPE-ΔglcD increased positively with increasing the CO$_2$ level (Fig. 4d). The increased growth and reduced glycolate production
of RPE-ΔglcD together indicated that supply of CO$_2$ enhanced the carboxylation reaction of RPE and consequently inhibited the oxygenation reaction.

**RPE Rubisco is located in the cytosol**

The enhanced glycolate production indicated an active oxygenation reaction catalyzed by RPE Rubisco and 4Pm Rubisco. This suggested that they are probably located in the cytosol rather than in the carboxysome as the O$_2$ concentration in cytosol is much higher. To provide direct evidence, we visualized their location *in vivo* by fluorescent labelling. We first tried to carry out the co-localization analysis by labelling RPE Rubisco with cyan fluorescent protein (CFP) and 6RBC with yellow florescent protein (YFP). RPE Rubisco was labelled with CFP at its C-terminal (termed as RPE-CFP). YFP was fused to the C-terminal of the large subunit of 6RBC (termed as 6RBCL-YFP). RPE-CFP and 6RBCL-YFP were individually expressed in the WT strain to give single fluorescent signal and co-expressed in the WT-strain to test whether these two fluorescent signals could be overlayed together. However, the fluorescent signals of RPF-CFP and 6RBCL-YFP were too week to give the location information (data not shown).

We next fused green fluorescent protein (GFP) to the C-terminal of RPE Rubisco or the large subunit of 6RBC Rubisco, termed as RPE-GFP and 6RBCL-GFP, respectively. RPE-GFP and 6RCBL-GFP were individually expressed in the WT strain to give single fluorescent signal. Meanwhile, the red fluorescence of endogenous chlorophyll-a of *Synechocystis* was used to indicate the shape of the whole cell (Cameron J et al. 2013). RPE-GFP gave rise to a large single fluorescent punctum at the cell polar, suggesting that RPE proteins intended to aggregate at the edge of cell (Fig. 5a). By contrast, 6RBCL-GFP intended to exhibit several fluorescent spots at a more central position within the cell, indicating the location of mature carboxysomes, which was in agreement with the previous report (Fig. 5b) (Cameron J, Wilson S et al. 2013). The different position of fluorescent signals between RPE-GFP and 6RBC-GFP indicated that RPE is not located in the carboxysome where 6RBC-GFP resides. The bacterial Form II Rubisco from *Rhodospirillum rubrum* was previously expressed in the Δrbc strain of *Synechocystis* (Durao P, Aigner H et al. 2015). The resulting mutant could not support the biogenesis of carboxysome and photoautotrophic growth at ambient CO$_2$ concentration (Durao P, Aigner H et al. 2015). Thus, it is conceivable that the aggregate of RPE-CFP observed here is most likely in the cytosol.

**Discussion**

The oxygenase function of Rubisco and the ensuing photorespiration have long been regarded as one of the obstacles to improve the photosynthesis efficiency (South PF et al. 2018, Hu GP et al. 2019, Luan GD et al. 2020). Cumulative studies have attempted to inhibit even avoid the occurrence of the oxygenation reaction of Rubisco but gained limited progress (Erb TJ and Zarzycki J 2018, Davidi D, Shamshoum M et al. 2020). Here, as the oxygenation product of Rubisco is involved in the overall carbon metabolism, we utilized the oxygenation activity of Form II Rubisco for production of glycolate, a versatile chemical with extensive industrial applications, from CO$_2$ in *Synechocystis*. 
In *Synechocystis*, glycolate can only be generated from 2PG, the direct product of the oxygenation reaction of Rubisco. Glycolate is then converted to glyoxylate and subsequently metabolized by three branched routes including the plant-like photorespiratory cycle, the bacterial glycerate pathway and the complete decarboxylation of glyoxylate to CO$_2$ (Eisenhut M, Ruth W et al. 2008). In the first instance, glycolate production was primarily achieved by inactivation of two forms of glycolate dehydrogenases which are responsible for converting glycolate to glyoxylate. As glycolate metabolism is completely inactivated, the resulting strain WT-ΔglcD produced glycolate irrespective of the provision of NaHCO$_3$. This indicated that Rubisco is performing the oxygenase reaction despite the active CO$_2$-concentrating mechanism (CCM) in carboxysome and the abundance of inorganic carbon, which is also proved in the earlier studies (Eisenhut M, Kahlon S et al. 2006, Eisenhut M, Ruth W et al. 2008). It is still under discussion whether cytosolic Rubisco, which is in the various stages of assembly during carboxysome biogenesis, is responsible for this oxygenase activity, or whether significant amounts of O$_2$ indeed enter the carboxysome (Espie GS and Kimber MS 2011, Burnap RL et al. 2015). Since overexpression of 6RBC Rubisco showed no effect on glycolate production, it is conceivable that the availability of O$_2$ is limited in carboxysome.

Additionally, inactivation of glycolate metabolism was reported to render a High CO$_2$ Requiring (HCR) phenotype which means the mutant was not able to grow at ambient CO$_2$ level (Eisenhut M, Ruth W et al. 2008). This HCR phenotype was presumably ascribed to the intracellular accumulation of toxic amounts of glycolate (Eisenhut M, Ruth W et al. 2008). It was reported that the intracellular glycolate concentration in the mutant increased to a much higher level within a few hours after the mutant was transferred from HC (5% CO$_2$) to LC (air, 0.035% CO$_2$) condition (Eisenhut M, Ruth W et al. 2008). Interestingly, strain WT-ΔglcD that we constructed did not exhibit the HCR phenotype (Fig. S3). Further investigation suggested that strain WT-ΔglcD did accumulate intracellular glycolate, but more than 99% of glycolate was excreted to the culture (Fig. 2 and Fig. S2). Glycolate excretion was previously observed in some filamentous cyanobacterial strains but not in *Synechocystis*, nor in mutant with HCR phenotype (Eisenhut M, Kahlon S et al. 2006, Eisenhut M, Ruth W et al. 2008). It is likely that glycolate excretion of strain WT-ΔglcD helped maintain the intracellular glycolate concentration at a low level, which allows the cell to grow normally at ambient CO$_2$ level, without displaying the HCR phenotype.

To further increase glycolate production, we identified the rate-limiting step by measuring the intracellular 2PG and glycolate concentrations of strain WT-ΔglcD. The result indicated that the conversion from 2PG to glycolate is fully active. As such, the oxygenase activity of Rubisco is the bottleneck of glycolate production, thus its activity needs to be increased. Accessibility to molecular oxygen is the prerequisite for the oxygenation reaction of Rubisco to occur. Overexpression of the native carboxysome-located 6RBC Rubisco of *Synechocystis* in strain WT-ΔglcD did not increase glycolate production, indicating that the oxygenation reaction of 6RBC Rubisco is hampered in the carboxysome which is a CO$_2$-rich but O$_2$-sheilding microcompartment (Price GD, Badger MR et al. 2008, Espie GS and Kimber MS 2011, Price GD 2011).
As compared to the carboxysome, the CO₂ concentration in the cytosol is much lower. To provide the gradient for inward diffusion of CO₂ and minimize its leakage from cell, cyanobacteria accumulate HCO₃⁻ but not CO₂ in the cytosol and maintained a chemical equilibrium in favor of HCO₃⁻ over CO₂ (Price GD, Badger MR et al. 2008, Price GD 2011, Burnap RL, Hagemann M et al. 2015). Thus, the low-CO₂-level cytosol might be a more favorable environment for the oxygenation reaction of Rubisco to occur. Additionally, as cyanobacteria perform oxygenic photosynthesis (Moroney JV, Jungnick N et al. 2013), the photosynthetic evolved O₂ from photosystem II located at the thylakoid membrane may also contribute to the glycolate production. Overexpression of an exogenous Form II Rubisco located in the cytosol indeed increased glycolate production. Among the three forms of Rubisco, there are three reasons why we consider Form II Rubiscos are promising candidates for glycolate production. First, the specificity of Form II Rubisco was reported to be extremely low, and thus can catalyze the oxygenation reaction more easily (Davidi D, Shamshoum M et al. 2020). Second, Form II Rubisco is not packaged in the carboxysome, as they do not support the carboxysome biogenesis (Baker SH, Jin S et al. 1998, Durao P, Aigner H et al. 2015). Third, Form II Rubiscos are structurally simple, comprising only a large subunit and commonly forming an L₂ or L₆ oligomer (Davidi D, Shamshoum M et al. 2020).

In this study, three form II Rubiscos were selected and individually overexpressed in strain WT-ΔglcD. Among them, both RPE Rubisco and 4Pm Rubisco increased glycolate production irrespective of carbon supplement. Strain RPE-ΔglcD produced the highest glycolate titer 2.8 g/L after 18 days of cultivation when supplied with 50 mM NaHCO₃ (Fig. 3b). Remarkably, it compares favorably over the majority of products synthesized from CO₂ in cyanobacteria (Oliver JWK and Atsumi S 2014, Gao XY et al. 2016). This indicated that the deceptively wasteful and undesired oxygenase activity of Rubisco has immense yet undeveloped ability with regard to photosynthetic bioproduction application.

It is interesting that supply of NaHCO₃ and CO₂ exhibits different effects on glycolate production by strain RPE-ΔglcD, as NaHCO₃ supply increased glycolate production while CO₂ supply decreased glycolate production. This could be related to the different manner of HCO₃⁻ and CO₂ entering the cell and the CCM applied by cyanobacteria. HCO₃⁻ is transported into the cytosol by the transporters located at the cytoplasmic membrane. The majority then enters the carboxysome and the sequestered carbonic anhydrase (CA) converts it to CO₂. RuBP enters the carboxysome and react with CO₂ catalyzed by the native Rubisco, generating two molecules of 3PGA. 3PGA escapes from the carboxysome and regenerate RuBP in the cytosol via CBB cycle (Fig. 6a). When supplied with NaHCO₃, the increased HCO₃⁻ availability generally facilitates the carbon fixation of the native Rubisco in the carboxysome and results in the enhanced of RuBP regeneration via CBB cycle (Fig. 6a). As regenerated in the cytosol, RuBP is preferentially oxygenated by RPE. Accordingly, less RuBP is channeled to biomass production, the growth of strain RPE-ΔglcD is impaired upon supplementation of additional NaHCO₃.

Furthermore, due to the absence of CA in the cytosol, the spontaneous conversion of HCO₃⁻ to CO₂ in the cytosol is much slower than the diffusion of CO₂ across the cytoplasmic membrane (Mangan NM et al.
2016). This means that supply of NaHCO₃ could not sharply raise the CO₂ concentration in the cytosol. The RPE Rubisco is identified from the chemolithoautotrophic symbiont in the trophosome of giant tubeworm *R. pachyptila* living at the deep-sea hydrothermal vents where the partial pressure of CO₂ can reach up to 2.9 kPa (Lutz RA et al. 1994). The internal total CO₂ concentration of *R. pachyptila* can approach up to 31 mM relying on the high concentration of CA in the worm's plume and trophosome tissue (Childress JJ et al. 1993). Therefore, it is possible that RPE Rubisco exhibits relatively low affinity to CO₂. Thus, the oxygenase activity of RPE is not inhibited even when supplied with 50 mM NaHCO₃.

As an uncharged small molecule, CO₂ can cross the cell membrane by diffusion (Price GD, Badger MR et al. 2008, Price GD 2011). Meanwhile, RPE Rubisco was not scattered inside the cell but aggregated near the cytoplasmic membrane. When supplied with CO₂, the relative concentration of CO₂ around RPE Rubisco is raised (as O₂ concentration is not changed) (Fig. 6b). Thus, oxygenation is inhibited and carboxylation is enhanced alone with the increased availability of CO₂. As a consequence, more RuBP is channeled to carbon fixation via CBB cycle, leading to increased cell growth and decreased glycolate production of strain RPE-ΔglcD when supplied with CO₂ (Fig. 6b).

In summary, we demonstrated that the oxygenase function of Form II Rubisco could be explored for production of chemicals, e.g. glycolate, from CO₂. Blocking the metabolism of photorespiration pathway led to glycolate production, and the efficiency for producing glycolate can be significantly improved when expressing Form II Rubisco in the cytosol. Thus, the Form II Rubisco with distinct peculiarity can exert their versatile extraordinary capability in photosynthetic biosynthesis applications.

**Declarations**

**Authors’ contributions**

Y.L. and J.Z. designed the research. F.Y., J.L.Z., C.Z. performed the research. Y.L. and F.Y. wrote the manuscript. All authors read and approved the manuscript.

**Funding**

This work was supported by Natural Science Foundation of China (31470231).

**Ethics approval and consent to participate**

Not applicable

**Consent for publication**
Acknowledgements

Not applicable

Authors’ information

1CAS Key Laboratory of Microbial Physiological and Metabolic Engineering, State Key Laboratory of Microbial Resources, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, China

2University of the Chinese Academy of Sciences, Beijing, China

3CAS Key Laboratory of Microbial Physiological and Metabolic Engineering, State Key Laboratory of Transducer Technology, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, China

Competing Interest

The authors declare no conflicts of interest in regards to this manuscript.

References

1. Baker SH, Jin S, Aldrich HC, Howard GT, Shively JM (1998) Insertion mutation of the Form I cbbL gene encoding ribulose bisphosphate carboxylase/oxygenase (Rubisco) in *Thiobacillus neapolitanus* results in expression of Form II Rubisco, loss of carboxysomes, and an increased CO₂ requirement for growth. J Bacteriol 180(16):4133-4139. https://doi.org/10.1128/JB.180.16.4133-4139.1998

2. Burnap RL, Hagemann M, Kaplan A (2015) Regulation of CO₂ concentrating mechanism in cyanobacteria. Life 5(1):348-371. https://doi.org/10.3390/life5010348

3. Cameron J, Wilson S, Bernstein S, Kerfeld C (2013) Biogenesis of a bacterial organelle: the carboxysome assembly pathway. Cell 155(5):1131-1140. https://doi.org/10.1016/j.cell.2013.10.044

4. Childress JJ, Lee RW, Sanders NK, Felbeck H, Oros DR, Toulmond A, Desbruyeres D, Kennicutt MC, Brooks JJN (1993) Inorganic carbon uptake in hydrothermal vent tubeworms facilitated by high environmental pCO₂. Nature 362(6416):147-149. https://doi.org/10.1038/362147a0

5. Davidi D, Shamshoum M, Guo Z, Bar-On YM, Prywes N, Oz A, Jablonska J, Flamholz A, Wernick DG, Antonovsky N, de Pins B, Shachar L, Hochhauser D, Peleg Y, Albeck S, Sharon I, Mueller-Cajar O, Milo R (2020) Highly active rubiscos discovered by systematic interrogation of natural sequence diversity. EMBO J 39(18):e104081. https://doi.org/10.15252/embj.2019104081
6. Durao P, Aigner H, Nagy P, Mueller-Cajar O, Hartl FU, Hayer-Hartl M (2015) Opposing effects of folding and assembly chaperones on evolvability of Rubisco. Nat Chem Biol 11(2):148-155. [https://doi.org/10.1038/nchembio.1715]

7. Eisenhut M, Hueje J, Schwarz D, Bauwe H, Kopka J, Hagemann M (2008) Metabolome phenotyping of inorganic carbon limitation in cells of the wild type and photorespiratory mutants of the cyanobacterium *Synechocystis* sp. strain PCC 6803. Plant Physiol 148(4):2109-2120. [https://doi.org/10.1104/pp.108.129403]

8. Eisenhut M, Kahlon S, Hasse D, Ewald R, Lieman-Hurwitz J, Ogawa T, Ruth W, Bauwe H, Kaplan A, Hagemann M (2006) The plant-like C2 glycolate cycle and the bacterial-like glycerate pathway cooperate in phosphoglycolate metabolism in cyanobacteria. Plant Physiol 142(1):333-342. [https://doi.org/10.1104/pp.106.082982]

9. Eisenhut M, Ruth W, Haimovich M, Bauwe H, Kaplan A, Hagemann M (2008) The photorespiratory glycolate metabolism is essential for cyanobacteria and might have been conveyed endosymbiontically to plants. Proc Natl Acad Sci U S A 105(44):17199-17204. [https://doi.org/10.1073/pnas.0807043105]

10. Erb TJ, Zarzycki J (2018) A short history of Rubisco: the rise and fall (?) of nature's predominant CO$_2$ fixing enzyme. Curr Opin Biotechnol 49:100-107. [https://doi.org/10.1016/j.copbio.2017.07.017]

11. Espie GS, Kimber MS (2011) Carboxysomes: cyanobacterial Rubisco comes in small packages. Photosynth Res 109(1):7-20. [https://doi.org/10.1007/s11120-011-9656-y]

12. Fernie AR, Bauwe H (2020) Wasteful, essential, evolutionary stepping stone? The multiple personalities of the photorespiratory pathway. Plant J 102(4):666-677. [https://doi.org/10.1111/tpj.14669]

13. Gao XY, Sun T, Pei GS, Chen L, Zhang WW (2016) Cyanobacterial chassis engineering for enhancing production of biofuels and chemicals. Appl Microbiol Biotechnol 100(8):3401-3413. [https://doi.org/10.1007/s00253-016-7374-2]

14. Hu GP, Li Y, Ye C, Liu LM, Chen XL (2019) Engineering microorganisms for enhanced CO$_2$ sequestration. Trends Biotechnol 37(5):532-547. [https://doi.org/10.1016/j.tibtech.2018.10.008]

15. Jensen RG (2000) Activation of Rubisco regulates photosynthesis at high temperature and CO$_2$. Proc Natl Acad Sci U S A 97(24):12937-12938. [https://doi.org/10.1073/pnas.97.24.12937]

16. Lindberg P, Park S, Melis A (2010) Engineering a platform for photosynthetic isoprene production in cyanobacteria, using *Synechocystis* as the model organism. Metab Eng 12(1):70-79. [https://doi.org/10.1016/j.menbin.2009.10.001]

17. Luan GD, Zhang SS, Lu XF (2020) Engineering cyanobacteria chassis cells toward more efficient photosynthesis. Curr Opin Biotechnol 62:1-6. [https://doi.org/10.1016/j.copbio.2019.07.004]

18. Lutz RA, Shank TM, Fomari DJ, Haymon RM, Lilley MD, Von Damm KL, Desbruyeres D (1994) Rapid growth at deep-sea vents. Nature 371(6499):663-664. [https://doi.org/10.1038/371663a0]
19. Mangan NM, Flamholz A, Hood RD, Milo R, Savage DF (2016) pH determines the energetic efficiency of the cyanobacterial CO$_2$ concentrating mechanism. Proc Natl Acad Sci U S A 113(36):E5354-E5362. https://doi.org/10.1073/pnas.1525145113

20. Moroney JV, Jungnick N, DiMario RJ, Longstreth DJ (2013) Photorespiration and carbon concentrating mechanisms: two adaptations to high O$_2$, low CO$_2$ conditions. Photosynth Res 117(1):121-131. https://doi.org/10.1007/s11120-013-9865-7

21. Oliver JWK, Atsumi S (2014) Metabolic design for cyanobacterial chemical synthesis. Photosynth Res 120(3):249-261. https://doi.org/10.1007/s11120-014-9997-4

22. Price GD (2011) Inorganic carbon transporters of the cyanobacterial CO$_2$ concentrating mechanism. Photosynth Res 109(1-3):47-57. https://doi.org/10.1007/s11120-010-9608-y

23. Price GD, Badger MR, Woodger FJ, Long BM (2008) Advances in understanding the cyanobacterial CO$_2$-concentrating-mechanism (CCM): functional components, Ci transporters, diversity, genetic regulation and prospects for engineering into plants. J Exp Bot 59(7):1441-1461. https://doi.org/10.1093/jxb/erm112

24. South PF, Cavanagh AP, Lopez-Calcagno PE, Raines CA, Ort DR (2018) Optimizing photorespiration for improved crop productivity. J Integr Plant Biol 60(12):1217-1230. https://doi.org/10.1111/jipb.12709

25. Spreitzer RJ, Salvucci ME (2002) Rubisco: structure, regulatory interactions, and possibilities for a better enzyme. Annu Rev Plant Biol 53(1):449-475. https://doi.org/10.1146/annurev.arplant.53.100301.135233

26. Zahoor A, Otten A, Wendisch VF (2014) Metabolic engineering of Corynebacterium glutamicum for glycolate production. J Biotechnol 192:366-375. https://doi.org/10.1016/j.jbiotec.2013.12.020

27. Zhan T, Chen Q, Zhang C, Bi CH, Zhang XL (2020) Constructing a novel biosynthetic pathway for the production of glycolate from glycerol in Escherichia coli. ACS Synth Biol 9(9):2600-2609. https://doi.org/10.1021/acssynbio.0c00404