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Current processes and future challenges of photoautotrophic production of acetyl-CoA-derived solar fuels and chemicals in cyanobacteria

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Summary

The production of fuels and other valuable chemicals via biological routes has gained significant attention during last decades. Cyanobacteria are prokaryotes that convert solar energy to chemical compounds *in vivo* in direct processes. Intensive studies have been carried out with the aim of engineering cyanobacteria as microfactories for solar fuel and chemical production. Engineered strains of photosynthetic cyanobacteria can produce different compounds on a proof-of-concept level, but few products show titers comparable with those achieved in heterotrophic organisms. Efficient genetic engineering tools and metabolic modeling can accelerate the development of solar fuel and chemical production in cyanobacteria. This review addresses the most recent approaches to produce solar fuels and chemicals in engineered cyanobacteria with a focus on acetyl-CoA-dependent products.

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Cyanobacteria, Photosynthesis, Acetyl-CoA, Metabolic engineering, Cultivation condition.

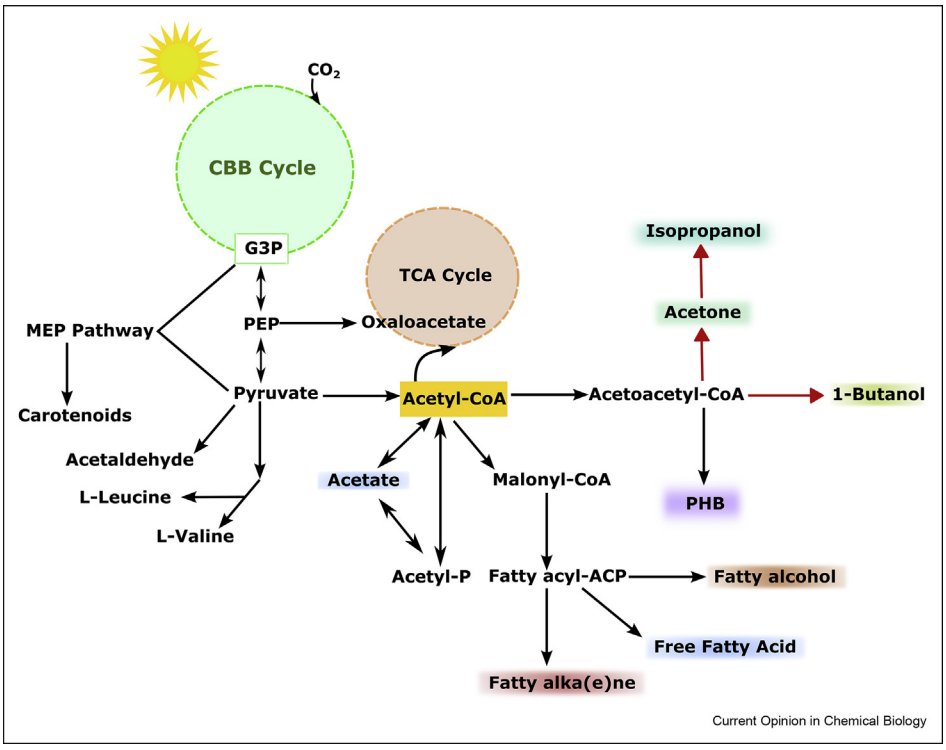
Introduction

Cyanobacteria, the ancestors of chloroplast in higher plants and algae, are capable of converting solar energy, CO_2, and H_2O, and in some species N_2 as well, into chemical energy while releasing O_2 to the atmosphere. They possess a high solar energy capturing efficiency, which is at least three times higher than that of plants. Their CO_2 concentrating mechanisms minimize the negative effects from photorespiration, therefore, accelerates the photosynthesis process. Owing to their rapid growth rate, modest nutrient requirements, and ability to grow using waste water resources and on nonarable land, cyanobacteria have emerged as potential hosts for sustainable production of valuable compounds directly from sunlight and CO_2. In the recent two decades, research has been directed toward modifying the metabolism of cyanobacteria for enhancing production of various chemicals or biofuels. Engineering strategies include optimizing light absorption capacity [1,2], increasing carbon fixation [3], rerouting metabolic flux to desired pathways [4,5], decoupling growth and production [6], and rebalancing cofactor supply [7,8]. Moreover, different metabolic models have been developed to provide better understanding of selected metabolic pathways and to suggest effective engineering approaches. In addition, some robust fast-growing cyanobacterial strains, for example, *Synechococcus* UTEX 2973 [9] and *Synechococcus elongatus* PCC11801 [10], have been characterized recently as potential efficient chemical producing chassis and metabolic engineering tool for the new strains are under developing. In this review, we highlight recent advances in metabolic engineering for enhanced chemical production in cyanobacteria, using production of acetyl-CoA-dependent chemicals as examples. Furthermore, we identify and discuss future challenges in this field.

Strategies for increasing the intracellular pool of metabolic precursor

An abundant precursor pool is essential for high level production of desired chemicals. Acetyl-CoA, a central metabolite directly involved in TCA cycle, is naturally produced from pyruvate decarboxylation catalyzed by a pyruvate dehydrogenase complex (PDHc) (Figure 1). When a biosynthesis pathway in cyanobacteria is introduced or overexpressed, titers of chemicals derived from pyruvate (*e.g.* 5.5 g L^{-1} ethanol [11]) are much higher than those derived from acetyl-CoA (*e.g.* 100 mg L^{-1} fatty alcohols [12]) because the intracellular content of acetyl-CoA is less than 5% of the abundant pyruvate

Figure 1

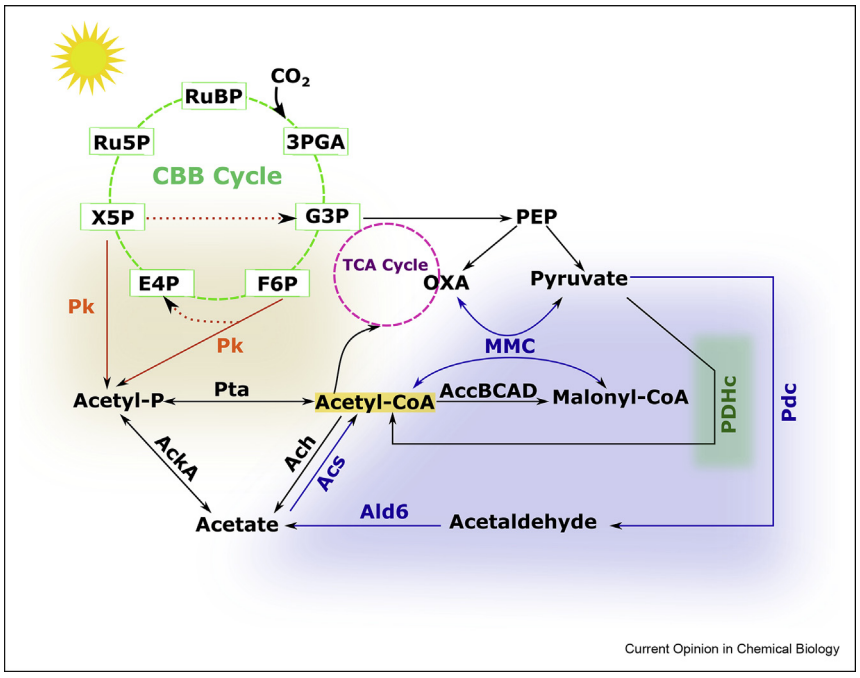


Scheme of biosynthetic pathways that have been demonstrated in *Synechocystis* PCC 6803 for acetyl-CoA derived chemical production. Native metabolic pathways are indicated in black lines and heterologous metabolic pathways are indicated in red lines. CBB cycle: Calvin–Benson–Bassham cycle; CcmM: carbon dioxide concentrating mechanism protein; G3P: glyceraldehyde 3-phosphate; PEP: phosphoenolpyruvate; TCA cycle: tricarboxylic acid cycle; PHB: polyhydroxybutyrate.

content [13,14]. The low acetyl-CoA content may be the result of a potential low catalytic efficiency of PDHc, or a weak driving force due to the generally low activity of the TCA cycle in cyanobacteria under photoautotrophic conditions [15]. Thus, enriching the flux to the acetyl-CoA pool is a key step to enhance the production of downstream products. Several earlier studies applied dark fermentation or nutrient deficient conditions to enhance the intracellular acetyl-CoA availability via activating glycogen degradation [16,17]. However, these cultivation strategies normally lead to reduced growth and productivity. More recently, attention has been focused on metabolic engineering of upstream metabolism to increase the flux towards the acetyl-CoA pool without using extreme cultivation conditions.

The pyruvate dehydrogenase complex consists of three enzymes, pyruvate dehydrogenase, dihydrolipoamide acetyltransferase, and dihydrolipoamide dehydrogenase. The expression and activity of this complex is tightly regulated [18]. Therefore, overcoming the regulation and increase flux from pyruvate to acetyl-CoA has been a strategy to enlarge the acetyl-CoA pool. A study in *Synechococcus elongatus* PCC 7942 showed that by overexpressing the native PDHc, total PDHc activity was increased 2-fold and intracellular acetyl-CoA content reached 2.6-fold of that in the control strain (Figure 2). When biosynthesis pathways for two acetyl-CoA-derived products, acetate and isopropanol, were introduced into the acetyl-CoA-enhanced strain separately, this resulted in a 7-fold and a 3.8-fold improvement in product titer, respectively [19]. In another proof-of-principle study, a synthetic acetate-acetyl-CoA/malonyl-CoA bypass (Figure 2) for overcoming the rate limitation of PDHc was examined in *Synechococcus elongatus* PCC 7942, using acetone titer as reporter. After integrating a pyruvate decarboxylase, an aldehyde dehydrogenase, and an acetylation-tolerant acetyl-CoA synthase into the WT strain, acetyl-CoA content was nearly 4-fold higher than that in the control strain on day 2 and more than 6.5-fold higher on day 8, whereas pyruvate content was about 2-fold higher than that in the control strain on both days [20]. However, when an acetone biosynthesis pathway was introduced into the engineered background strain, the intracellular acetyl-CoA content decreased from 138 μg g^{-1} DCW^{-1} to 5 μg g^{-1} DCW^{-1} on day 2 while pyruvate content decreased by more than half. By contrast, on day 8, the significant decrease in acetyl-CoA and pyruvate pools

Figure 2



Scheme illustrating the strategies of increasing acetyl-CoA pool. Different colored backgrounds indicate different strategies. Blue: AAM-bypass. Pdc: pyruvate decarboxylase; Ald6: aldehyde dehydrogenase; Acs: acetylation-tolerant acetyl-CoA synthase; MMC: methyl malonyl-CoA carboxyltransferase; Green: overexpression of PDHc. PDHc: pyruvate dehydrogenase complex. Orange: Carbon redirecting from CBB cycle. Pk: phosphoketolase. OXA: oxaloacetate; 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; PEP: phosphoenolpyruvate; acetyl-P: acetyl-phosphate.

disappeared. Although the authors did not extensively discuss this difference from different dates, our hypothesis is that the initial decrease of acetyl-CoA and pyruvate pool was because of the constant production of acetone and cell growth. From day 6, cells stopped growing and less acetone was produced. Thus, more acetyl-CoA was accumulated in the cells because the flux from acetyl-CoA to acetone slowed down [20]. Furthermore, the expression of a methyl malonyl-CoA carboxyltransferase significantly increased acetyl-CoA pool even on day 2 and the fitness of the cells was better during stationary phase while more acetone was produced compared with the control strain. This may be due to a higher turnover of TCA cycle and an efficient conversion from malonyl-CoA to acetyl-CoA. With the help of acetate-acetyl-CoA/malonyl-CoA bypass, acetone titer was successfully increased to 0.41 g L^{-1} [20]. Notably, it is not always easy and accurate to follow the change of acetyl-CoA pool because the change in a central metabolite can disperse to many downstream biosynthesis pathways instantaneously. Thus, when more acetyl-CoA is produced in the cell, we may not be able to monitor it before it quickly goes into other pathways. Therefore, concerning the metabolic engineering strategy, it would be more efficient to optimize the target biosynthesis pathways first; then the effects from modifications on central metabolism can be observed using final products as reporters.

Despite the modification made on the reaction from pyruvate to acetyl-CoA, investigations on directing carbon flux toward acetyl-CoA from other routes have also been demonstrated in studies aiming for enhancing butanol production. In photoautotrophic conditions, the introduction of phosphoketolase allows carbon to be pulled directly from the Calvin–Benson–Bassham (CBB) cycle without going through Embden-Meyerhof-Parnas pathway (Figure 2). This bypass requires less CO_2 and RubisCO turnover. Flux balance analysis was used to predict the effect of introducing phosphoketolase on acetyl-CoA-dependent 1-butanol production in *Synechocystis* PCC 6803. Based on the positive modeling result, a phosphoketolase from *Bifidobacterium breve* was introduced into WT *Synechocystis* PCC 6803, resulting in a 6-fold increase of acetyl-CoA under nitrogen replete condition [21]. When the phosphoketolase was introduced into the 1-butanol producing strain, 1-butanol titer was increased 1.7-fold in N^- condition, but change of acetyl-CoA was not reported. A similar strategy resulted in a 1.4-fold increase of acetone production in *Synechococcus* PCC 7942 where a phosphoketolase from

Aspergillus nidulans and a phosphotransacetylase from *Bacillus subtilis* were used [22].

Although many studies have been carried out on modifying precursor pool in the aim of improving final chemical production, more metabolomics studies are needed to provide comprehensive overview on metabolic changes happening in the cell when the central pathways are modified or redirected. For example, when the acetyl-CoA pool is modified, we would like to know more about the effects on carbon fixation efficiency, carbon storage, TCA cycle turnover rate, and intracellular content of other acetyl-CoA-derived metabolites, for example, fatty acids, and PHB. With more metabolomics data, better metabolic models can be built for more reliable guidance for future engineering designs.

Optimizing and rewriting biosynthesis pathways

Biosynthesis pathways can be optimized via various approaches, such as improving enzyme expression levels, increasing enzyme activities, eliminating or down-regulating competing pathways, accelerating transportation of intermediates between enzymes, and improving the excretion of end products.

Significant progress has been made on developing more predictable and efficient genetic elements for enhanced and stable expression of key enzymes within interested biosynthesis pathways in cyanobacteria. Many new artificial and endogenous promoters [23], terminators [23], ribosome binding sites [24], riboregulators [25], and riboswitches [26] have been developed and characterized in a number of cyanobacterial strains. The utilization of these new genetic tools has led to noticeable improvement in pathway expression and product titers. For instance, in a study aimed to increase the production of (R)-3-hydroxybutyrate (3HB) in *Synechocystis* PCC 6803, a ^{13}C kinetic flux profiling method was first used to identify the bottleneck enzyme of the pathway. Then the ribosome binding site of the identified bottleneck enzyme, acetoacetyl-CoA reductase, was optimized to be fully complementary to the 3′-terminal sequence of 16S rRNA in *Synechocystis* PCC 6803. This modification resulted in a 2.2-fold increase in enzyme activity and 3.5-fold higher 3HB production [27]. Although intense efforts have been put in discovering, developing, and optimizing different genetic tools on transcriptional and translational levels, a fine-tuning expression system has not been established, because of the limited option of inducible elements and the lack of knowledge on the mechanism behind the existing repression and induction systems. For example, the widely used IPTG-induced series of LacI-repressed promoters that work well in *Escherichia coli*, yeast, and even in *Synechococcus elongatus* 7942, can only be used as strong constitutive promoters in *Synechocystis* PCC6803 because of significant leakage for which the mechanism remains unclear [28].

Clustered regularly interspaced short palindromic repeats (CRISPR) system has also been successfully established in both unicellular and filamentous cyanobacterial strains for genome editing [29]. A system for multiplex gene repression through CRISPR interference (CRISPRi) using a modified Cas9 was developed for *Synechocystis* PCC6803 [30], and later used for increasing fatty alcohol production [31]. A simultaneous repression of six genes involved in alkane synthesis pathway and lipid synthesis pathway was achieved efficiently and the fatty alcohol content increased two-fold compared with the control strain [31]. A heterocyst-specific conditional gene repression of glutamine synthetase via CRISPRi was also generated in the filamentous cyanobacterium *Anabaena* PCC 7120, resulting in enhanced ethanol production [32]. By using a CRISPR system, multiple gene regulation or genetic modifications can be done without the obstacle of limited choices of antibiotics. Nonetheless, the expression of CRISPR system is not always easy because of the toxicity or the large size of the enzymes, Cas9 or Cpf1.

Finding efficient enzymes is always an important consideration when the aim is to establish a well-performing synthetic metabolic pathway. The initial step for identifying suitable enzymes is to screen numerous isoforms from different organisms [33]; then rational design or directed evolution can be done on the selected candidates for further improving their catalytic capacities. Unfortunately, there are not many protein engineering studies using cyanobacteria as host organisms, even when cyanobacterial enzymes were explored. For example, to generate an aldehyde-deformylating oxygenase (ADO) with better catalysis capacity for hydrocarbon production, Kudo et al. successfully compared the activity and solubility of ADO isoforms from 10 different cyanobacterial strains by overexpressing and extracting them in *E. coli*. Then 37 single amino acid substitutions were examined to create an ADO isoform with high activity as well as high solubility [34]. For protein engineering study, both *in vitro* and *in situ* assays are essential for a clear understanding of the engineered enzyme. There has been evidence showing that an *in vitro* assay cannot provide a comprehensive reflect on *in vivo* or *in situ* performance of the engineered enzymes [35], because even when the differences in the amino acid sequence between two enzymes are small, the differences in expression levels may be large.

It is worth noting that when modifying metabolic pathways which branch to different products, it may be unpredictable how each product will be affected. Recently, Yunus et al. attempted to increase hydrocarbon production in *Synechocystis* PCC 6803 by

overexpressing the native ADO that synthesizes alkane from fatty aldehyde, and introducing a heterologous carboxylic acid reductase from *Mycobacterium marinum* which converts free fatty acids to fatty aldehyde [36]. This modification, however, resulted in enhanced level of fatty alcohols but decreased level of alkanes. To overcome this obstacle, a light-dependent fatty acid photodecarboxylase from *Chlorella variabilis* [37] was expressed instead of carboxylic acid reductase and ADO, resulting in a 19-fold higher alkane production compared with that of wild-type *Synechocystis* PCC 6803 strain [36]. A reliable metabolic model can be helpful for guiding an efficient and predictable metabolic engineering design. In cyanobacteria, considerable progress has been made in establishing different models, for example, genome-scale modeling and kinetic modeling, although confined to the model species. This topic has been recently reviewed in Hendry et al. 2020 [38].

In a comprehensive study on metabolic engineering for 1-butanol production via an acetyl-CoA-dependent route in *Synechocystis* PCC 6803, many of the systematic approaches mentioned previously were implemented to enhance product titer [39]. First of all, screening of different enzyme candidates including engineered variants was performed to enable more efficient catalysis of each step of the 1-butanol synthesis pathway. Then, to route carbon flux directly from the CBB cycle, nine phosphoketolases from different organisms were examined and the best performer among these 9 candidates, a phosphoketolase from *Pseudomonas aeruginosa* which contributed in a 53% increased titer, was selected. Furthermore, another 2.3-fold increase of 1-butanol production was observed after enhancing protein transcription and translation levels by using different strong promoters and genetic insulators. In the end, an optimized 1-butanol-producing strain was cultivated in a modified condition with product removing and pH adjustment, which resulted in a cumulative photoautotrophic production of 4.8 g L^{-1}, with a maximal rate of 302 mg L^{-1} day^{-1}, a titer comparable with those achieved in heterotrophic production hosts [39,40].

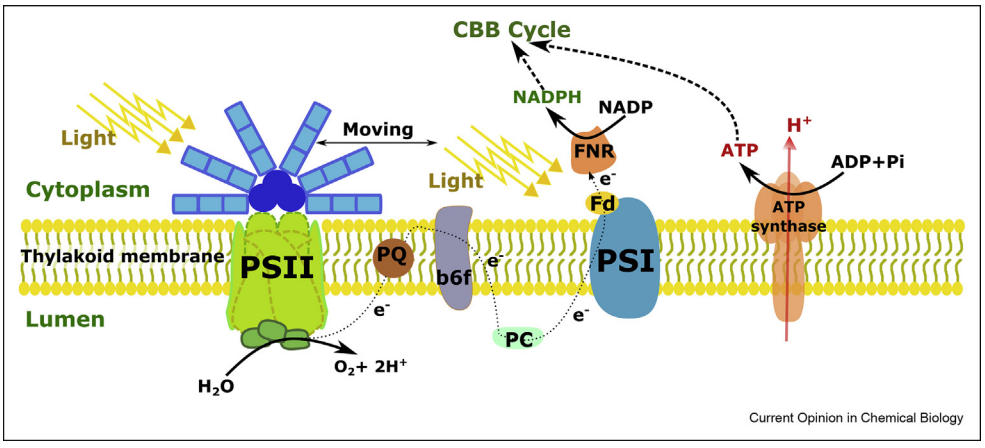
A single manipulation of the metabolism may result in multiple physiological changes

Numerous proof-of-concept studies have demonstrated the possibility to engineer cyanobacteria to produce interesting and valuable chemicals [41–43]. However, the absolute majority focus on production rates and levels, and do not report physiological effects of the engineered strains beyond growth assessment, resulting in a lack of information to guide further metabolic engineering design. In cyanobacteria, physiological studies are especially important because all introduced metabolic modifications may affect the functionality of photosystems and the CBB cycle. In some studies, it has been shown that the overproduction of target metabolites in cyanobacteria may result in a significant change in photochemistry. When an engineered strain shows a retarded growth or unhealthy phenotype, product or intermediate toxicity is often considered as a potential cause. However, it is not always reliable to evaluate cell tolerance level by adding a high concentration of the chemical of interest externally into the growth media because when the engineered cells themselves produce the chemical, they may also be able to gradually adapt to increasing concentrations. When detrimental effects are observed in an engineered strain, it would be helpful to examine more physiological parameters, such as cell morphology, cell division, efficiency of the CBB cycle, and effects on the expression and function of the photosynthetic apparatus. The results of such investigations may lead to the discovery of better metabolic engineering and cultivation strategies. For example, free fatty acids, fatty alcohols, and alka(e)nes are popular biofuel compounds that have been overproduced in different cyanobacterial strains. However, the overproduction of these compounds is not innocent to the entire cell because their biosynthesis pathways are closely connected to the inherent lipid metabolism. Lipid content and composition are important for maintaining and adjusting membrane structure, fluidity, and rigidity. In photosynthetic organisms, the complete photosynthetic apparatus is located on the thylakoid membrane (Figure 3), so minor changes on the thylakoid membrane may affect the photosynthesis drastically. This was demonstrated by Kaczmarzyk et al. in a study of fatty alcohols production in *Synechocystis* PCC 6803. They found that as fatty alcohols accumulated, cell growth was impaired, the membrane composition varied, and more reactive oxygen species were produced [31].

Conclusion and outlook

Progress has been made in recent years in engineering cyanobacteria as cell factories to produce acetyl-CoA-derived valuable chemicals that can be used as biofuels, bulk chemicals, pharmaceutical compounds, cosmetics, and more. The development of synthetic genetic tools and metabolic modeling opens new opportunities to make significant improvements in metabolic manipulation. For example, the utilization of various transcriptional and translational regulators, as well as CRISPRi, enables more precise modification and tuning of gene expression, which allows us to redesign essential metabolic pathways to benefit the balance of cell fitness and production of downstream metabolites. Investigations on photosynthesis and carbon fixation will provide better understanding of cell physiology, energy supply, and transfer mechanisms, which can indicate further engineering directions. However, studies of combined perspectives are needed for more comprehensive improvement in metabolic engineering of cyanobacteria

Figure 3



Photosynthetic apparatus located on the thylakoid membrane of cyanobacteria. PSI: photosystem I; PSII: photosystem II; PQ: plastoquinone; b6f: cytochrome b6f complex; PC: plastocyanin; Fd: ferredoxins; FNR: ferredoxin NADP-reductase. Phycobilisome moves rapidly on top of the thylakoid membrane between PSII and PSI.

as efficient cell catalysts. Until now, many metabolic engineering studies, not only in cyanobacteria, lack in systematic design. In the future, transcription and translation models may be developed to be used together with or even fused into the metabolic models to estimate the whole cell metabolism change with the chosen transcription and translation elements. The goal is to develop cyanobacteria as energetically and commercially viable production hosts. This means being able to fine-tune the cell metabolism to increase productivity and lower the costs of production, while maintaining fitness, something that will require a combined approach as outlined previously. For increasing productivities, enhancement of photosynthesis as well as carbon fixation efficiencies will be needed, and expanding the catalog of strains used to include naturally faster growing variants is another strategy which is receiving increased attention and should be further explored in the future.

Conflict of interest statement

Nothing declared.

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** of outstanding interest

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