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Current advances in engineering cyanobacteria and their applications for photosynthetic butanol production

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Cyanobacteria are natural photosynthetic microbes which can be engineered for sustainable conversion of solar energy and carbon dioxide into chemical products. Attempts to improve target production often require an improved understanding of the native cyanobacterial host system. Valuable insights into cyanobacterial metabolism, biochemistry and physiology have been steadily increasing in recent years, stimulating key advancements of cyanobacteria as cell factories for biochemical, including biofuel, production. In the present review, we summarize the current progress in engineering cyanobacteria and discuss the achieved and potential utilization of these advances in cyanobacteria for the production of the bulk chemical butanol, specifically isobutanol and 1-butanol.

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Introduction

Cyanobacteria, prokaryotic microorganisms with oxygenic photosynthesis, are able to convert solar energy, CO_2 and water into chemical energy [1,2]. They have emerged as potential green cell factories for sustainable generation of carbon neutral renewable chemicals and fuels. Butanol is a four-carbon alcohol (C_4H_9OH), an important bulk chemical and excellent blend-in fuel produced from fossil resources [1,2]. Additionally, there are biological routes for fermentative butanol production, mainly to produce isobutanol or 1-butanol [2]. Natural strains of cyanobacteria produce neither isobutanol nor 1-butanol, indicating that the butanol biosynthetic pathways and necessary genes are absent. A native 1-butanol forming pathway from the genus *Clostridium*, the clostridial pathway, was introduced into *Escherichia coli* for heterotrophic 1-butanol production [3]. Besides, an artificial biosynthetic pathway, the 2-keto acid pathway, was constructed in *E. coli* for isobutanol production [4]. The clostridial and 2-keto acid pathways resulted in 30 g L^{-1} 1-butanol titer and 22 g L^{-1} isobutanol titer in fermentative system, respectively [3,4]. Following the success of producing isobutanol and 1-butanol in *E. coli*, the two corresponding pathways (hereafter isobutanol and 1-butanol pathway, respectively) have been constructed and assessed in cyanobacteria [5–14,15••] (Figure 1).

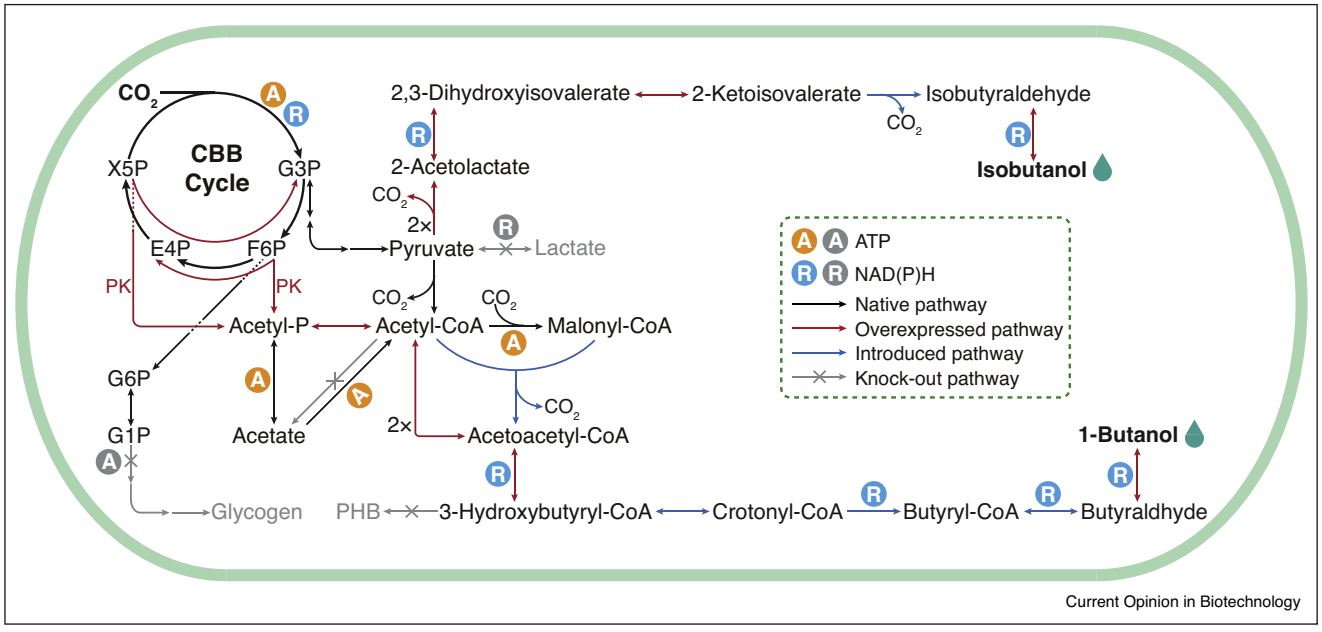
Including the two butanol pathways, most of biosynthetic pathways introduced in cyanobacteria have previously been constructed in heterotrophic microbial models (e.g. *E. coli* and *Saccharomyces cerevisiae*). Therefore, most of the metabolic engineering work in cyanobacteria is based on straight-forward metabolic pathway information in *E. coli* and *S. cerevisiae*. However, it is particularly important to recognize that cyanobacterial metabolism differs from other heterotrophic microbes in many aspects. Cyanobacteria have robust photoautotrophic systems and sophisticated metabolism networks, making them more challenging production systems compared to other heterotrophic microbes. Research efforts have been directed towards engineering cyanobacteria in different fields, including carbon metabolism, reducing equivalent, cell organization, protein localization, chemicals transportation, photosynthesis, environmental stress, product tolerance, cultivation system, cell division and growth. As the research in engineering cyanobacteria has progressed, impressive advances have emerged for designing the cell with emphasis on products formation and increased carbon partitioning to the biosynthetic products. Nevertheless, the strategies of performing these advances for production in cyanobacteria are complex, relying on the crucial aspects of different biosynthetic pathways, such as substrates, driving forces, carbon conservation, metabolites toxicity and products secretion.

In this review, we summarize and discuss the current advances in engineering cyanobacteria, focusing on their achieved and prospective applications for photosynthetic butanol production.

Optimizing biosynthetic pathway and editing native carbon flux

A traditional metabolic engineering strategy developed in heterotrophic microbial models, the 'push–pull strategy', has been extended to chemical production in other microbes, including cyanobacteria. This production

Figure 1



Schematic diagram of engineered metabolic pathway for biosynthesis of isobutanol and 1-butanol from CO_2 in cyanobacteria. Based on [5–14,15••]. Metabolite abbreviations: G3P, glyceraldehyde-3-phosphate; F6P, fructose-6-phosphate; E4P, erythrose-4-phosphate; X5P, xylulose-5-phosphate; G6P, glucose-6-phosphate; G1P, glucose-1-phosphate; Acetyl-P, acetyl-phosphate; PHB, poly-3-hydroxybutyrate.

strategy, pushing the carbon flux towards the substrate(s) by editing native carbon metabolism and pulling the substrate(s) towards the product by optimizing the product forming pathway, is extremely relevant to the biosynthetic pathway design and carbon partitioning. The major focus of engineering the two introduced butanol biosynthetic pathways in cyanobacteria is based on this strategy (Figure 1).

First, research towards engineering cyanobacteria for butanol production has been mainly focused on two unicellular model strains *Synechocystis* PCC 6803 (hereafter *Synechocystis*) and *Synechococcus elongatus* PCC 7942 (hereafter *Synechococcus*) [5–14,15••], which are more amenable to genetic manipulation. To optimize the butanol biosynthetic pathways, various butanol forming enzymes were introduced, overexpressed and evaluated [8–12,15••]. Because of the natural NADPH and ATP abundance in oxygenic cyanobacteria, which are generated in photosynthesis, NADPH favored, ATP driven and/or oxygen tolerant enzymes showed stable and higher activity for butanol production [8,11,12,15••]. On the other hand, the native metabolic flux was modified for improved isobutanol and 1-butanol production via increasing the formation of their precursors, which is pyruvate and acetyl-CoA, respectively (Figure 1). The competing pathways of isobutanol and 1-butanol biosynthesis were knocked-out by homologous recombination to increase the pyruvate and acetyl-CoA content, respectively [7,8,13,15••]. In another study, CRISPR interference (CRISPRi) was employed to repress specific enzymes which were predicted to compete metabolic flux with 1-butanol biosynthesis in *Synechocystis* [14]. In addition, the phosphoketolase (PK) pathway, one of the native acetyl-CoA supporting pathways in *Synechocystis* [16], was enhanced for 1-butanol production by overexpressing a heterogenous PK [13,14,15••]. However, for isobutanol production in cyanobacteria, enhancement of direct pyruvate supporting pathways has not been reported to date. Instead, overexpression of three enzymes of the three native steps between the Calvin–Benson–Bassham (CBB) cycle and pyruvate increased total carbon yield by 1.8-fold and 2,3-butanediol production by 2.4-fold in *Synechococcus* [17]. This approach has potential to improve pyruvate pool for isobutanol biosynthesis.

At present, the maximal reported isobutanol and 1-butanol production achieved in *Synechocystis* during long-term cultivation is 0.9 $g\,L^{-1}$ in 46 days [10] and 4.8 $g\,L^{-1}$ in 28 days [15••], respectively. Corresponding maximal rates are 43.6 $mg\,L^{-1}\,day^{-1}$ [10] and 302 $mg\,L^{-1}\,day^{-1}$ [15••] for isobutanol and 1-butanol, respectively. Recently, 600 mg 1-butanol L^{-1} day^{-1} was reported [1]. These numbers for photosynthetic butanol are still low compared to titers and rates obtained for heterotrophic butanol [3,4]. However, cyanobacteria represent more challenging production systems compared to heterotrophic microbes with, as discussed below, numerous potential

engineering strategies still to be explored. Additionally, photosynthetic butanol is derived directly from CO_2 with a reported carbon partitioning of 60% from CO_2 to 1-butanol [1], while, in comparison, CO_2 is converted to sugars/biomass at a very low efficiency before being used to generate heterotrophic butanol.

Rewriting central carbon metabolism and redistributing reductive forces

Chemical production from cyanobacteria requires not only optimizing the metabolic pathway for production titer, but also minimizing the loss of fixed carbon for productivity. The carbon loss in forming acetyl-CoA from decarboxylation of pyruvate limits the maximum carbon yield of photosynthesis. Drawing carbon flux away from this natural process requires insertion of a non-native or artificial pathway to bypass this enzymatic step to form acetyl-CoA. This can be achieved by utilizing the PK pathway, which is the best-developed rewriting pathway for expansion of sugar catabolism in *E. coli* and *S. cerevisiae*. PK catalyzes a direct and non-decarboxylating conversion of sugar phosphate in the central carbon metabolism to acetyl-P and further on to acetyl-CoA [13,14,15••,16,18] (Figure 1). However, as discussed above, PK is an endogenous enzyme in *Synechocystis*, with contribution only to heterotrophic metabolism [16]. Thus, critically speaking, overexpressing the PK pathway in *Synechocystis* may rewrite the photoautotrophic central carbon metabolism in the photosynthetic environment. Although PK was exploited in cyanobacteria to improve the production of acetyl-CoA-derived chemicals [13,14,15••,18], such as 1-butanol, acetone and fatty acid ethyl esters, the glycolysis pathway is still natively expressed. To address this issue, the glycolysis pathway would be either knocked-out or knocked-down to lower the carbon loss, coupling the PK pathway to overcome the productivity limitation.

Recently, in a proof-of-principle study, the acetyl-CoA concentration was successfully increased by designing and implementing a synthetic malyl-CoA-glycerate (MCG) pathway in *Synechococcus* [19]. This pathway converts a three-carbon metabolite, phosphoenolpyruvate, to two acetyl-CoA by an addition of CO_2, or assimilates glyoxylate, a photorespiration intermediate, to produce acetyl-CoA without any carbon loss. In another recent study, to reduce the effect(s) of native regulation and the rate limitation of the pyruvate decarboxylation, a synthetic acetate-acetyl-CoA/malonyl-CoA (AAM) bypass was designed and examined in *Synechococcus* [20•]. This pathway utilizes acetate assimilation and carbon rearrangements to enlarge the acetyl-CoA pool from pyruvate, resulting in acetyl-CoA-derived acetone titer effectively increased to 0.41 g L^{-1}. The MCG and AAM pathways constructed in cyanobacteria can also be applied to high-level production of chemicals derived from acetyl-CoA, such as 1-butanol.

Apart from carbon conservation, chemical production in cyanobacteria is also limited by reducing cofactor ratios. As discussed above, it has become apparent that coupling steps to the major reducing power carrier NADPH in cyanobacteria significantly enhances the products biosynthesis [8,11,15••,21], whereas the NADH-specific reductase is still involved in some metabolic steps. The co-expression of a soluble bidirectional NAD(P) transhydrogenase, linking the pools of NADPH/$NADP^+$ and NADH/NAD^+, has been demonstrated to increase titers of lactate and 3-hydroxypropionic acid in cyanobacteria [21]. Thus, modifying cofactors balance and supply should also be considered when further improving butanol biosynthesis in cyanobacteria.

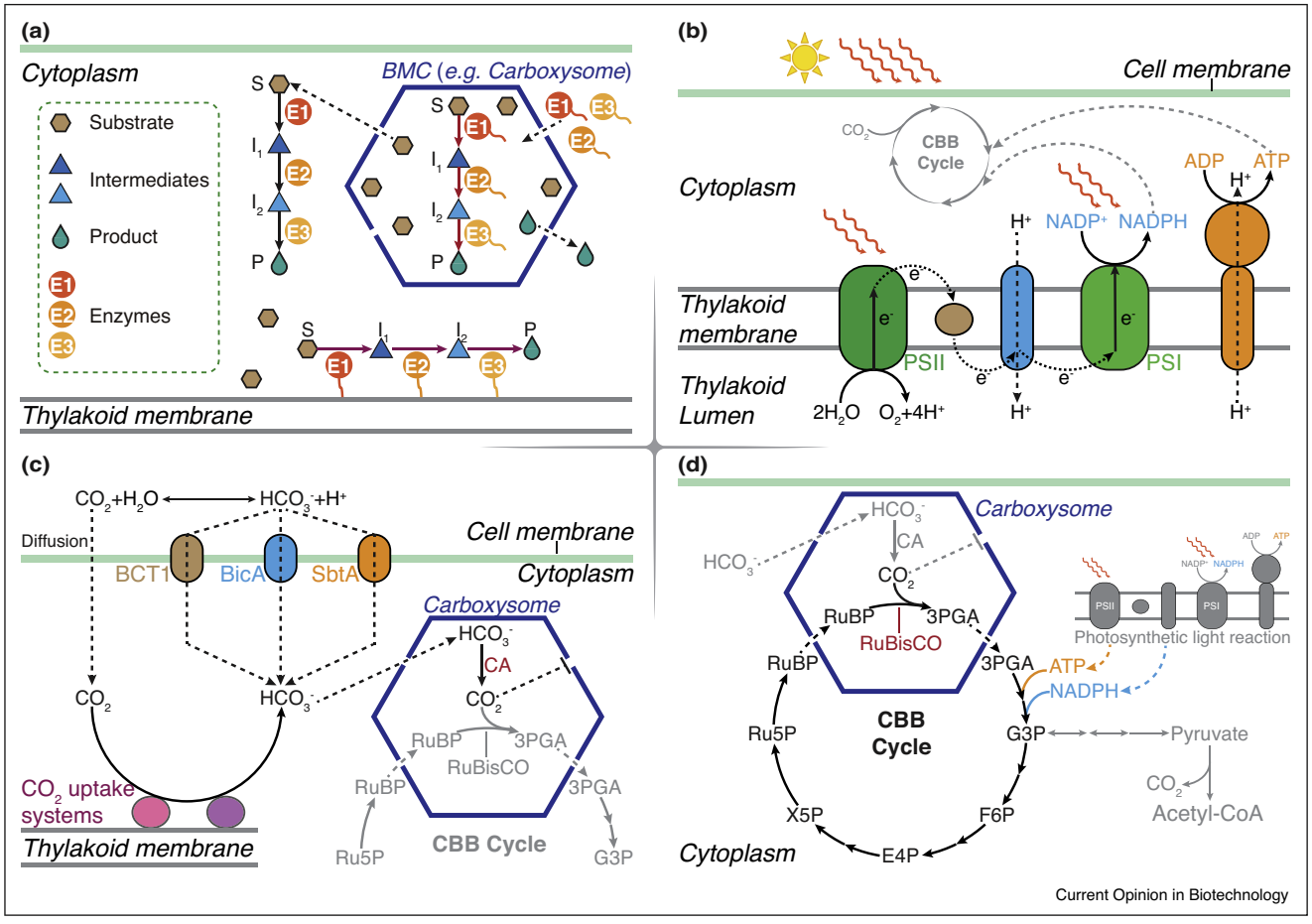
Harnessing biosynthetic pathway in subcellular compartments

Efforts to improve production in cyanobacteria have mainly involved expression of enzymes in the cytoplasm (Figure 2a). Instead, an avenue towards compartmentalizing partial or complete biosynthetic pathway in membrane-enclosed organelles has been explored to increase chemical production in eukaryotes like yeast, fungi and higher plants. For example, since yeast mitochondria has an abundant source of 2-ketoisovalerate, which is the precursor of last two steps of isobutanol pathway, re-localizing the last two-step enzymes using a targeting tag into mitochondria increased the isobutanol production by 260% in *S. cerevisiae* [22]. This strategy possesses desirable properties for production (Figure 2a): 1) Concentrating intermediates and enzymes in smaller volume to favor faster reaction rates; 2) Reducing the loss of intermediates to competing pathways, thereby significantly decreasing accumulation of by-products; 3) Avoiding repressive regulation and toxic effects of intermediates to cytoplasmic metabolism; and 4) Removing the need to transport substrate out of organelles.

As prokaryotes, cyanobacteria contain diverse protein-bound organelles, termed bacterial microcompartments (BMCs), such as carboxysome and phycobilisome [23]. However, unlike eukaryotes, much smaller additional efforts have been carried out in targeting signal study of cyanobacterial BMCs. Maybe due to this limit, the first study of expanding cyanobacterial BMC as a nanoreactor for production appeared in 2020 [24••]. In this study, the [FeFe]-hydrogenase was incorporated within an empty carboxysome built in *E. coli*, leading to 5.5-fold higher hydrogen production than that of cytosolic hydrogenase. However, reprogramming BMCs for production in cyanobacteria is only suitable for the products with availability of both substrates in particular BMCs and corresponding targeting tag (Figure 2a).

In addition to the shell-based BMCs, cyanobacteria also exhibit highly differentiated internal membrane system like the thylakoid membrane [25,26]. For *Synechocystis*,

Figure 2



Simplified schematics of **(a)** Harnessing biosynthetic pathway in subcellular compartments, **(b)** Photosynthetic light reaction, **(c)** Inorganic carbon transportation, and **(d)** CO_2 fixation. Metabolite abbreviations: Ru5P, ribulose-5-phosphate; RuBP, ribulose-1,5-bisphosphate; 3PGA, 3-phosphoglycerate; G3P, glyceraldehyde-3-phosphate; F6P, fructose-6-phosphate; E4P, erythrose-4-phosphate; X5P, xylulose-5-phosphate.

information regarding thylakoid-specific targeting is available already [26]. This provides possibility for translocation of the biosynthetic enzymes from the cytosol to the thylakoid membrane for improved butanol biosynthesis (Figure 2a).

Improving photosynthetic light reaction

Unlike sugar fed heterotrophic production in *E. coli* or *S. cerevisiae*, cyanobacterial systems are often limited by energy or carbon. Unique solutions aiming to enhance photosynthesis are required for improving production titers. During the light phase of photosynthesis in cyanobacteria, the thylakoid-located photosystems capture and transfer light energy to split water, with oxygen being released and both ATP and NADPH generated [27] (Figure 2b). These two energetic intermediates are subsequently utilized to power CO_2 fixation. The capability of cyanobacterial photosynthetic light reaction is limited by multiple factors, including selective capture of visible range of solar radiation (400–700 nm), excessively absorbing photons by cells in the surface layer of cultures under high-intensity light, and inefficient electron transport [27]. Consequently, three engineering strategies have been carried out to increase the photosynthetic efficiency of cyanobacteria: 1) Broadening the absorption spectra to maximize light energy harvesting; 2) Downsizing the light-gathering antenna to penetrate excess light deeper into the cultures; and 3) Optimizing the electron transport chain to enhance electron flux [27].

Most recently, three studies have been shown to positively impact photosynthetic activity in cyanobacteria. In one study, the NADPH supply was engineered by integration a NADPH-dependent reductase into downstream of the photosynthetic electron transport in *Synechocystis* [28]. Channeling of electrons toward the heterologous reduction sink provided highly efficient electron chain flux and cofactor regeneration via

photosynthesis. In another study, two heterologous metabolic pathways were co-expressed and acted as a photosynthetic sink in *Synechococcus*, exhibiting improved photosynthetic capacity and photosystem I oxidation [29•]. In the third study, adaptive laboratory evolution of *Synechocystis* strains under high illumination led to dramatically enhancements of photosynthesis and high light tolerance, allowing cyanobacteria to cope with altered light conditions [30•].

All above approaches will strengthen the photosynthetic light reaction in cyanobacteria and may improve product yields from nearly all biosynthetic pathways, including the butanol pathways.

Enhancing inorganic carbon transportation

The role of inorganic carbon transportation is crucial for supplying intracellular inorganic carbon (Ci; that is, CO_2 and HCO_3^-) for CO_2 fixation (Figure 2c). Cyanobacteria have evolved a carbon concentrating mechanism (CCM) to accumulate internal Ci from low atmospheric CO_2 level in their native environments [27,31–35]. The CCM process can be divided into two stages (Figure 2c). In the first stage, the dissolved CO_2, that is HCO_3^-, is pumped through plasma membrane into the cytoplasm by bicarbonate transporters, while the gaseous CO_2 freely diffuses into the cytoplasm and then is hydrated to HCO_3^- by CO_2 uptake systems. All these steps create an increased HCO_3^- pool in the cytoplasm. In the second stage of CCM, the cytoplasmic HCO_3^- is imported to the carboxysome, and then dehydrated back to CO_2 by carboxysomal carbonic anhydrase (CA). The carboxysomal protein shell is permeable to HCO_3^- and relevant metabolites, but prevents CO_2 to escape. All these operations allow a highly concentrated CO_2 for fixation in the carboxysome.

So far, there is only one study successfully enhancing the cyanobacterial Ci uptake capacity by identifying and overexpressing the operational components in CCM. Overexpressing an endogenous bicarbonate transporter BicA in *Synechocystis* almost doubled growth rate and biomass as the wild-type strain under atmospheric CO_2 pressure [31]. Additionally, in a heterologous expression study, homologs of another bicarbonate transporter SbtA from different cyanobacterium species were expressed in *E. coli*, resulting in an increased HCO_3^- transportation rate and internal Ci pool [32]. Recently, several studies advance our knowledge of structural mechanism and functions of BicA and SbtA from *Synechocystis* [33,34,36], as well as regulation of CCM [35], which may guide further engineering towards enhancement of Ci uptake process. We propose that the Ci uptake enhancement may be employed for a production increment of almost any biosynthetic pathway in cyanobacteria, which has so far not been explored for any product, including butanol.

Improving CO_2 fixation

Efficient CO_2 fixation ensures maximized utilization of photosystem-generated energy and is also a key factor determining the production of cyanobacteria. In light-independent phase of photosynthesis, NADPH and ATP are used to fix CO_2 into central carbon metabolites through the Calvin–Benson–Bassham (CBB) cycle [27,37–39] (Figure 2d). In CBB cycle, ribulose-1,5-bisphophate carboxylase/oxygenase (RuBisCO) catalyzes the CO_2-fixating reaction inside the carboxysome, which is considered as a rate-limiting step. Then, a series of cytoplasmic enzymes catalyze the remaining reactions of the CBB cycle to re-generate the substrate of RuBisCO. Therefore, improving expression levels of the enzymes responsible for CBB cycle is a straightforward way to reinforce CO_2 assimilation in cyanobacteria, which has already been achieved by overexpressing individually selected enzymes of CBB cycle, especially the low-activity RuBisCO [37]. Following this strategy, studies towards engineering biosynthetic pathways together with separately overexpressing cyanobacterial RuBisCO or other CBB enzymes resulted in increased production of isobutyraldehyde, ethanol and free fatty acids in cyanobacteria [5,37,38]. Most recently, Rousou *et al.* effectively improved ethanol production in *Synechocystis* by combined overexpression of selected native CBB enzymes compared to overexpression of a single CBB enzyme [39]. Therefore, co-overexpression of multiple CBB enzymes in cyanobacteria could be a promising direction to improve the turnover rate of the CBB cycle and thereby further increase the production of a chemical like butanol.

Even more intriguingly, pioneer efforts have been made to introduce an entire cyanobacterial carboxysome or even proteobacterial CCM and CBB system into *E. coli* that are not capable of assimilating CO_2 in their native forms, achieving CO_2 assimilation ability [40,41•]. These studies provide a hypothesis that heterogeneous assembly and generation of CCM and CBB system in cyanobacterial host strains might pave the way for improving photosynthetic production, as the strength of natural regulation is proved very high on endogenous CO_2 fixation [35,42]. Moreover, to minimize the native regulation, constructing synthetic pathways for efficient CO_2 fixation in cyanobacteria is another promising strategy which may facilitate chemical production. The synthetic MCG pathway discussed above improved internal acetyl-CoA pool and CO_2 assimilation rate by roughly twofold in *Synechococcus* [19]. It is worth noting that the CBB cycle produces C3 metabolites, not C2 acetyl-CoA (Figure 2d). Thus, the MCG pathway augments the CBB cycle, as might be expected for higher carbon partitioning into production in cyanobacteria.

Stress responses

In addition to altering the metabolic pathways, it is also possible to divert carbon flow under certain stress conditions like nutrient deprivation and salt shock, and therefore stress conditions are routinely applied to stimulate product biosynthesis in cyanobacteria. For instance, salt stress was found to increase production of sucrose, hydrogen, ethanol, acetate and formate in cyanobacteria [43,44]. In a butanol production study in *Synechocystis*, nitrogen starvation improved the acetyl-CoA level by twofold, thereby increasing the specific 1-butanol productivity up to threefold, but cessation of cell growth limited the total 1-butanol titers [13]. Additionally, a preprint (DOI: 10.21203/rs.3.rs-155437/v1) discovered that high salinity stress significantly enhanced isobutanol production in *Synechococcus*. Thus, further optimizing stress conditions is a practical strategy to improve butanol production in cyanobacteria.

Products toxicity is another concern that can greatly restrict the metabolic output of cyanobacteria. Although some products like isobutanol and 1-butanol can be directly exported out of the cyanobacterial cells via secretion or simple diffusion, accumulation of toxic products in the cultivation medium may inhibit cell growth [5,6,12,21,45]. Thus, *in situ* removal of the products or improvement of cellular tolerance to the target products would benefit cyanobacterial cells to prolong the production phase. In two previous studies, a gas stripping method and a solvent trap by oleyl alcohol were employed during the cultivation process to efficiently remove the produced isobutyraldehyde from *Synechococcus* and isobutanol from *Synechocystis*, respectively [5,6]. Alternatively, improving butanol tolerance of cells is another strategy that has been achieved in *Synechocystis* by adaptive laboratory evolution or overexpression of the native transcriptional regulators involved in butanol tolerance [45]. Although the tolerance engineering has not been combined with butanol biosynthesis in engineered cyanobacteria so far, it is a promising strategy for further increasing the butanol production.

Modulating growth rate

An additional issue in engineering cyanobacteria is that common cyanobacterial model strains grow much slower than industrially utilized microbes. To promote industrial applications of cyanobacteria, an urgent need arises to search for and engineer faster-growing cyanobacterial strains. So far, four single-celled strains with substantially faster doubling time have been identified, including *Synechococcus elongatus* UTEX 2973 [46], *Synechococcus elongatus* PCC 11801 [47], *Synechococcus elongatus* PCC 11802 [48], and *Synechococcus* PCC 11901 [49]. Basic molecular-genetic techniques and genetic background have been developed for these strains. Thus, these strains can replace slower-growing strains by transferring the biosynthetic pathways into these new metabolic engineering hosts, which would take considerably less time and effort to culture and likely contribute to more efficient production.

The limited growth rates of commonly studied cyanobacterial strains can also be resolved by genetic editing of their cell metabolism. Since low photosynthesis and carbon assimilation rate is one of the main reasons and bottlenecks for slow growth, the above-discussed studies for improving photosynthetic light reaction, inorganic carbon transportation and CO_2 fixation successively improved the growth rates of cyanobacteria. In a different study, genes associated with the rapid growth of *S. elongatus* UTEX 2973 were identified and introduced into *Synechococcus*, remarkably reducing the doubling time from 6.8 to 2.3 hours [50]. These studies help to bridge the gap between current engineered strains and fast-growing strains and, in turn, might boost metabolic production.

When cyanobacterial cultures grow to exceed an optimal cell density, the light and carbon will become insufficient to supply to all cells. Therefore, engineering cyanobacteria to approach maximal production requires two phases to function in tandem — one for growth to accumulate biomass and metabolites, and a second for growth arrest to increase carbon partitioning into products. In a related study, through inducible CRISPRi repression of essential genes for cell growth, a two-phase cultivation was performed which increased the 1-butanol titer by 70% in *Synechocystis* [14]. Thus, there is excellent scope for improving production performance by modulating the coupled growth and production of cyanobacteria. Besides the genetic strategies, photo-bioreactor is a standard device for controlling cell growth and chemical production, as well as a large-scale cultivation system for expanded biomass and chemical production [1,23]. Nevertheless, the cyanobacterial butanol-producing studies reported to date were all performed under small-scale laboratory conditions, such as closed flasks. By contrast, there might be large improvement for butanol titers by cultivating engineered cyanobacterial strains in photobioreactors which provide high intensity light and concentrated CO_2.

Conclusion and outlook

Using the above outlined strategies to design, engineer and analyze cyanobacteria for improved production, the well-characterized system or synthetic biology techniques are required, including the metabolic model, genome-scale model, omics studies, genetic tools and genetic manipulation. Indeed, sets of metabolic and genome-scale models have been developed for cyanobacteria [51]. These models have followed behind high-throughput omics analysis in the cyanobacteria, including application of nearly all omics technologies in the representative strain *Synechocystis* [51]. Applying extensive

strategies needs multiple genes to be expressed, which has enabled the development of more efficient genetic tools and genetic manipulation in recent years, such as CRISPR-based systems [51]. Especially with a CRISPRi gene repression library completed [52••] and an improved natural transformation approach developed [53] recently in cyanobacteria, the potential applications of the research advances for biosynthesis can be accelerated. Ultimately, we anticipate that the most successful strategies will be those that combine a variety of the advances described throughout this review. Achievements from applying more comprehensive approaches and technologies are promising to establish efficient cyanobacterial systems for production.

Conflict of interest statement

Nothing declared.

Acknowledgements

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- • of special interest
- •• of outstanding interest

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