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Biofuels from cyanobacteria - a metabolic engineering approach

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Abstract

The concern about the limited availability of petroleum-based fuels and their role in increasing CO_2 levels in the atmosphere has sparked significant attention toward biofuel and bioenergy production. The global pursuit of sustainable energy sources has catalyzed innovative research into alternative biofuel production strategies. Transforming CO_2 into usable fuels and chemicals is gaining even more prominence. Cyanobacteria, renowned for their photosynthetic ability, have emerged as promising candidates for biofuel synthesis. Their ability to convert solar energy and carbon dioxide into valuable biofuels makes them a compelling avenue for sustainable energy solutions. Using metabolic engineering principles, researchers have endeavored to optimize cyanobacterial metabolic pathways, enhance photosynthetic efficiency, and redirect carbon flux toward biofuel precursors. Numerous species of cyanobacteria offer genetic and metabolic traits that facilitate manipulation, and their photosynthetic characteristics imply that carbohydrates, fatty acids, and even alcohol could serve as potential renewable sources for biofuels. This review showcases cyanobacteria's ability as a biofuel source and emphasizes the transformative influence of metabolic engineering employed in the creation and production of "cyanofuels".

Keywords

Biofuel production; CRISPR; cyanobacteria; genetic engineering; genes; secondary metabolites

Introduction

Cyanobacteria or blue-green algae are prokaryotic, unicellular, aquatic, gram -negative bacteria and are ancient photosynthetic microorganisms that are widespread (1). These single-celled creatures can produce a wide range of biologically active compounds with antagonistic effects including toxins. Cyanobacteria can produce poisons, enzymes, vitamins, pigments, amino acids, and several fluorescent dyes (2). As a photoautotrophic organism, it requires fewer organic nutrients for biochemical production and is an ideal host for large-scale biotechnological applications (3). These bacteria have received special attention as an alternative source for biofuel generation. The most advantages of cyanobacteria rather than the traditional sources are the minimal requirements for their culture, the short generation time, the high oil content, and the ease of genetic manipulation. Cyanobacteria are currently considered an integral part of innovative energy-efficient designs for biofuel production (4). The success of the application of cyanobacteria in biofuel synthesis depends on their genetic engineering. Through the expression of the various foreign genes in cyanobacteria, the production of

many valuable chemicals is possible by redirecting metabolic pathways (5). Only cyanobacteria and microalgae are capable of producing oxygen and hydrogen. Photo-biological synthesis of H_2 in microbes is of public interest because it acts as a renewable energy carrier from nature's most abundant resources: solar energy and water. They have been explored to produce carbohydrates for ethanol, hydrocarbons, and isoprenoids for gasoline, and hydrogen and lipids for biodiesel and biofuel production (5). The synthesis of lipids by cyanobacteria as a feedstock for biofuels has tremendous promise (6). Cyanobacteria have a photosynthetic and biomass production efficiency of up to 10%, which is more than that of both plants and algae (which have efficiencies of about 1% and 5%, respectively) (7, 8).

Like fossil fuels, cyanobacterial biofuels combine alkanes, fatty acids, and fatty alcohols (9). Therefore, biofuel appears to be the best alternative to current transportation fuels without requiring significant engine modifications (10). Despite all the benefits, using genetically modified (GM) cyanobacteria to produce biofuels raises certain environmental issues. Natural ecosystems may be impacted by horizontal gene transfer and competition between GM cyanobacteria and other microbes (11). In 1999, an engineered *Synechococcus elongatus* PCC 7942 strain that had alcohol dehydrogenase II from *Zymomona mobilis* generated ethanol for the first time (12). The first organization to successfully manufacture biofuels from altered cyanobacteria was Algenol, established in 2006 and based in Fort Myers, Florida, in the United States (13).

Engineering cyanobacteria for improved secondary metabolite synthesis

Natural products are low-molecular-weight organic compounds with a variety of biological functions, many of which are quite strong. They are also known as secondary metabolites. Secondary metabolites are not necessary for an organism's proper development, growth, or reproduction. They provide defense mechanisms against stress, aid in the reproductive process, and enable the generating organism to withstand interspecies competition (14). Secondary metabolites are often categorized according to structural classes that relate to their production. This categorization has several limitations because some substances contain building blocks from many biosynthetic pathways and some substances that seem to be closely related may have entirely separate biosynthetic origins. Polyketides and non -ribosomal peptides are significant types of secondary metabolites, while additional structural classes include alkaloids, terpenoids, compounds generated from shikimate, and aminoglycosides (14). In this review, we introduce a range of secondary metabolites that can be produced from genetically modified cyanobacteria (Table 1).

Primary and secondary metabolites found in cyanobacteria include non-ribosomal proteins, polyketides, terpenes, and alkaloids. Several of these are known to have anticancer, antiviral, and UV protective activities as well as hepatotoxicity and neurotoxicity (15). Figure 1 shows the structure of some secondary metabolites synthesized by genetically engineered cyanobacteria. The use of cyanobacteria as hosts for photosynthetic chemical synthesis has advanced greatly in recent years. Numerous cyanobacterial secondary metabolites have been identified including non-ribosomal peptides (NRPs), polyketides (PKs), NRPs-PKs hybrids, ribosomal peptides (RPs), and post-translationally modified peptides (RiPPs) produced during secondary metabolism by non-ribosomal peptide synthetases (NRPS) and polyketide synthases (PKS) (16). Major biosynthetic pathways followed by cyanobacteria for the production of secondary metabolites such as malonate pathway, methylerythritol-phosphate pathway, mevalonate pathway, and shikimate pathway (17). The functionality, productivity, and efficiency of metabolic pathways have all been enhanced by genetic tools like CRISPR/Cas9 and riboswitches, which have also assisted in removing metabolic bottlenecks in native metabolism (18).

The CRISPR/Cas system is an interesting method for genetically modifying cyanobacteria and microalgae. The target design is substantially easier and there are many chances for target modification using the CRISPR/Cas system. The insertion of CRISPR/Cas9 gene editing on a plasmid backbone can result in temporary gene expression with less toxicity to the entire system and enable focused genetic engineering because Cas9 nuclease demonstrates potential lethality in cyanobacteria (18). For metabolic engineering, deactivated Cas9 and Cas12a can also be used to repress genes. To express the cryptomaldamide biosynthetic gene cluster from the marine cyanobacterium *Moorea producens*, a strain of segregated double recombinant *Anabaena* PCC 7120 including *Synechococcus*

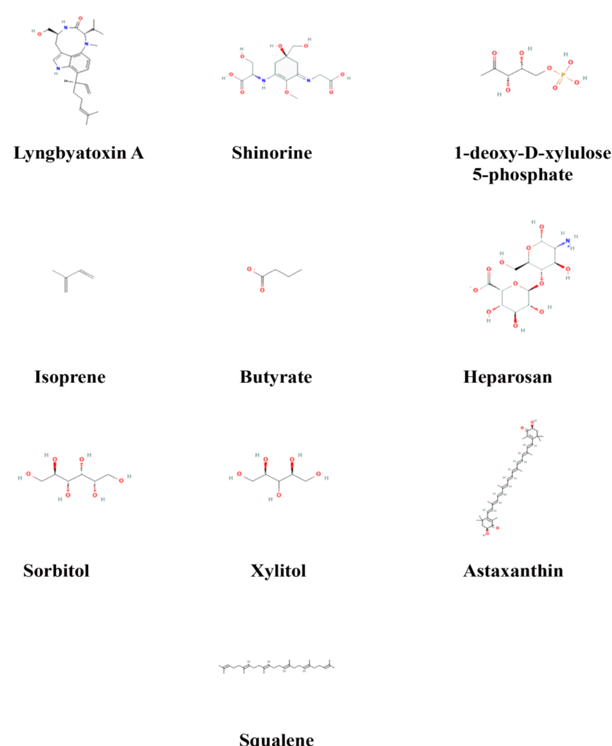

Figure 1. Structure of various secondary metabolites synthesized by genetically engineered cyanobacteria

Table 1. Various secondary metabolites synthesized by genetically engineered cyanobacteria

Sr. No.	Donor bacteria	Gene of interest	Recipient bacteria in which the gene of interest is genetically engineered	Name of the desired product synthesized by genetically engineered bacteria	Result
1	*Escherichia coli* strain GB05- red	Fosmid fos-DE3-86	*Moorea producens*	Non-ribosomal peptide lyngbyatoxin A	Heterologous expression of this pathway afforded high titers of both lyngbyatoxin A (25.6 mg L−1) and its precursor indolactam-V (150 mg/l) (21)
2	*Saccharomyces cerevisiae* CEN.PK2-1C	DDGS (*NpR5600*), O-MT 105 (*NpR5599*), ATP-grasp ligase (*NpR5598*), and D-ala-D-ala ligase (*NpR5597*)	*Nostoc punctiform*	Shinorine, mycosporine-like amino acid	Increase Sedoheptulose 7-phosphate (S7P), an intermediate of the pentose 32 phosphate pathway, is a key substrate for shinorine biosynthesis (22)
3	*Escherichia coli* BL21 (DE3)	*psbA2*, *dxs*	*Synechocystis sp.* PCC6803	1-deoxy-D-xylulose 5-phosphate synthase	Over-expression of a key enzyme in 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway, 1-deoxy-D-xylulose 5-phosphate synthase (*DXS*) (23)
4	*Synechocystis sp.* PCC 6803	*ispGS, idi, dxr*	*Synechococcus elongatus* PCC 7942	Isoprene	Engineered the MEP pathway in the cyanobacterium *S. elongatus* for photosynthetic production of isoprene from CO_2. Engineered strains achieved a final titer of 1.26 g L^{-1} isoprene and an average production rate of 4.26 mg L^{-1} h^{-1} (24)
5	*Streptomyces sp.* strain CL190, *Ralstonia eutropha, Aeromonas caviae*	*NphT7, phaBJ*, *Ptb*, *buk*, *pte2*, *tesB*, *yciA*	*Synechococcus elongatus* PCC 7942	Butyrate	Cumulative butyrate titer of around 1.1 g/L with 38 mg/L and 541 µg/L of acetate and crotonate, respectively, secreted as by-products (25)
6	*Pasteurella multocida*	*galU, PmHS2*	*Synechococcus elongatus* PCC 7942	Heparosan	pgp7942 cells yielded a maximum heparosan production 0.5µg/g-DCW with a titer of 0.44µg/l (26)
7	*Escherichia coli* JM109	*had1, fbp, trc-s6pdh, pnt*	*Synechocystis sp.* PCC 6803	Sorbitol	The highest production level of sorbitol observed was 2387 mg/l for 432 h (27)
8	*Escherichia coli* (BW25113), *Candida boidinii* (ATCC 18810), *Pichia stipites* (ATCC 58785)	*XylE, XylFGH, XR, XI, and XDH*	*Synechococcus elongatus* PCC 7942	Xylitol	Overexpression of *XylE* successfully increased the rate of xylose consumption and allowed efficient reduction to xylitol when coupled to the NADPH-dependent *XR* (28)
9	*Brevundimonas sp.* SD212, *Erwinia herbicola*	*CrtWD, CrtZA, RuBisCO, F/SBPase, Rpe, TktA*	*Synechocystis sp.* PCC 6803	Astaxanthin	Engineered strain produced astaxanthin up to 29.6 mg/g (dry cell weight) directly from CO_2 (29)
10	*Escherichia coli* DH5α	*acnB, dxs, cpcB2, SQS, idi*	*Synechococcus elongatus* PCC 7942	Squalene	Developed Cas12a-mediated CRISPRi for metabolic engineering, photosynthetic squalene production was improved by repressing the essential genes of either *acnB* encoding for aconitase or *cpcB2* encoding for phycocyanin b-subunit in *Synechococcus elongatus* PCC 7942 (30)

elongatus homologous sequences was developed (19). On the other hand, the lack of vital metabolites might inhibit cell development. To avoid lethality caused by the knockout of essential genes, CRISPR interference (CRISPRi) is a promising option for the temporary repression of competing essential metabolic pathways. CRISPRi can repress gene expression without causing nucleic acid strand excision (20).

Cyanobacteria have unique connections with other micro and macro species due to their intricate genetic pathways that produce secondary metabolites (31). Shestakov and Khyen, 1970 first documented exogenous DNA transformation in cyanobacteria, and recombinant DNA technology enabled genetically designed cyanobacteria (32). Genetic engineering's biggest challenge is transferring foreign DNA into the host (33). Several cyanobacterial species can transfer DNA through the cell membrane (34, 35, 36). The best cyanobacterial host strains for genetic engineering include naturally transformable *Synechococcus* sp. PCC 7002, *Synechococcus elongatus* PCC 7942, and *Synechocystis* sp. PCC 6803 (37). Understanding cyanobacterial cellular systems allows us to use synthetic biology to create new systems by integrating elements or controlling them. Genetic engineering can be useful to produce valuable molecules by creating novel biosynthetic pathways (38). Researchers have created cyanobacteria that utilize solar energy, CO_2, and water to make a range of compounds, such as ethanol, isobutanol, and isoprene (39). These substances might be used to make biofuels and other industrial goods. An ongoing field of study with encouraging outcomes is the engineering of cyanobacteria for the generation of fuels and chemicals (40, 41).

Synechococcus elongatus PCC 7942 and *Synechocystis* sp. PCC 6803 was genetically engineered with an ethylene-forming enzyme expressing a gene from *Pseudomonas syringae* (42, 43). The ethylene-producing genes 1-aminocyclopropane-1-carboxylate ACC synthase and aminocyclopropane carboxylate oxidase were inserted into *S. elongatus* PCC 7942 (44). *Synechocystis* cells were designed to produce and secrete glucosyl glycerol (GG) by disrupting GG uptake transporter genes, ggtC and ggtD, and a repressor gene, ggpR, for GG synthesis (45). *Aphanocapsa* (46), *Anabaena* (47), *Nostoc* (48), *Oscillatoria* (49), *Synechococcus* (9), *Gloeocapsa* (50), *Agmenellum* (51), *Arthrospira* (52), and *Haplosiphon* (53) have been utilized in genetic engineering research to produce biofuels.

Enhancing biofuel synthesis through genetic modification of cyanobacteria

Cyanobacteria, are ancient creatures that have been on Earth for at least 3.5 billion years. Cyanobacteria have a wide range of morphologies, including filamentous, unicellular, and colony-forming forms. They are regarded as biotechnological gems for high-temperature operations and are present in various geothermally heated regions of the Earth. They are used for CO_2 capture, the manufacture of biofuels, and bioremediation processes such as the elimination of phenolic chemicals. Both naturally occurring and artificial cyanobacteria create a wide range of chemical substances (54). Biofuels worldwide focus on biohydrogen, bioethanol, biodiesel, and biogas. The genetic modification of cyanobacteria in biofuel production is easier and more well-developed than other eukaryotic algae (55). Table 2 contains a list of the production of biofuels in genetically engineered cyanobacteria. Figure 2 shows the structure of some secondary metabolites mentioned in Table 2.

Based on the comparison of the fatty acid and biodiesel qualities of cyanobacterial strains in various mediums two strains, *Synechocystis* sp. CACIAM05 and *Microcystis aeruginosa* CACIAM03 showed better production of fatty acids and biodiesel quality in BG-11 medium (66). *Synechocystis* sp. and *Limnothrix* sp. had higher biodiesel quality metrics at low light intensity and NaNO3 concentration (67). A thermostable lipase gene from *Fervidobacterium nodosum* Rt17-B1 was introduced into *Synechocystis* sp. PCC 6803 genome via double-crossing over, causing cytoplasmic membrane hydrolysis and FFA release (68). *Synechococcus elongatus* PCC 7942 produced more fatty acids by overexpressing the era gene, a GTP-binding protein involved in fatty acid and hydrocarbon metabolism (69). The lipid profile of cyanobacterial strains *Cyanobium* sp., *Limnothrix* sp., and *Nostoc* sp. and fatty acid content showed that *Limnothrix* sp. was better for biodiesel generation (70).

Bio-oil is considered to be a very promising biofuel and can be used as a fuel for heat, power, or as feedstock in the chemical industry (71). Pyrolysis temperature, particle size, and nitrogen flow rate affected bio-oil production from blue-green algal blooms (BGAB). BGAB bio-oil had a high heating value of 31.9 MJ kg−1 and an O/C molar ratio of 0.16 at optimum circumstances, with a maximum oil production of 54.97% (wt%) at a final pyrolysis temperature of 500°C, particle size below

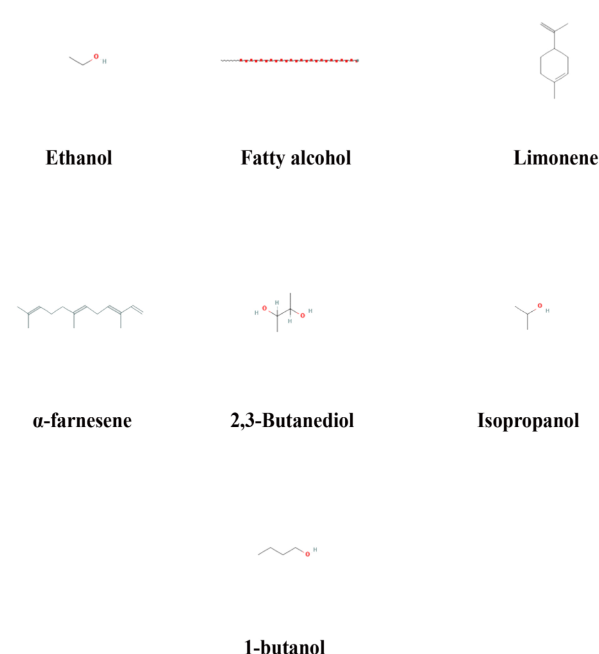

Figure 2. Structure of various biofuels synthesized by genetically engineered cyanobacteria

Table 2. Various biofuels synthesized by genetically engineered cyanobacteria

Sl No	Donor bacteria/other source	Gene of interest	Recipient bacteria in which the gene of interest is genetically engineered	Quantity of the product synthesized	Result
1	*Escherichia coli*	*fbaA, glpX, tktA, fbp, pdc, adh*	*Synechocystis* PCC 6803	1.2 g/l, corresponding to 28 mM ethanol	Over-expression of two selected CBB enzymes (*FBA + TK*, *FBP*/SBPase + *FBA*, or *FBP*/ SBPase + *TK*) enhances ethanol formation (56)
2	*Acinetobacter baylyi*	*ActesA*	*Synechocystis* sp. PCC 6803	Extracellular fatty acids produced up to 171.9 ± 13.22 mg/l	mAcT strain (engineered strain with *AcTesA* on its membrane) secreted 60% of total fatty acids was monounsaturated (C18:1) which is the preferable biodiesel component (57)
3	*Marinobacter aquaeolei* VT8	*Ptrc, far, plsX*	*Synechocystis sp*. PCC 6803	Fatty alcohol production up to 10.3 mg/g (dry cell weight)	CRISPRi allowed repression of *slr1510* increased octadecanol productivity threefold over the base strain and gave the highest production (58)
4	*Pseudomonas mendocina*	*Ole, Und, fap*	*Synechocystis sp*. PCC 6803	4 to 77 mg/g cell dry weight of fatty alkene	Replacement of CAR and ADO with *Pseudomonas mendocina UndB* resulted in the high-yield conversion of thioesterase-liberated FFAs into corresponding alkenes (59)
5	*Pueraria montana*	*cpcB, IspS*	*Synechocystis sp*. PCC 6803	28.9 ± 1.06 μg/l/h of isoprene accumulation	The direct fusion of the *IspS* to the *cpcB* gene (*cpcb***IspS*) substantially enhanced (275 -fold) the concentration of the *IspS* protein in the cells (60)
6	*Mentha spicata*	*lims, SomB, CrtE, dxs, idi*	*Synechococcus elongatus* UTEX 2973	Engineered strain produced 16.4 mg/l of limonene at a rate of 8.2 mg/l/day	Limonene titer in the *dxs* and *idi* coexpression strains increased significantly to 13 mg L-1 under 0.01 mM IPTG conditions (61)
7	*Anabaena sp*. PCC 7129	*ASF*	*Synechococcus elongatus* PCC 7942	Production of α-farnesene (4.6 ± 0.4 mg/l in 7 days) from CO2	α-farnesene production rates increased during the growth period to 480.3 μg/L/OD730/d 195 from days 3-5 and to 625.2 μg/L/OD730/d from days 5-7 (62)
8	*Clostridium beijerinckii*	*als, aldc adh, luc, lacZ*	*Synechococcus elongatus* PCC 7942	2,3-Butanediol production of titer 496 ± 42 mg/L	Optimum construction (B-*alsS*, *B-alsD*, *D-adh*) produced 496 mg-1 after 72 h, achieving a 180% increase over the non-optimized strain (63)
9	*Synechocystis sp*. PCC 6803	*aceEF, lpd, pdh*	*Synechococcus elongatus PCC 7942*	Production titer of isopropanol up to 1.9 mM, 114.2 mg/l	Enhanced flux to acetyl-CoA improved photosynthetic acetate/isopropanol production (64)
10	*Escherichia coli*	*pha, Fad, nphT7*	*Synechocystis* sp. PCC 6803	The cumulative 1-butanol titer of 4.8 g/l with a maximal rate of 302 mg l/day	Optimizing the 5′-regions of expression units for tuning transcription and translation, rewiring the carbon flux and photosynthetic central carbon metabolism to enhance the precursor supply, and performing process development for 1-butanol production (65)

0.25mm, and nitrogen flow rate of 100 mL min−1 (72). Cyanobacterial carbohydrates can be utilized to produce bioethanol or biohydrogen production, and lipids are considered for bio-oil production (73). One of many different kinds of biofuels, which also include solid, liquid, and gaseous fuels derived from biomass, is biogas. A biofuel is any combustible fuel made from recent (non-fossil) living matter (biomass), such as ethanol made from plant products, biodiesel made from plant or animal oils, or biogas made from biomass (74). Biogas production reached a maximum value of 0.4 m3 biogas/kgCODi in *Arthrospira platensis* (75). *A. platensis* NIOF17/003 entered the late exponential phase on the eighth day of development, with a dry weight (DW) of 0.845 g L^{-1} (76).

CRISPR technology and metabolic engineering in cyanobacteria

CRISPR is used for regulating metabolic production *via* editing genes in the way of substitutions, point mutations, and creating gene knockouts and knock-ins. CRISPR/Cas improves the effectiveness of genome editing by generating double-stranded cleavage of the genome, which in turn stimulates homologous recombination through a DNA repair mechanism called homology-directed repair. Genome editing effectiveness is increased

by using CRISPR/Cas to cause double-stranded cleavage of the genome, which stimulates homologous recombination in a DNA repair procedure known as homology-directed repair. The CRISPR/Cas9 technology was used to demonstrate this increase in effectiveness for the first time in cyanobacteria (77). Recently, both CRISPR-dCas9 and -dCas12a systems have demonstrated successful applications in several model cyanobacteria, such as *Synechocystis* 6803 (58), *Synechococcus* 7942 (30) and *Anabaena* 7120 (78).

In these applications, CRISPRi was successfully employed for the dynamic up/down-regulation of the target genes in various synthetic pathways for improved productivity of biofuels (e.g., fatty acids and fatty alcohols) and other important metabolites (e.g., amino acids, succinate, lactate, and pyruvate) (79). There are other applied advantages of using CRISPR/Cas9 for genome editing in cyanobacteria other than in genome editing efficiency. One is shorter homology arms of 400bp up to 700bp is enough for effective homologous recombination. This leads to opening up the possibility of integrating genes within small genomic target sites, thereby avoiding the risk of unwanted recombination events that can significantly alter host behavior and phenotype. Another advantage is reducing the template plasmid amount for homologous recombination (80). Due to the apparent toxicity of Cas9 nuclease, CRISPR technology does have certain disadvantages. With the use of CRISPR technology and the revolutionary RNA-directed dsDNA nuclease, Cpf1, which is harmless to cyanobacteria, a very effective and accurate tool for producing many marker-less alterations in cyanobacteria was created to overcome this impediment. Because it only requires a 42 nt RNA component significantly less expensive to produce than the >100 nt gRNA needed by cas9 systems, the cpf1 system is regarded as being more economically sound in synthetic biology. Because the cpf1 genome editing tool lacks markers, it may modify complicated genomes in ways that were previously impractical. The next stage in enabling the development of cyanobacteria as extremely promising bio-factories will undoubtedly be scaling up the quantity and effectiveness of alterations to compete with those of *E. coli* and *S. cerevisiae* (81).

Conclusions

By fine-tuning cyanobacterial metabolism, recent advances in metabolic engineering and synthetic biology using sophisticated and cutting-edge tools have demonstrated noteworthy progress in making cyanobacteria a promising photosynthetic platform for the production of biofuels and many commodity chemicals. Although there have been multiple successful proof-of-concept studies, there is presently only a small amount of work being done to scale up this technology. Since titer, productivity, and stability are the characteristics that can only be attained by cyanobacterial strains that have undergone genetic engineering, these qualities are crucial for commercial realization. In this case, the theoretical production rates are to be experimentally determined. A considerable portion of the fixed carbon must be directed or redirected toward the desired products, which will need several coordinated research initiatives. The technology for harvesting the end products must be devised in addition to upgrading the infrastructure of open ponds or low-cost bioreactors. Despite several obstacles to the economic viability of cyanobacterial systems, the unique viewpoint of these photosynthetic microorganisms is piquing the interest of continuous metabolic engineers as a green and sustainable production system.

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Authors' contributions

PP and LKE carried out conceptualization and original draft preparation; NSP supervised and edited the final draft. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

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