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Review

Carbon-negative synthetic biology: challenges and emerging trends of cyanobacterial technology

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Global warming and climate instability have spurred interest in using renewable carbon resources for the sustainable production of chemicals. Cyanobacteria are ideal cellular factories for carbon-negative production of chemicals owing to their great potentials for directly utilizing light and CO_2 as sole energy and carbon sources, respectively. However, several challenges in adapting cyanobacterial technology to industry, such as low productivity, poor tolerance, and product harvesting difficulty, remain. Synthetic biology may finally address these challenges. Here, we summarize recent advances in the production of value-added chemicals using cyanobacterial cell factories, particularly in carbon-negative synthetic biology and emerging trends in cyanobacterial applications. We also propose several perspectives on the future development of cyanobacterial technology for commercialization.

Highlights

Excessive CO_2 emissions are one of the most widely discussed challenges in the 21st century. Carbon-negative production of value-added chemicals by cyanobacterial cell factories has been considered a top priority for solving such problems.

Emerging synthetic biology tools, such as clustered regularly interspaced short palindromic repeats (CRISPR)/cpf1, riboswitch, and metabolic network reprogramming circuits, have accelerated the industrial applications of cyanobacterial cell factories.

The synthesis of a range of biochemicals has been demonstrated in cyanobacteria; however, low product titers are the biggest barrier to commercialization of cyanobacterial biotechnology. To further increase production and meet industry demands, attention needs also be given to the integration of polymer biosynthesis with flocculation by cyanobacteria, thereby bypassing this unsolved problem.

Recent efforts are underway to characterize and develop high-value-added applications of cyanobacteria in biomedicine, power production, and human habitation on Mars.

Three birds with one stone: producing value-added products from CO_2

Excessive CO_2 emissions from industrial societies exacerbate global warming and climate instability. Annual CO_2 emissions from burning fossil fuels have increased every decade, from an average of 3 billion tons of carbon (11 billion tons of CO_2) per year in the 1960s to 9.5 billion tons of carbon (35 billion tons of CO_2) per year in the 2010s [1]. If no measures are taken, it will be highly harmful to the environment once the temperature rises above the critical point of 1.5°C (https://www.ncei.noaa.gov/cag/). Inexpensive CO_2 is a carbon resource with abundant natural reserves. However, fossil resource reserves are significantly limited and will gradually deplete with extensive mining. Therefore, the development and utilization of renewable carbon resources for the sustainable production of chemicals and fuels have become increasingly important [2].

Currently, the **third-generation (3G) biorefineries** (see Glossary) expand the range of raw materials and can utilize atmospheric CO_2 to produce biological chemicals using microbial cell factories [3]. **Carbon-negative synthetic biology** has inspired much attention from researchers [4]. Cyanobacteria are among the largest unicellular prokaryotes capable of oxygen-producing photosynthesis [5]. They have attracted widespread attention owing to their excellent abilities to convert CO_2 into biofuels and high-value compounds [6]. For example, Betterle and colleagues reported that the 10-carbon monoterpene β-phellandrene was produced through systematic overexpression at the protein level of terpenoid biosynthetic pathway genes in *Synechocystis* sp. PCC 6803 [7]. Producing bioproducts directly from CO_2 can significantly avoid the utilization of crop-based feedstocks, thereby avoiding the competition for grain and farmland resources between industrial manufacturers and agriculture. In addition, cyanobacteria account for 20–30% of the earth's photosynthetic productivity to produce O_2. Cyanobacteria have a phenomenal carbon absorption rate, with each hectare soaking up one ton of CO_2 [8]. Furthermore, oxygen supply is one of the challenges in supporting humans on Mars [9]. With this emphasis, we can reasonably

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anticipate that the development of an *in situ* resource utilization (ISRU) strategy for Mars would unlock the full potential of cyanobacteria. In summary, it is of great significance that applying cyanobacteria for efficient utilization of CO_2 and developing efficient *Synechococcus* chassises can realize the 'light-driven cell factories' of green synthetic chemical production and simultaneously ease urgent environmental and energy pressures (Figure 1). Therefore, it is a promising strategy to simultaneously address excessive CO_2 emissions and substitution of nonedible feedstocks, which would shed light on green and sustainable biocatalysts, with the manner of 'killing three birds with one stone.' Here, we review the latest development of carbon-negative **synthetic biology** and emerging trends in cyanobacterial applications. We also put forward several perspectives on future developments of cyanobacterial technology for commercialization.

Chemical production and associated challenges

For several decades, cyanobacteria have been engineered as 'green' chassises to produce fuels and value-added chemicals, such as polylactic acid (PLA) [10] and polyhydroxybutyrate (PHB) [11], squalene [12], α-farnesene [13], limonene [14], sucrose [15], and ethylene [16]. However, despite many successful proof-of-concept studies, using cyanobacteria in this way remains a significant challenge owing to the low yield of bioproducts. Specifically, low carbon fixation efficiency challenges bioprocess scale-up for biochemicals using cyanobacterial chassis [6], and cyanobacterial chassises lack sufficient tolerance to many products such as fuels and chemicals [17]. Many products, such as bioethanol and lactic acid, are toxic to cyanobacteria and decrease the growth rate of the host, thereby limiting industrial production. For example, cyanobacteria cultivated in the wild are easily contaminated by chemical factors. Additionally, low chemical productivities of cyanobacterial chassises lead to product harvesting difficulties [10]. Harvesting bioproducts at such low concentrations is laborious and time consuming.

Several attempts have been made to overcome the difficulties of low productivities in cyanobacteria. The first aspect of low productivity is the development of robust cyanobacterial chassises. Wendt and colleagues discovered and characterized the fastest growing cyanobacterial species, *Synechococcus elongatus* UTEX 2973, with a replication time of 1.5 h, as a strong candidate for large-scale applications [18]. Wlodarczyk and colleagues reported that the newly discovered *Synechococcus* sp. PCC 11901 is a robust cyanobacterial strain for high biomass production under high light intensities with a short doubling time of approximately 2 h [19]. Apart from the discovery of the naturally robust chassises, we can reasonably anticipate that adaptive evolutionary strategies could also be exploited to artificially domesticate super cyanobacterial chassises that can tolerate high light intensity and salt concentration with high photosynthetic efficiency. With the development of these effective strategies, the photosynthetic efficiencies of cyanobacteria can be significantly improved in the future, thus accelerating commercialization.

The second aspect of low productivity is light penetration, a fundamental limitation for cyanobacterial bioproduction. Long and colleagues partially overcame this challenge by developing machine learning-informed and synthetic-enabled semicontinuous algal cultivation to unleash renewable fuel productivity [20]. This well-developed strategy highlights the commercialization limitations caused by mutual shading and high harvest costs.

The third direction in enhancing productivity is to improve photosynthesis. Li and colleagues found that plants often deplete the pool of C5 intermediates shunted by glucose-6-phosphate (G6P) in photosynthesis dark reactions, resulting in an extremely slow ramp-up of productivity in subsequent photosynthesis light reactions [21]. Shinde and colleagues demonstrated that the existence of a G6P shunt to increase C5 pools allows more dark reaction intermediates in

Glossary

Carbon-negative synthetic biology: carbon negativity is the reduction of an entity's carbon footprint to less than neutral. Carbon-negative synthetic biology is a potentially significant new option for capturing CO_2 and using it to create materials and products for human consumption by synthetic biology toolkits.

Clustered regularly interspaced short palindromic repeats (CRISPR): it is a family of DNA sequences found in the genomes of prokaryotic organisms such as bacteria.

CRISPR/Cpf1: Cpf1 represents a novel single RNA-guided CRISPR/Cas endonuclease system suitable for genome editing with distinct features compared with Cas9.

Cyanobacterial cell factories: cyanobacteria are a group of photoautotrophic prokaryotes engineering the cyanobacteria as cell factories for the sustainable production of chemicals from CO_2 and sunlight.

Markerless genome editing: a markerless genome editing system that uses counter-selectable genes to promote the death of microorganisms and also uses a second single exchange ensures that the edited microorganism no longer carries other unwanted genes.

Synthetic biology: a multidisciplinary area of research that seeks to create new biological parts, devices, and systems, or redesign systems.

Third-generation (3G) biorefinery: 3G biorefineries aim to utilize microbial cell factories to convert renewable energies and atmospheric CO_2 into fuels and chemicals.

Zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs): synthetic proteins whose DNA-binding domains enable them to create double-stranded breaks in DNA at specific points.

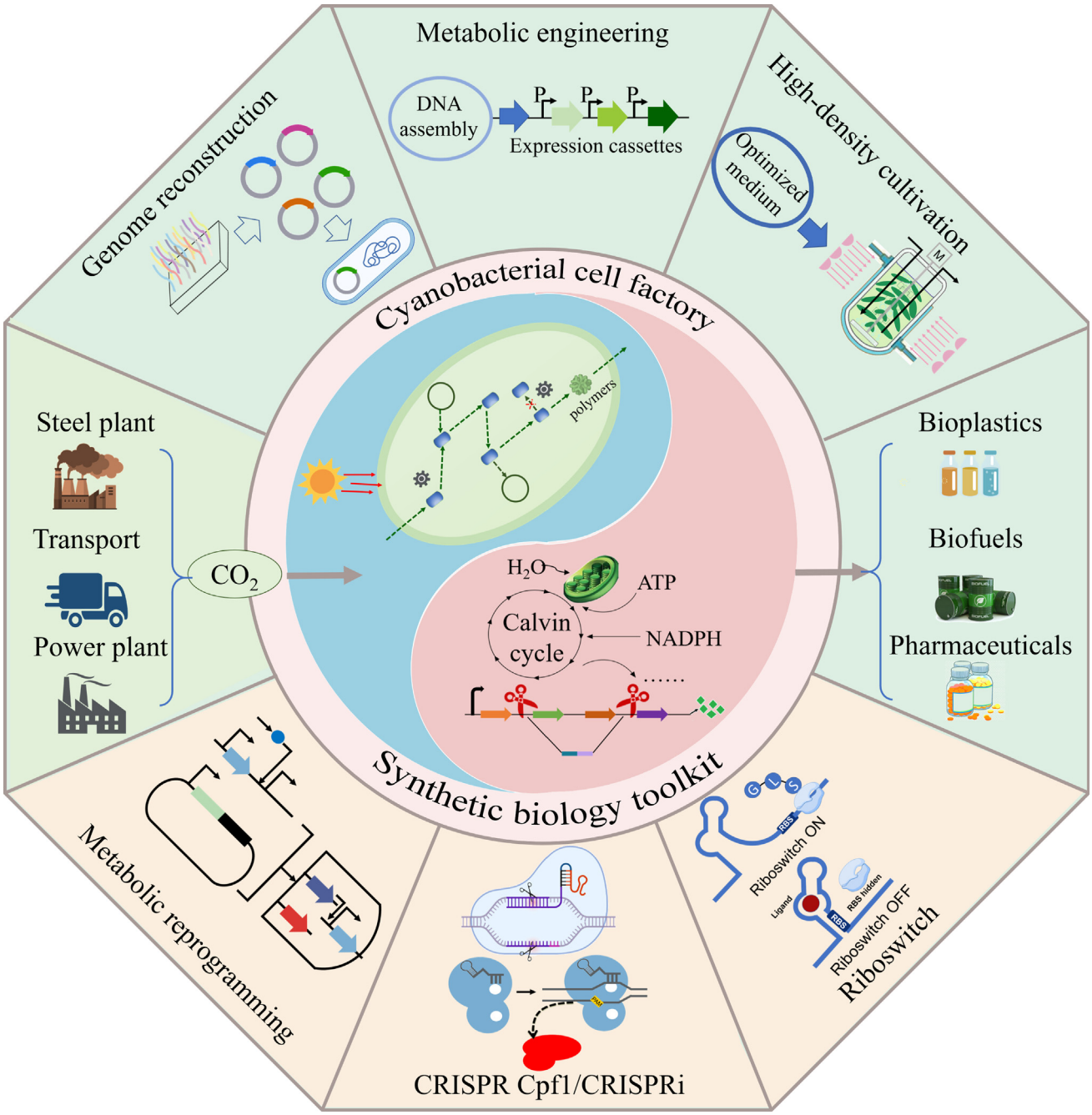

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Figure 1. Applications of synthetic biology toolkits in metabolic network reprogramming for producing value-added products like biofuels and bioplastics by cyanobacterial cell factories directly from CO_2. Cyanobacteria can directly use the CO_2 from the Calvin cycle like high plants. CO_2 is produced from the combustion of fuel in steel and power factories and means of transportation, and the largest human source of CO_2 emissions is the combustion of fossil fuels. Many synthetic biology toolkits, such as CRISPR Cpf1/CRISPRi and the biosensors of the riboswitch, can be used to reprogram the cyanobacterial metabolic network for desired products. A combinational strategy of metabolic engineering and high-density cultivation can be used to improve production using cyanobacterial cell factories. Abbreviation: CRISPR, clustered regularly interspaced short palindromic repeats.

cyanobacteria, rendering photosynthesis more rapid and with productivity improvements [22]. They demonstrated that a smooth metabolic transition from dark to light is crucial for fitness and overall photosynthetic productivity. With the latest developments involving carbon-negative synthetic biology in cyanobacteria, we can reasonably anticipate that large-scale bioproduction applications by cyanobacteria can be achieved in the future.

What are the most suitable bioproducts for cyanobacteria to produce?

Cyanobacteria have been successfully demonstrated to produce many value-added bioproducts, such as squalene [12], α-farnesene [13], limonene [14], ethylene [16], resveratrol, p-coumaric acid, caffeic acid, and ferulic acid [23], directly via carbon capture. These chemicals are high-value-added products and have been widely used in multiple domains. In addition, cyanobacteria can also be used to produce many other products with low value but high consumption, such as biodegradable plastics [10]. Easy harvesting is crucial in terms of commercial applications for this type of product. The following three types of products may essentially provide the solutions to solve the problem of product harvesting for cyanobacterial water-soluble chemicals: (i) polymer products (Box 1), such as polyhydroxyalkanoates (PHAs) [24], starch [25], bacterial cellulose [26]. A low product titer is the most significant barrier to commercializing

Box 1. Production of polymers and hydrocarbons by cyanobacteria

Global contamination caused by nondegradable plastics is a huge environmental burden. PHA and PLA are the most promising 'green plastics' because of their outstanding properties, such as biocompatibility and biodegradability [10]. Currently, the production of biodegradable plastics relies on crop-based feedstocks and poses a potential threat to human food supplies. Engineering cyanobacterial cell factories to produce biodegradable plastics directly from CO_2 has emerged as a promising strategy to simultaneously address plastic pollution and excessive CO_2 emissions. Koch and colleagues reported an increased PHB production to 81% per dry cell weight using a series of systematic metabolic engineering strategies, including knocking out the regulatory protein PirC (production of sll0944) in *Synechocystis* sp. PCC 6803 [11]. The high amounts of PHB generated demonstrate the potential of cyanobacteria for sustainable industrial production of PHB. Tan and colleagues developed a cyanobacterial cell factory for *de novo* biosynthesis of PLA directly from CO_2 using combinational strategies of metabolic engineering and high-density cultivation [10]. As presented in Table 1 in the main text, the production of water-soluble chemicals by cyanobacteria is usually relatively low, which is a major barrier to the commercialization of cyanobacterial cell factories [6]. Cyanobacterial cells comprise only a small proportion of the culture broth volume. *In vivo* production of PHA polymers can solve the problem of product harvesting in cyanobacteria because the cellular inclusion granules formed can easily be harvested together with the cells.

Bacterial cellulose, a polysaccharide consisting of many β (1→4) linked D-glucose units, exhibits several excellent properties, such as high purity, strength, moldability, and water-holding capacity [26]. It has various current and potential future applications in multiple fields, including the food industry, medicines, commercial and industrial products, and other technical areas [73]. Cellulose is an abundant biopolymer and can be easily separated from aqueous media owing to its solubility. Microbial synthesis of cellulose is an effective method for its production. Gao and colleagues developed an *in situ* microbial fermentation method to produce functional bacterial cellulose. They demonstrated that microbial synthesis is more efficient, controllable, and environmentally friendly than traditional modification methods [74]. Huang and colleagues proposed a new concept for regulating the structure of bacterial cellulose to provide a theoretical basis for its applications in several fields [26]. Therefore, producing cellulose through cyanobacterial cell factories directly from CO_2 is feasible and promising. Considering its ease of harvest, we believe that it would be a suitable candidate for industrial applications in cyanobacterial cell factories.

Hydrocarbons are ideal fuel components that serve as raw materials for the production of plastics, fibers, rubbers, solvents, and industrial chemicals. Cyanobacteria have emerged as ideal cell factories to produce low molecular weight chemicals directly from CO_2. The bioproducts of low molecular weight chemicals are usually water soluble. Harvesting and purifying water-soluble chemicals when they are at extremely low concentrations is time consuming and laborious. Notably, medium- to long-chain hydrocarbons are usually insoluble in water, making them easier to harvest by centrifugation than low molecular weight soluble chemicals. Yunus and colleagues engineered four metabolic systems for converting CO_2 into hydrocarbons using cyanobacteria [75]. This approach has three characteristics: (i) natural secretion from cells, (ii) easy separation from aqueous media, and (iii) ready for use without further processing. Therefore, direct carbon capture for the production of medium- to long-chain hydrocarbons is a promising and sustainable strategy for solving the product harvesting difficulties.

cyanobacterial biotechnology. **Cyanobacterial cell factories**' production of biodegradable plastics, such as PHAs, could perfectly integrate polymer biosynthesis with flocculation, circumventing this unsolved problem. (ii) Water-insoluble liquid products, such as medium- to long-chain hydrocarbons (Box 1). Hydrocarbon biosynthesis by cyanobacteria directly from carbon capture has gained much attention as an alternative to fossil fuels, plastics, and solvents [27]. Hydrocarbons can be easily separated from aqueous media and are ready for use without further processing. (iii) Volatile gas products such as limonene [14] and ethylene [16]. Durall and colleagues reported that ethylene production was increased through the genetic rewiring of cyanobacterial central carbon metabolism to enhance carbon supply to the tricarboxylic acid (TCA) cycle. These products are water insoluble and can be easily harvested without further process engineering. In summary, producing these value-added bioproducts by cyanobacteria directly from CO_2 would be a promising strategy to solve the problem of product harvesting difficulties. Therefore, we expect that they represent suitable bioproducts for commercial cyanobacterial applications.

Synthetic biology toolkits

Cyanobacteria-based production is one of the most carbon-efficient solutions for sustainable chemicals and CO_2 capture and utilization. Despite considerable efforts, the economy of cyanobacterial bioproducts has been hindered by the lack of synthetic biology toolkits involving cyanobacteria as these lag far behind toolkits available for bacteria such as *Escherichia coli* and *Bacillus subtilis* [28]. Based on engineering biology concepts, synthetic biology aims to be able to redesign and transform existing or natural biological systems through *de novo* design and construction of DNA components [29]. The notion of synthetic biology toolkits for cyanobacteria is an abstraction overlaid on physical processes that could process inputs to produce outputs by techniques such as metabolic network reprogramming, genome editing, and dynamic metabolic flow regulation (Figure 2). In this section, we have elucidated the mechanisms, by which synthetic biology toolkits help address some challenges in enhancing CO_2 uptake and fixation in cyanobacteria.

Currently, several synthetic biology toolkits have been developed for use in cyanobacteria. For instance, Niu and colleagues designed and improved **clustered regularly interspaced short palindromic repeats (CRISPR)-Cpf1** genetic strategies in cyanobacterium *Anabaena* PCC 7120, realizing genetic experiments that have thus far been difficult to perform in cyanobacteria [30]. Yamamori and colleagues have obtained a stress resistance chassis by introducing stress resistance elements and an artificial adaptive evolution toolkit [31]. Shabestary and colleagues developed a novel strategy for arresting growth and activating lactate synthesis to increase the cyanobacterial bioproduction of lactate using CRISPR interference (CRISPRi), and lactate titer reached 1 g/l [32]. In addition to the CRISPR-based genetic toolbox, other aspects of synthetic biology toolkits also serve as valuable tools for identifying and mitigating metabolic bottlenecks. For example, Wang and colleagues systematically characterized promoters and ribosome bind site (RBS) design strategies to effectively manipulate genetic circuits in cyanobacteria [33]. They demonstrated a strong promoter and a strategy complementary to RBS prediction software to facilitate the rational design of an RBS library to optimize gene expression in cyanobacteria. Consequently, the development of synthetic biology has accelerated the synthesis of high-value-added products by cyanobacteria. For instance, a 'self-remodeling' mechanism of the photosynthetic chain in *S. elongatus* was obtained, accelerating the synthesis of 2-phenyl ethanol [34]. In addition, cyanobacterial cell factories have been engineered as kernels to produce the C3 platform chemicals of glycerol, dihydroxyacetone, 1,3-propanediol, and 3-hydroxy propionic acid [35], as well as other fine chemicals for manufacturing foodstuffs and pharmaceuticals, such as vanillin and 4-vinyl phenol [36], D-lactate [32], p-coumaric acid [37], and L-lysine [38]. These efforts

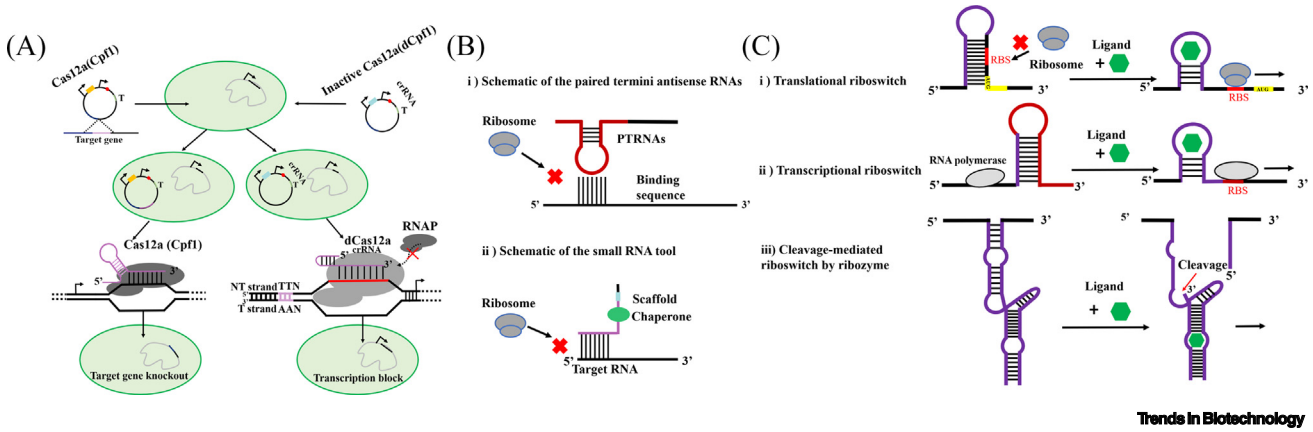

Figure 2. Schemes of cyanobacteria synthetic biology toolkits. (A) Overview of the Cas12a and dCas12a (dcpf1)-mediated CRISPR systems that have been used for genome editing and metabolic flux regulation in cyanobacteria, respectively. The functions and principle of the two CRISPR-based systems are shown to edit the target genes and redirect the metabolic flux. Adapted from [44]. (B) Schematic of the paired-termini antisense RNA (named PTRNA) tool and the small RNA (sRNA) tool that have been used in cyanobacteria. Function and design principles were shown for the PTRNA and sRNA tools. The chaperone can expedite annealing of sRNA to its target mRNAs and recruit the major endo-ribonuclease RNase E for cleavage of the target. Adapted from [76]. (C) Three kinds of riboswitches are used in cyanobacteria. The riboswitch comprises two sections: (i) two tandem aptamers and (ii) an expression platform part. The riboswitch could change its secondary structure and activate/repress the expression of its downstream gene in response to its corresponding legends (metabolites). Abbreviations: RBS, ribosome bind site; RNAP, RNA polymerase.

demonstrate that cyanobacteria are ideal cellular factories and excellent self-supporting platforms for the sustainable production of chemicals [39].

CRISPR-based efficient genome editing

CRISPR-based genome editing has been a technology for specific DNA modification of targeted genes since it was initially discovered in the bacterial immune system [40]. The CRISPR system can be divided into three categories based on CRISPR/Cas proteins. Among the three types of CRISPR, the class II type II system from *Streptococcus pyogenes* has been widely used for genome editing, particularly the representative Cas9 protein [41]. The CRISPR/Cas9 system guides Cas9 protein to specifically bind to a target sequence to perform DNA cleavage through a specific single-guide RNA (sgRNA) [42]. Compared with **zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs)**, two traditional gene-editing technologies, the CRISPR/Cas9 system has the advantages of simplicity, high efficiency, and low cost [43]. It is increasingly used in plants, animals, humans, and cyanobacteria [44].

CRISPR-based systems have enormous potentials for developing synthetic biology in cyanobacteria to synthesize high-value-added products because it permits **markerless genome editing**, simultaneous manipulation of multiple genes, and transcriptional regulation of genes [44]. For instance, markerless genome editing has been achieved by targeting *nblA*, which encodes an essential protein for its vital role in biological responses to nitrogen-deprivation conditions [45].

Moreover, to repurpose CRISPR/Cas9 for fine-tuning gene regulation rather than genome editing, catalytically inactive Cas9 (dcas9) has been used as an essential platform for RNA-guided transcriptional regulation [46]. This modified CRISPRi system can yield specific silencing of the targeted gene without Cas9 nuclease cleavage activity by repressing RNA transcription, which can involve the simultaneous manipulation of multiple genes through multiple sgRNAs [47]. CRISPRi, a titratable, trans-acting regulatory gene toolkit for value-added products, was achieved in *Synechocystis* sp. PCC 6803 [47] and *S. elongatus* UTEX 2973 (a fast-growing unicellular cyanobacterium) [48].

However, the Cas9 protein is toxic even when Cas9 expression is at a medium level in *S. elongatus* UTEX 2973. Thus, the CRISPRi system cannot be stably maintained for prolonged periods [45]. Therefore, other CRISPR/Cas protein-mediated CRISPR systems have been applied to achieve more stable multiple gene knockdown with improved stability in cyanobacteria. Another CRISPR-Cas protein (Cpf1, formerly known as type V Cas12a protein [49]), which belongs to class II CRISPR endonucleases, has been widely used for genome editing in cyanobacteria [50]. Cpf1 endonuclease binds to and cleaves specific sites of a target gene under the guidance of sgRNA [44]. The CRISPR/Cpf1 system has many advantages over the CRISPR/Cas9 system: (i) the CRISPR/Cpf1 system is more convenient and simpler than the CRISPR/Cas9 system because only crRNA is required in the Cpf1 system. (ii) Unlike Cas9 protein, the protospacer adjacent motif (PAM) sequence recognized by Cpf1 is thymidine-rich (T), which can expand the editing range of CRISPR [51]. (iii) Cas12a cleaves target DNA to produce sticky ends, which is beneficial for inserting target genes [52]. Thus, the Cpf1 exhibits a lower off-target rate than Cas9 under the premise of similar editing efficiency [49,50]. Unlike Cas9, Cpf1 lacks the HNH domain but contains the Nuc domain (RnvC-like domain) to cleave the target strand [50]. Moreover, Cas12a mutations D917A and E1006A result in the loss of cleavage of the DNA strand. Therefore, the CRISPR/'dead' Cas12a system has been successfully applied in CRISPRi for tunable repression of multiplex genes in *E. coli* [50] or *Synechococcus* sp. UTEX 2973 [49].

Strategies for metabolic network reprogramming

Synthetic biology and metabolic engineering of photosynthetic organisms offer a promising route for producing high-value-added products directly from CO_2. However, the development of microbial cell factories for chemicals in cyanobacteria is limited by low-flux carbon metabolic pathways

Box 2. Gene regulation by biosensors of riboswitches in cyanobacteria

The riboswitch, also known as the RNA molecular switch (RNA switch), is a regulatory component of mRNA and is located in the 5' untranslated region (UTR) [77]. The riboswitch has two parts: an aptamer domain (AD), which is responsible for binding metabolites, and an expression platform (EP). The AD can perceive structural changes and modulate gene expression through transcription termination, translation initiation, and intron splicing [78]. Several different types of riboswitches have been discovered [79]. These riboswitches can be used to identify essential metabolites, such as coenzymes, amino acids, nucleotide bases, and sugars. Most riboswitches are widespread in eubacteria and are usually involved in regulating feedback inhibition of metabolite biosynthesis or transport-related genes. Most riboswitches bind to specific ligands and inhibit the expression of downstream target genes (the 'off' switch), except for adenine and glycine riboswitches, which can activate the expression of downstream target genes after binding to their specific ligands (the 'on' switch) [76]. Nine types of metabolites have been discovered: adenosylcobalamin (AdoCbl), thiamine pyrophosphate (TPP), flavin mononucleotide (FMN), guanine, adenine, S-adenosylmethionine (SAM), lysine, glycine, and glucosamine-6-phosphate (GlcN 6P) [80].

Performing a common gene trial for cyanobacteria takes longer than that in rapidly growing bacteria. Currently, a small number of riboswitches are functional in cyanobacteria. In 2013, a modified theophylline-dependent riboswitch was introduced into cyanobacteria for the first time to achieve strict control of protein expression in *S. elongatus* 7942 [81]. These theophylline-dependent riboswitches can control the translational initiation of target genes in cyanobacteria after binding to the aptamer of theophylline, allowing the maximum magnitude of induction to be 190-fold [81]. Singh and colleagues have analyzed the distribution of riboswitch families in various cyanobacterial genomes and observed that four riboswitch classes were abundant in cyanobacteria, including B12-element (Cob)/AdoCbl/AdoCbl-variant riboswitch [82]. Additionally, a vitamin B12 (cobalamin)-dependent riboswitch was recently discovered in *Synechocystis* sp. PCC 7002, which cannot synthesize cobalamin [83]. However, it is unclear whether it also functions in other cyanobacteria that can synthesize cobalamin, such as *Synechococcus* sp. 7942. A recent study showed that glutamine could tightly regulate the expression of glutamine synthetase in *Synechocystis* sp. PCC 6803 [84]. Sun and colleagues provided an *N*-acetylneuraminic acid-based sensing and responding switch for orthogonal gene regulation in *S. elongatus* UTEX 2973 and *S. elongatus* PCC 7942 [85]. They integrated the orthogonality between the switch and theophylline-responsive riboregulatory system to achieve the coordination or binary regulation of target genes in cyanobacterial *Synechococcus* strains. Synthetic riboswitches can be used to detect the presence or absence of a metabolite in solution, providing a molecular tool for improving the productivity of value-added chemicals. Further studies are warranted to discover new functions of riboswitches or engineer them for better use in other kinds of cyanobacteria. The well-developed synthetic biology toolkits would have a wide range of applications for promoting the metabolic regulation of cyanobacteria in the future.

Table 1. Summary of biochemicals obtained using different cyanobacterial chassis

Cyanobacterial chassis	Strategy	Biochemical	Titer	Refs
S. elongatus PCC7942	Increase the expression of a fusion CpcB1-SQS protein	Squalene	79.2 mg/g dry cell weight (DCW)	[12]
	Engineer carbon metabolism to improve glucose utilization, enhance CO_2 fixation	2,3-BDO	12.6 g/l	[6]
	Improve β-carotene flux toward zeaxanthin synthesis by cloning CrtR (β-carotene oxygenase) gene	Zeaxanthin	9.02 ± 1.1 mg/g DCW	[86]
	Overexpression of carbonic anhydrase and bicarbonate transporter genes in carbon concentrating mechanism (CCM)-deficient strains	α-Farnesene	5.0 ± 0.6 mg/l	[13]
	Overexpressing the primary sigma factor, together with computational modeling and wet lab analyses	Limonene	19 mg/l in 7 days	[14]
Synechocystis sp. PCC 6803	Heterologously expressing feedback-inhibition-resistant AroG and TyrA enzymes from *E. coli*	Phenylalanine and tyrosine	580 ± 34 mg/l; 41 ± 2.3 mg/l	[87]
	Rate-limiting step from β-carotene to astaxanthin was identified and improved and eliminated bottlenecks	Astaxanthin	29.6 mg/g DCW	[88]
	Improved flux toward valencene; downregulated the formation of carotenoids; increased the spatial proximity of the two enzymes	Valencene	19 mg/g DCW	[89]
	Overexpressing two bottleneck enzymes and enhancing the Embden–Meyerhof–Parnas pathway together with high-density cultivation	Bisabolene	180 mg l^{-1}	[90]
	Extra copies of the native *pepc*, heterologous expression of phosphoenolpyruvate synthase (PPSA) and PEPc	Ethylene	16.77 ± 4.48 μg mL^{-1} OD^{-1} day^{-1}	[16]
	Knockout of the regulatory protein PirC and introduced *phaA* and *phaB* under the control of the strong promoter PpsbA2, grown in a nitrogen medium	PHB	81% per DCW	[11]
Synechocystis sp. PCC 7002	Overexpression of the riboflavin biosynthesis genes ribDGEABHT and the flavin transporter *pnuX*	Riboflavin (vitamin B2)	41 μM	[91]
	Addition of UDP-GlcNAc or UDP-GlcUA synthesis enzyme	Hyaluronic acid	112 mg/l	[70]
Synechococcus sp. PCC 11901	Discovered a new cyanobacterial strain with a short doubling time of ≈2 hours that accumulates up to ≈33 g DCW	Fatty acids	1.5 g l^{-1}	[19]
S. elongatus UTEX 2973	Expression of a feedback-resistant copy of aspartate kinase and a lysine exporter via plasmid-based expression; modularly extending the lysine pathway to glutarate and cadaverine	L-Lysine, cadaverine, and glutarate	556.3 ± 62.3 mg/l; 55.3 ± 6.7 mg/l; 67.5 ± 2.2 mg/l	[92]
	Reduction of intracellular glycogen accumulation in S2973 using dCas12a method	Glycogen	35–50% of DCW	[49]

such as the shikimate pathway [34]. Metabolic network reprogramming can redirect carbon flux to enhance the production of chemicals and biofuels [53]. Several studies have been conducted to remodel carbon flux. For example, Ni and colleagues established a powerful metabolic sink, an artificial feedback-inhibition-resistant cassette, in *S. elongatus*, thus accelerating electron transport and reducing energy wastage in the shikimate pathway [34]. Kanno and colleagues introduced a modified glycolytic pathway. They deleted *cp12*, a regulatory gene of the Calvin–Benson cycle, to increase carbon flux and redirect it toward carbon fixation, yielding 12.6 g/l of 2,3-butanediol (2,3-BDO) [6]. Gao and colleagues alleviated carbon catabolite repression and constructed a protease-based dynamic regulation circuit to fine-tune metabolic flow, thus achieving a 12.63 g/l shikimate titer [54].

Consortium engineering of cyanobacteria can overcome many metabolic bottlenecks that are inherent to cyanobacteria and eventually allow high productivities and titers. For example, Zuñiga and colleagues provided a robust modeling framework for the rational design of synthetic communities of heterotrophs and phototrophs with optimized growth sustainability [55]. Kruyer and colleagues used the cyanobacteria to convert CO_2 into sugars that were upgraded by engineered *E. coli* into

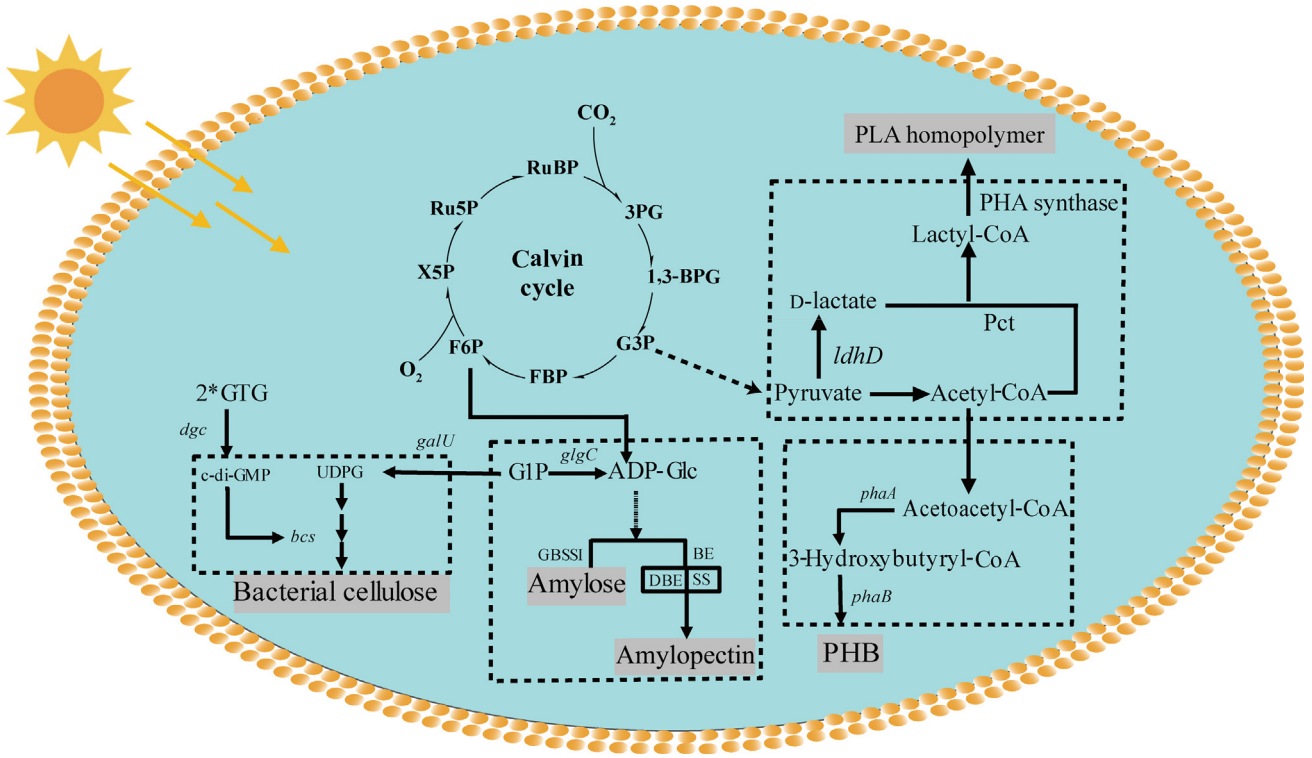

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Figure 3. Overview of the synthetic pathway of polymers in cyanobacteria. The heterologous and native enzymes of cyanobacteria that participate in the biosynthesis of bacterial cellulose, PLA, poly-β-hydroxybutyric acid, and starch are shown. PLA and bacterial cellulose are homopolymers made of lactic acid and glucose subunits, respectively. Starch, a storage form of carbohydrate, is generally produced from photosynthesis in higher plants and it can be divided into amylose and amylopectin. Cyanobacteria could direct carbon capture for chemical production through the Calvin cycle like a high plant. UDPG and acetyl-CoA are the essential intermediates for producing bacterial cellulose, PLA, and poly-β-hydroxybutyric acid. The unbroken arrow indicates that the catalysis step can be achieved by one step, and the broken arrow indicates that the catalysis step contains several steps. Abbreviations: ADP-Glc, ADP glucose; BE, debranching enzyme [66]; *bcs*, bacterial cellulose synthase [26]; DBE, starch debranching enzyme; *dgc*, diguanylate cyclase; F6P, fructose 6-phosphate; G3P, glyceraldehyde-3-phosphate; GBSS, granule-bound starch synthase [69]; galU, UDP-glucose pyrophosphorylase; RuBP, ribulose-1,5-bisphosphate; PEP, phosphoenolpyruvate; Pct, propionate CoA-transferase; PHA, polyhydroxyalkanoate synthase; PHB, polyhydroxybutyrate; PLA, polylactic acid; SS, soluble starch synthase; UDPG, uridine diphosphate glucose.

2,3-BDO [56]. Weiss and colleagues reported synthetic light-driven consortium cyanobacteria and heterotrophic bacteria for stable PHB production [57]. With this emphasis, cyanobacteria can export photosynthetically fixed carbon as sucrose, which is utilized by heterotrophic bacteria for high yields of chemicals. Therefore, fine-tunable synthetic biology toolkits and enabling technologies can be used to redirect carbon flux into the target bioproduct pathways such as riboswitch-mediated biosensors (Box 2), thus improving the economy of cyanobacteria for large-scale bioproduction.

Conducting metabolic engineering and regulation is challenging because of the two relatively independent systems involving cyanobacterial energy and material metabolisms. With the development of synthetic biology, a new idea of metabolic network reprogramming has been proposed, which starts from the node elements of carbon metabolism and uses synthetic biology regulator parts, including RBS and promoter cassettes to optimize metabolic flux in synthetic pathways. Meanwhile, we can also combine two or three advanced metabolic reprogramming design toolkits for improving metabolic flux management; for example, Ni and colleagues combined 'metabolic sink' biotechnology and an artificial feedback-inhibition-resistant cassette into *S. elongatus* to redirect >30% of the carbon to the shikimate pathway for synthesizing 2-phenyl ethanol [34]. Xiong and colleagues enabled efficient ethylene production from the TCA cycle by enhancing photosynthetic activity and redistributing the carbon flux [58]. Regarding synthetic biology toolkits, Gibbons and colleagues advanced some new directions involving a double crossover approach to inactivate target genes in *Anabaena* PCC 7120 [59], which may be broadly applicable to the study of gene function in cyanobacteria and other prokaryotic organisms.

Apart from flux redirection, metabolic network modeling-based approaches are also significant for improving the economy to achieve high bioproduction by cyanobacteria. The latest developments in the modeling field have focused on using mathematical modeling to identify metabolic bottlenecks to improve bioproduction. Zhao and colleagues developed a dynamic system model of C4 photosynthesis based on maize to identify features associated with high photosynthetic efficiency in NADP-malic enzyme-type C4 photosynthesis [60]. These findings provide promising strategies for improving C4 photosynthesis. Shinde and colleagues increased limonene titer to 19 mg/l in 7 days after characterizing the primary sigma factor in *S. elongatus* PCC 7942 using computational modeling and wet lab analysis [14], providing a novel strategy for future terpenoid engineering in phototrophs. Machine-learning-based frameworks have advanced new directions for modeling-based work. Wu and colleagues developed a machine-learning-based framework for ^{13C}-fluxomics, highlighting advances in the design–build–test–learn cycle in synthetic biology [61]. Wang and colleagues expanded the knowledge of natural guanidine degradation metabolic pathways and demonstrated that biological applications could support ethylene bioproduction through comparative proteomics and experimental validation [62]. These designs enable cyanobacteria to reprogram carbon flux to enhance the carbon metabolic pathway. This will significantly reduce operational workloads and increase the efficiency of cyanobacteria in synthesizing high-value compounds.

Concluding remarks and future perspectives

Cyanobacterial cell factories have been shown to produce dozens of value-added products, including low molecular weight chemicals such as lactic acid, squalene, and valencene (Table 1). The production by cyanobacteria is lower than that of heterotrophic cell factories such as *E. coli*. In addition, harvesting low molecular weight and water-soluble chemicals is time consuming and laborious, which poses a major barrier to industrial applications (see Outstanding questions). In addition to insufficient resistance to stress, low product export efficiency, and poor cellular regulation, the realization of industrial applications involving cyanobacterial cell factories is limited. These limitations must be addressed and overcome in the future.

Outstanding questions

Several emerging synthetic biology toolkits have been developed and utilized for cyanobacteria. Which of these tools have the considerable potentials to improve commercialization of cyanobacterial bioproduction on a large scale?

The high cost of phototrophic cultivation partially hinders the commercial production of cyanobacterial bioproducts. Are there any engineering strategies to augment growth rate and biomass along with the reduced cost of large-scale cultivation?

The latest developments in cyanobacterial research have focused on developing new robust chassises. How can cyanobacterial tolerance and photosynthetic efficiency be balanced? What are the upper limits of light and salt tolerance in cyanobacteria?

Would be instrumental for expanding the spectrum of light available for photosynthesis in cyanobacteria?

Emigration to Mars has become a popular topic in recent years. Cyanobacteria can absorb CO_2 and produce O_2, which is crucial to Earth's ecology. Could cyanobacteria play a vital role in supporting human habitats on Mars? How feasible is that?

Cyanobacteria are an ideal class of photosynthetic microorganisms because they do not need fertile soil to grow; therefore, they do not interfere with the cultivation of food and crops [63]. Cyanobacteria are commonly accepted as excellent candidates for synthesizing biofuels or high-value-added chemicals [64]. However, the low product export by cyanobacteria challenges their commercialization. It is well known that the polymers synthesized *in vivo* by cyanobacteria are a type of polymer granule that serve as stores of carbon, such as glycogen [63] and sucrose [65] that are easy to harvest. The polymers synthesized by cyanobacteria from CO_2 serve as intracellular carbon and energy reserves. They are environmentally friendly and easy to harvest, such as PHAs [24] and PLA [10] (Figure 3). Considering the characteristics of easy separation from medium and fewer further handling processes, polymer products would be promising candidates for cyanobacterial industrial applications.

In addition to these successfully demonstrated polymer products, starch would be a strong promising candidate polymer product in cyanobacteria. Starch is a primary food supply for humanity and is produced mainly in seeds, rhizomes, and roots [25]. Unlike glycogen particles, starch aggregates into insoluble semicrystalline granules, which are almost entirely composed of two main polysaccharides: amylose and amylopectin. Starch granules are polymeric carbohydrates containing α-(1,4)-linked D-glucose residues [66]. The marine cyanobacterium (strain CLg1) has been reported to synthesize glycogen and starch [67]. Starch biosynthesis requires the coordinated activities of several enzymes, including adenosine 5'-diphosphate-glucose (ADP-Glc), pyrophosphorylase (AGPase), and granule-bound starch synthase (GBSS) [68]. The GBSS enzyme produces amylose from ADP-Glc, which requires the binding of semicrystalline polysaccharides to be active [67,69]. The cyanobacterium *Synechococcus* sp. contains ADP-Glc [70], thus making it possible to engineer cyanobacteria to produce amylose. Kato and

Box 3. Emerging trends of cyanobacterial applications

Cyanobacteria have biomedical application owing to their ability to generate O_2. Hypoxia can alter the tumor microenvironment, leading to tumor progression and metastasis. Photodynamic therapy is a promising strategy to inhibit tumor formation by catalyzing O_2 to produce cytotoxic reactive oxygen species (Figure I). Sun and colleagues created a bio-oxygen pump to alleviate hypoxia, achieving almost 100% inhibition of tumor tissues using *S. elongatus* UTEX 2973 [93]. Cardiovascular disease is a leading cause of death worldwide. Many patients succumb to heart failure secondary to residual microvascular perfusion deficits, indicating an urgent need to discover novel approaches to treat myocardial injury. Cohen and colleagues developed a novel system that rescues the myocardium from acute ischemia through the intramyocardial delivery of cyanobacteria [94]. Notably, the cyanobacterial therapy increased tissue oxygenation, preserved myocardial metabolism, and enhanced cardiac output and is nontoxic and does not elicit an immune response. Currently, 25% of patients with diabetes are at risk of chronic nonhealing wounds [95]. Chen and colleagues developed an oxygen-producing patch using a cyanobacterium that naturally produces oxygen in sunlight through photosynthesis. They demonstrated that it could provide oxygen to accelerate wound healing in mice with diabetes, helping them to heal as quickly as healthy mice [96]. Cyanobacteria have great potential for biomedical therapeutic applications.

Exploration of Mars has been widely considered the next step in interplanetary exploration. One of the solutions to the supply problem for human-crewed spacecraft is to deliver them directly from Earth via launch vehicles. Cyanobacteria consume CO_2 to produce O_2, food, biopolymers, and pharmaceuticals. For example, *Spirulina* is rich in high-quality protein, γ-linolenic acid, fatty acids, carotenoids, vitamins, and a variety of trace elements such as iron, iodine, selenium, and zinc [97]. Hence, cyanobacteria could be used for large-scale food production in bioreactors of spacecraft (Figure I). The success of this kind of technology would significantly reduce the operating costs of spacecraft. Kruyer and colleagues advanced our prospects for interplanetary space travel by designing a rocket propellant, 2,3-BDO, from CO_2, via engineered photosynthetic cyanobacteria and *E. coli* [56]. They demonstrated that the developed ISRU strategy for 2,3-BDO production used 32% less power and 2.8-fold higher payload mass than the proposed chemical ISRU strategy. Therefore, cyanobacteria could play crucial roles in supporting human habitats on Mars.

Cyanobacteria are strong candidates for converting solar energy to generate electricity using their light-harvesting system through photosynthesis. Cyanobacteria have great potential to be used for sustainable, affordable, and decentralized power generation (Figure I). Bombelli and colleagues developed a bio-photovoltaic energy harvester system using cyanobacteria that can power a microprocessor [98]. The newly developed energy harvester charges the microprocessor for over 6 months in a domestic environment under ambient light. Cyanobacteria can also be used in 3D printing owing to their ability to generate electricity. Chen and colleagues developed an aerosol jet printing method by rewiring photosynthetic bokmakieries to electrodes [99]. They demonstrated that cyanobacteria have the distinct advantages of being inexpensive, easily accessible, and environmentally beneficial for electricity generation. Joshi and colleagues created a bionic mushroom to generate photosynthetic bioelectricity by intertwining cyanobacterial cells with graphene nanoribbons onto the umbrella-shaped pileus of the mushroom using a 3D printing technique [100]. They demonstrated that 3D printed cyanobacterial colonies resulted in an eightfold increase in photocurrent compared with isotopically cast cyanobacteria at similar seeding densities. Collectively, these studies highlight the potential for developing photosynthetically powered devices.

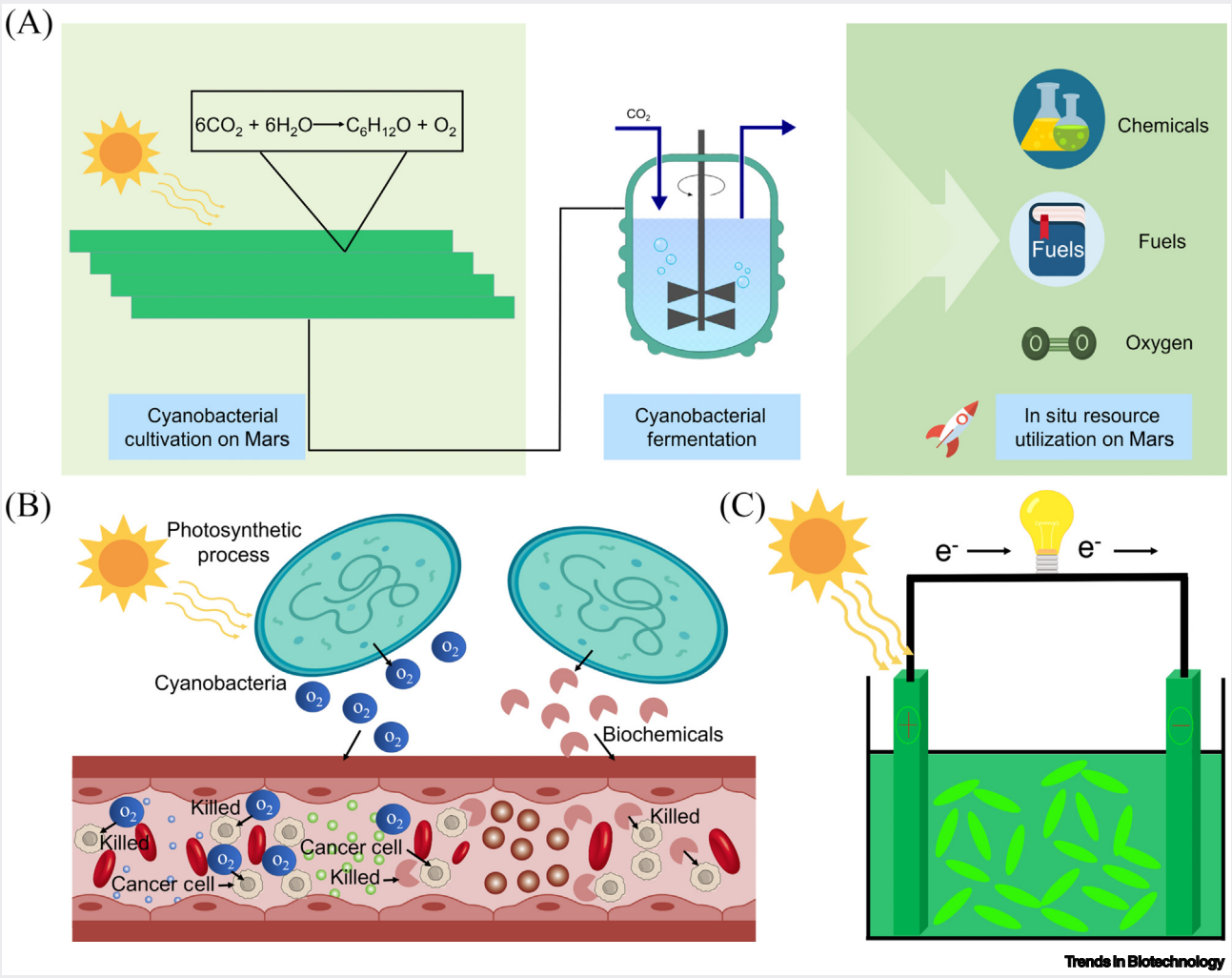

Figure I. Emerging trends of cyanobacterial applications. (A) Schematic of the proposed strategy of supporting human habitation on Mars using cyanobacteria. The engineered cyanobacteria that are cultivated in photobioreactors or outdoors could produce O_2, fuels, and chemicals by *in situ* utilization of atmospheric CO_2, water, and nutrients from regolith under sunlight or artificial light. The O_2 produced by cyanobacteria could be directly used for life support, the fuels for Mars-specific propellants, and the chemicals for therapeutics. Cyanobacteria cultivation by a photobioreactor in a spacecraft could also provide O_2 for spacecraft launches or other aspects of Martian exploration. (B) Schematic illustration of proposed biomedical applications of cyanobacteria. *In vitro* cyanobacterial cellular oxygenation could assist in killing cancer cells. Sustained photosynthetic O_2 evolution by cyanobacteria could be used for tumor therapy *in vitro* and *in vivo* upon 660 nm laser irradiation. Cyanobacteria can also be used to produce or deliver chemicals for killing cancer cells. (C) Schematic illustration of proposed cyanobacterial power generation purpose by photosynthesis. Cyanobacteria possess a unique ability for photosynthetic energy conversion with an unmatched internal quantum efficiency through their light-harvest systems, resulting in great potential for photosynthetic bioelectricity generation.

colleagues proved that the increased flux of CO_2 through glycolytic intermediates is directly associated with the enhanced formation of small starch-like particles by light/dark-conditioned screening in the lipid-rich mutant *Chlamydomonas* sp. KOR1 [71]. Cai and colleagues developed a hybrid system in which CO_2 is reduced to methanol by an inorganic catalyst and then converted by enzymes to three- and six-carbon sugar units and then to polymeric starch [72]. This is a huge step forward for the production of starch because it constructs an artificial starch synthesis pathway involving only 11 reactions, significantly reducing synthesis complexity compared with the more than 60 steps required for natural starch synthesis. Cell-free chemoenzymatic starch

synthesis needs to produce the intermediate methanol through chemical hydrogenation. By contrast, cyanobacterial cell factories could produce the bioproducts directly from CO_2. Therefore, there is a huge potential for the industrial production of starch by cyanobacteria directly from CO_2 owing to its characteristics of cost-effective and sustainable development.

Based on the cyanobacterial characteristics of converting CO_2 to O_2, several emerging high-level applications may also be considered as the solutions to the commercialization of cyanobacteria, for instance, biomedical applications, supporting human habitation on Mars, and power production (Box 3). In summary, we summarized the latest development in cyanobacterial carbon-negative synthetic biology and the emerging trends in cyanobacterial applications. We also put forward several perspectives on future development for cyanobacterial commercialization, which may be considered a crucial foundation for further research.

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Declaration of interests

The authors declare no competing interests.

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