



**International Journal of Biology, Pharmacy
and Allied Sciences (IJBPAS)**

'A Bridge Between Laboratory and Reader'

www.ijbpas.com

HARNESSING PHOTOSYNTHETIC EFFICIENCY OF *SYNECHOCOCCUS ELONGATUS* PCC 7942 FOR SUSTAINABLE BIOFUEL PRODUCTION: RECENT ADVANCES AND CHALLENGES

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Received 16th April 2026; Revised 19th May 2026; Accepted 1st Aug, 2026; Available online 1st Nov. 2026

<https://doi.org/10.31032/IJBPAS/2026/15.11.10617>

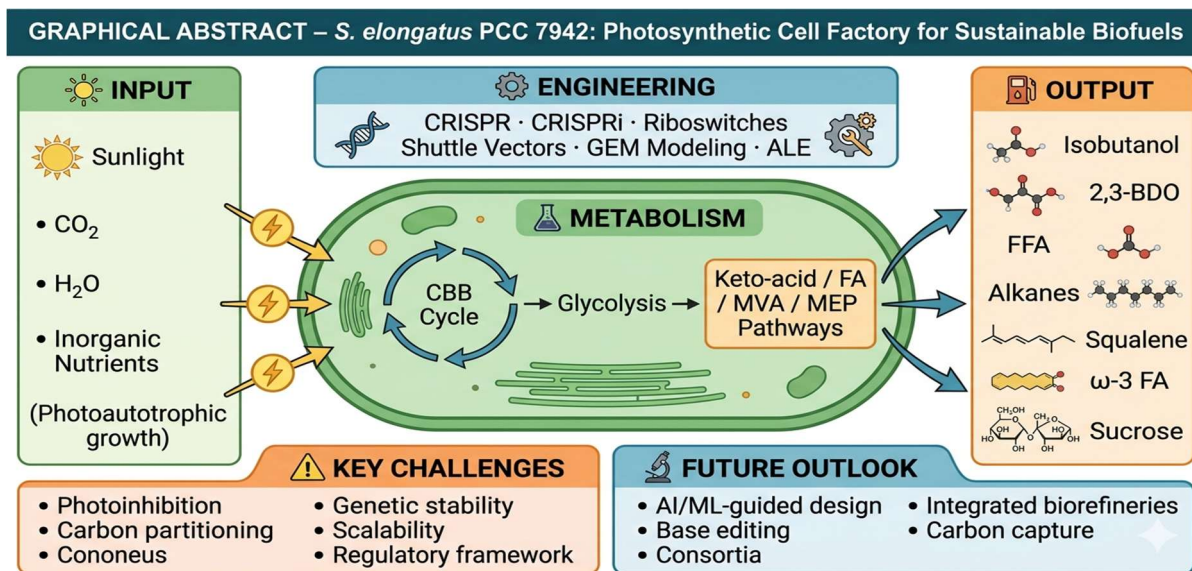
ABSTRACT

The accelerating global energy crisis, combined with an urgent need to curtail greenhouse gas emissions, has positioned photosynthetic microorganisms as compelling platforms for next-generation biofuel production. Among these, *Synechococcus elongatus* PCC 7942 - a well-characterized, genetically tractable freshwater cyanobacterium - stands out as one of the most intensively studied model systems for harnessing solar energy and atmospheric CO₂ to synthesize a diverse portfolio of biofuel molecules. This review provides a comprehensive and critical synthesis of recent advances (2015-2026) in the engineering of *S. elongatus* PCC 7942, covering its photosynthetic machinery, metabolic flux optimization, synthetic biology toolbox, and photobioreactor systems. We compare key cyanobacterial chassis organisms and systematically catalogue biofuel molecules produced in engineered strains, from short-chain alcohols and fatty acids to alkanes and terpenoids. We also examine the persistent bottlenecks - photoinhibition, carbon partitioning, genetic stability, and technoeconomic scalability - that currently limit industrial deployment. The review concludes by proposing an integrated research agenda combining adaptive laboratory evolution,

genome-scale metabolic modeling, and advanced photobioreactor designs to bridge the gap between laboratory demonstration and commercially viable photosynthetic biofuel production.

Keywords: *Synechococcus elongatus* PCC 7942; cyanobacteria; biofuel; photosynthesis; metabolic engineering; CRISPR; synthetic biology; carbon fixation

GRAPHICAL ABSTRACT



Graphical Abstract on Central Overview: Engineering *Synechococcus elongatus* PCC 7942 as a Photosynthetic Biofuel Cell Factory

1. INTRODUCTION

Energy security along with climate change is two of the most important issues facing humanity in the 21st century. This is evidenced by the recent surging levels of carbon dioxide (CO₂) in our atmosphere (above 420 parts per million) as measured globally. Despite the continued burning of fossil fuels around the globe, which are responsible for more than 75% of all greenhouse gas emissions worldwide [1], countries around the world are still failing at meeting their international commitments to reduce emissions under the Paris Agreement. Traditional biofuels (corn, sugar cane, palm

oil) are commercially viable on large scales, yet continue to raise legitimate issues regarding food vs. fuel competition, land use changes, and water consumption [2]. Photosynthetic microbes occupy a unique position among biomass feedstocks, as they do not require any arable land and can grow rapidly through the use of sunlight as well as inorganic nutrients. These microorganisms have also been engineered to convert atmospheric CO₂ directly into fuel molecules using metabolic pathways [3].

Cyanobacteria, known as the oldest oxygen-producing organisms capable of using

sunlight for energy on the planet Earth, could have a theoretical ability to achieve photo efficiencies of 10%, compared to the 3-4% limits of most of our land-based crops, with such advantages being the consequence of their prokaryotic genetic configurations that possess high capabilities for performing synthetic biological methods and developing and using capitalized tools for genetical manipulation of synthetic biological structures [4]. Natural species' compatibility with synthetic biology methods has allowed scientists to develop a great variety of molecular instruments to genetically modify them at the genomic level using site-directed genome editing technologies or semi-directed methods using a wide spectrum of organism as fall-back options [5].

With nearly 2,000 cyanobacteria species known to date [6], *S. elongatus* PCC 7942 has become the favorite research organism for biofuels for a combination of the following significant factors: (1) sequencing of its genome has been fully completed (2.7 million nucleotides), (2) fast growth (average generation time of 6-8 hours under ideal conditions), and (3) an extensive accumulate amount of genetic information to better understand its biology or aid with genetic modifications for biofuel applications. *S. elongatus* PCC 7942 exhibits unique features

that make it an ideal candidate for biofuel research and production: (1) high rates of CO₂ fixation through the Calvin-Benson-Bassham (CBB) cycle using the key enzyme RuBisCO (2) higher rates of photosynthesis through a photosynthetic electron transport system coupling both Photosystems I and II (3) low-cost energy reserves as glycogen which can provide addition sources for the biosynthesis or other desired precursors of biofuels, and (4) cell walls in *S. elongatus* PCC 7942 that permit the efficient secretion of the biofuels produced; thus making the cyanobacterium an affordable and sustainable feedstock for producing biofuels directly from the sun through the use of photosynthesis [7]. In 2009, because of the significant achievement by Atsumi *et al.* in which *S. elongatus* PCC 7942 was successfully modified to produce isobutanol directly from CO₂, it became apparent that the cyanobacterium has true potential as a photosynthetic cell factory for biofuels [8].

Significant advancements have occurred over the past decade, including the introduction of genome editing technologies based on CRISPR/Cas9 and their application to create genome-scale metabolic models (GEM) in many microorganisms; through novel strains generated by Adaptive Laboratory Evolution (ALE) strategies and the development of

lignocellulosic co-substrates for the production of biofuels, these technologies have driven product titers, strain robustness, and process scalability to unprecedented levels [9–11]. As this has also led to a better understanding of the physiological limits or constraints of these cells, such as photoinhibition of photosynthesis, metabolic burden, redox imbalance, and product toxicity, it will facilitate the rational design of improved engineering approaches [12].

This paper is organized into the following sections: Section 2 describes the photosynthetic machinery of *S. elongatus* PCC 7942 and potential approaches for improving its performance. Section 3 discusses the various metabolic engineering methods used to produce particular biofuels. Section 4 reviews the tools of synthetic biology that are currently accessible for this organism. Section 5 provides detailed descriptions and examples of photobioreactor designs and their limitations. Section 6 compares *S. elongatus* PCC 7942 with other cyanobacteria. Section 7 discusses the continued obstacles to successful use of cyanobacterial photosynthetic pathways for biofuel generation, while Section 8 provides a vision for future research in this area. To support understanding of the content, three tables summarizing the information provided

and a graphical abstract have been included throughout the paper.

2. PHOTOSYNTHETIC MACHINERY AND CARBON FIXATION IN *S. ELONGATUS* PCC 7942

2.1 LIGHT HARVESTING AND ELECTRON TRANSPORT

The photosynthesis process of *S. elongatus* PCC 7942 occurs within thylakoid membranes using two main photo-reaction centers - Photosystem II (PSII) and Photosystem I (PSI) - connected via the cytochrome b6f complex and the plastocyanin/ferredoxin carriers [13]. Light energy is captured by phycobilisome antenna complex (phycocyanin, allophycocyanin, phycoerythrin) then directed to PSII for oxidation of water, providing Reducing Equivalents (NADPH) and ATP used to produce the CBB cycle [14]. Of note, the phycobilisome is the primary light-harvesting antenna in this species and is composed of a large amount of total protein, contributing significantly to the overall photosynthesis process as its dynamics under nutrient/light stress is crucial to determining total rates of photosynthetic flux [15]. If exposed to high light intensities typical for outdoor and dense photobioreactor cultures, photoinhibition can be of great concern - excess photons cannot be accommodated by the electron transport chain

and create Reactive Oxygen Species (ROS), damage the PSII D1 protein leading to reduced carbon fixation rates [16]. The species achieves photoprotection through Non-Photochemical Quenching (NPQ) involving the Orange Carotenoid Protein (OCP) that dissipates excessive energy [17]. Key differences comprise elevated expression of PSI, cytochrome b6f, and plastocyanin, resulting in a higher electron flux during the thylakoid membrane and a correspondingly enhanced ability for NADPH and ATP generation [18].

2.2 CARBON FIXATION VIA THE CBB CYCLE

The CBB cycle is responsible for the carbon fixation process in *S. elongatus* PCC 7942 exclusively under phototrophic conditions. A major limitation of this cycle is RuBisCO's (Form IB) rate-limiting plant expression (NA), low rates of catalysis ($\sim$ three s^{-1}), and RuBisCO's (form IB) and the competing metabolism of CO₂ (RuBisCo): O₂ through the conducting reaction [19]. The has studied the overproductive along the directed routes of the two enzymes in the glgA, glgB and glgC RBS involved in the biosynthesis of glycogen (younger? and products, e.g., 2,3-butanediol), with promising but still moderate gains relative to the carbon reduction in glucose and sucrose from that site

[20]. The most significant interior carbon storage polymer is glycogen, and it is a major junction of the carbon pathway and the biomass / biofuels objective (e.g., carbon diverted away from biofuels). Both the synthesis and glycogen synthesis are genetic disruptions at one of the three glycogenic enzyme locations in the glgABC (sucrose synthesis and storage): carbon flux towards those projects in the glgABC (e.g., sucrose synthesis or 2,3-butanediol) is occurring, but at that cost, they slow growth and reduce stress tolerance [21]. The use of ribosome switches (via signalling with riboswitches such as theophylline responsive regulatory elements) in this context leads to more elegant flow of carbon processing than the traditional approach of regulated biosynthetic growth rates, which eventually leads to no growth-related penalties [22].

3. METABOLIC ENGINEERING FOR BIOFUEL PRODUCTION

3.1 SHORT-CHAIN ALCOHOLS: ISOBUTANOL AND 2, 3-BUTANEDIOL

The engineering of *S. elongatus* PCC 7942 for producing short-chain alcohols was by far the most researched example of cyanobacterial synthetic biology. One example is the production of isobutanol (a four-carbon, high-energy-density alcohol that has lower vapor pressure than ethanol) by

using the keto-acid pathway and adding five heterologous genes to the wild-type strain: *alsS* (the acetolactate synthase gene from *B. subtilis*); *ilvC* and *ilvD* (the ketol-acid reductoisomerase and dihydroxy-acid dehydratase genes, respectively); *kivd* (the alpha-ketoisovalerate decarboxylase from *L. lactis*); and *yqhD* (the alcohol dehydrogenase from *E. coli*). Refinements to improve the cofactor balance (NADPH/NADH ratios), reduce isobutanol toxicity, and increase the carbon flux through the pathway have occurred subsequently [23].

Another high-value platform chemical that can be further processed into useful compounds like methyl ethyl ketone (a gasoline additive) and 1,3-butadiene (a feedstock for polymers) is 2,3-butanediol (2,3-BDO). Research conducted by Oliver *et al.* demonstrated 2,3-BDO production in *S. elongatus* PCC 7942 by expressing *alsS*, *budA* (the acetolactate decarboxylase gene), and *bdh* (the secondary alcohol dehydrogenase gene). They obtained titers of 2.38 g/L, one of the highest titers reported for any cyanobacterium at that time [24]. More recently, expressing heterologous glucose and xylose transporters in photomixotrophic conditions using lignocellulosic hydrolysates as co-substrates has further increased production rates of 2,3-BDO and demonstrated

the potential to couple cyanobacterial fixation of CO₂ with waste [25].

3.2 FATTY ACIDS AND LIPID-DERIVED BIOFUELS

Biodiesel (as fatty acid methyl esters, FAMES) and jet fuel (as fatty alcohols and alkanes) are important uses for fatty acids, the major example of this is triacylglycerols as a lipid storage form. Fatty acids are mainly produced by cyanobacteria as membrane glycerolipids and are not found in eukaryotic microalgae. The engineering techniques for directing the fatty acid pathway towards the outside include: (i) heterologous expression or overexpression of thioesterases (*tesA* from *E. coli*, plant acyl-ACP thioesterases) to release fatty acids from the acyl carrier protein; (ii) knockout of fatty acid activation genes (*aas* encoding acyl-ACP synthetase) to prevent reconstitution of released fatty acids into membrane lipids; (iii) overexpression of fatty acid biosynthetic genes (*fabH*, *fabF*) to increase the overall flux in the pathway [26]. The updated GEM analysis data showed that the second-most critical class of reactions, besides fatty acid biosynthesis, that needed improvement to enhance ALA yield were the photosynthetic electron transport chain (very interestingly demonstrating that photosynthesis and lipid-anabolism are tightly coupled) and that *S. elongatus* PCC 7942 would be an

attractive platform for the production of nutraceutical omega-3 fatty acids - as an exciting biofuel, and a high-value bioactive compound; [27-28].

3.3 TERPENOIDS AND ALKANES

Terpenoids are a major category of natural products, with some members of this wide-ranging group including isoprene, farnesane, limonene, and squalene having potential fuel or fuel additive applications. The biosynthetic pathway that produces terpenoids in cyanobacteria is called the methylerythritol phosphate (MEP) pathway, which produces the common building blocks of terpenoids isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) [29]. In 2008, Choi *et al.* produced squalene by engineering *S. elongatus* PCC 7942 through the overexpression of a heterologous squalene synthase. This allowed for additional improvements to be made through both metabolic engineering and photobioreactor

scale-up; ultimately producing approximately 11.9 mg/L and demonstrating that terpenoid production in this microorganism is achievable, but still well below commercial standards [30]. Cyanobacterial alkane biosynthesis occurs through a two-enzyme pathway consisting of an acyl-ACP reductase (AAR) enzyme that converts fatty acyl-ACP into fatty aldehydes followed by the decarbonylation of those fatty aldehydes into alkanes through an aldehyde deformylating oxygenase (ADO) enzyme [31]. *S. elongatus* PCC 7942 naturally produces small quantities of pentadecane and heptadecane; however, engineering efforts to increase the titers of these minor products have utilized increased overexpression of both AAR and ADO, as well as increased substrate flux through the fatty acid biosynthetic pathway, and have been successful in producing approximately 26 mg/L of both pentadecane and heptadecane [32].

Table 1: Biofuel molecules and value-added chemicals produced by engineered *Synechococcus elongatus* PCC 7942

BIOFUEL / PRODUCT	METABOLIC PATHWAY	KEY GENES / ENZYMES	TITER / YIELD	PRODUCTION CONDITIONS
Isobutanol	Keto-acid pathway	alsS, ilvC, ilvD, kivd, yqhD	450 mg/L (6 days)	CO ₂ , light, 30°C
2,3-Butanediol (2,3-BDO)	Pyruvate → acetolactate pathway	alsS, budA, bdh	2.38 g/L	Photomixotrophic, 30°C
Sucrose	Calvin cycle → sucrose phosphate synthase	sps, spp, cscB	2.1 g/L/day	Phototrophic, salt-induced
Free Fatty Acids (FFA)	Fatty acid biosynthesis	tesA, fabH overexpression; ΔAAT	~197 mg/L	Thioesterase overexpression
Ethylene	Ethylene-forming enzyme (EFE)	efe from <i>Pseudomonas syringae</i>	~171 μL/L/h	CO ₂ , continuous light
Alkanes/Alkenes	Acyl-ACP reductase + ADO	aar, ado	~26 mg/L	Phototrophic, WT background
Squalene	MVA / MEP isoprenoid pathway	SQS (squalene synthase) overexpression	~11.9 mg/L	Engineered terpenoid flux
ω-3 Fatty Acids (ALA)	Fatty acid desaturation pathway	desA, desB, fabF overexpression	~23% of total lipids	Genome-scale model guided

4. SYNTHETIC BIOLOGY TOOLBOX FOR *S. ELONGATUS* PCC 7942

4.1. TRANSFORMATION AND CHROMOSOMAL INTEGRATION

S. elongatus PCC 7942 is able to take up DNA naturally, which means that it is convenient to introduce foreign DNA into the organism through homologous recombination at chromosomal integration loci (NS1, NS2, NS3) (Commonly called "neutral sites" that allow for the insertion of transgenes without disrupting essential genes) [33]. The parameter of completeness of segregation (i.e., the removal of all wild-type copies of the target locus from the polyploid genomic DNA of cyanobacteria) is essential, and the application of antibiotic selection over several passages is needed to ensure fully segregated mutants are obtained [34]. Almeida found that the use of linear DNA fragments and the presence of a DNase inhibitor improved transformation efficiency, thereby producing a substantial increase in integration frequency with a minor change to procedure [35]. Both pANS and the RSF1010-derived shuttle vectors provide higher copy number and faster initiation of transgene expression than chromosomal integration; however, the shuttle vectors provide less segregation stability than do the chromosomal integrants due to the lack of continued antibiotic

selection. Chen *et al.* developed and characterized a series of self-replicating pANS-based shuttle vectors that provide stable moderate copy expression platforms suitable for the production of heterologous enzymes that are metabolically costly to synthesize at [36].

4.2 CRISPR-BASED GENOME EDITING AND REGULATION

Research on the use of CRISPR-Cas9 methods for genome editing in cyanobacteria is improving both how quickly genome edits can be achieved and how accurately those edits are made. The researchers Ungerer and Pakrasi have developed a new tool to deliver CRISPR in a way that is able to perform multiple gene disruptions in one experiment and to do so in many different species of cyanobacteria, including *Synechococcus elongatus* PCC7942 [37]. They were able to successfully demonstrate the use of another CRISPR tool, Cpf1 (Cas12a), to deliver CRISPR in different types of cyanobacteria accomplishing multiplexed disruption in a single transformation [37]. When CRISPR is combined with the use of CRISPR interference (CRISPRi), a method where dCas9 or dCas12a are fused to transcriptional repressors, researchers can transiently or reversibly diminish the expression of target genes without making permanent mutations.

This aspect is very important when scientists use this technology to study essential genes and to conditionally modulate metabolic pathways by the repression of essential metabolic genes [38]. A significant accomplishment in CRISPRi is the generation of a genome-wide CRISPRi library (21,705 unique guide RNAs) derived from from *Synechocystis* sp. PCC6803 (hereafter referenced as PCC6803), which targets all annotated genes (21,705 guide RNAs represented 21,705 genes) with five or more unique targeting sites. The base for generating the CRISPRi library was developed in PCC6803; however, they are directly translatable to PCC7942, because of the genomic relationship of these two cyanobacterial strains. These and other gene libraries will allow unbiased, pooled fitness screening across a variety of perturbations. These changes will also allow scientists to rapidly identify genes that negatively affect beneficial traits (i.e., biofuel production or

stress tolerance) through the use of these libraries and resultant CRISPR tools [39].

4.3 INDUCIBLE PROMOTERS AND POST-TRANSCRIPTIONAL REGULATION

To separate biomass creation from product creation requires accuracy of transgenic expression with precision timing. High levels of expression of the necessary biosynthetic enzymes during the growth increase burdens placed upon metabolism and, therefore, decrease cell yield [40]. The P_{trc} (IPTG-regulated) promoter system is commonly utilized for strong, chemically induced transgene expression in *S. elongatus* PCC 7942. The P_{pcpB} promoter (phycocyanin operon) provides phototropic-coupled expression based upon solar energy and will therefore correlate product production timewise with photosuccessful precursors. Theophylline is a low-cost, non-toxic alternative to IPTG-induced processes for use as an inducer in the economic processes and acceptable by regulators [41].

Table 2: Synthetic biology tools and strategies developed for *Synechococcus elongatus* PCC 7942

Tool / Strategy	Type	Application in <i>S. elongatus</i> PCC 7942	Advantages / Limitations
Natural Transformation	Genetic transformation	Chromosomal integration via double homologous recombination	Simple, stable integration; low efficiency with large fragments
RSF1010 / pANS Shuttle Vectors	Plasmid-based expression	Episomal gene expression; replicating vectors for metabolic pathway introduction	High copy number; mild segregational instability
CRISPR-Cas9 / Cpf1	Genome editing	Precise gene knockout; multiplex editing; targeted insertions	Versatile and efficient; requires PAM site; toxicity at high expression

CRISPRi (dCas9 / dCas12a)	Gene repression	Downregulation of competing pathways (e.g., glycogen synthesis)	No permanent mutation; reversible; potential polar effects
Riboswitches (theophylline)	Post-transcriptional regulation	Flexible, inducible control of glycogen metabolism and carbon flux	Low-cost inducer; dose-responsive; requires optimization
Inducible Promoters (Ptrc, PcpCB)	Transcriptional control	IPTG-inducible or light-driven heterologous gene expression	Strong expression; metabolic burden possible at high induction
Adaptive Laboratory Evolution (ALE)	Strain improvement	Tolerance to high light, temperature, and biofuel products	Generates robust strains; time-consuming; unpredictable mutations
Genome-Scale Metabolic Modeling (GEM)	Computational / Systems biology	Flux balance analysis for predicting optimal gene targets and yield	Powerful for in silico strain design; requires experimental validation

5. PHOTOBIOREACTOR SYSTEMS AND SCALE-UP CONSIDERATIONS

Designs for photobioreactors (PBRs) that optimize volumetric productivity and minimize both capital and operational costs are required for the commercial-scale conversion of laboratory-scale biofuel production in the cyanobacterium *S. elongatus* PCC 7942 into viable production processes. There are three major configurations of PBRs that have been evaluated for use with cyanobacteria: (i) open raceway ponds; (ii) flat-panel PBRs; and (iii) tubular PBRs [42]. These three different types of PBRs each have unique trade-offs with respect to light utilization efficiency, CO₂ supply, temperature control, contamination risk, and scalability [43]. Open raceway ponds are the most economical per volume; however, open raceway ponds have poor light penetration in dense cultures, significant evaporation of water, and they are also at risk of being

contaminated by competitive microorganisms; these concerns are amplified in the case of genetically modified organisms (GMOs), which in many jurisdictions are subject to containment regulations [44]. Flat-panel PBRs provide better distribution of light and mixing resulting in higher volumetric productivity but cost significantly more to construct and operate at larger scales [43]. Closed tubular systems have the highest environmental control and allow for the operation of outdoor facilities with the addition of CO₂ from industrial flue gases to the production process; this configuration has been explored by Algenol during the company's commercial scale ethanol production test program [45]. Comparing the above to the equivalent scenario using solar photovoltaic (PV) systems to power direct air capture (DAC) using relatively large amounts of CO₂ from DAC facilities to supply biomass-based carbon removal via cultivation

of cyanobacteria would appear competitive for the removal of CO₂ in a biomass based manner. However, for biofuel production specifically, economic feasibility will require significantly larger product titers and extraction efficiencies than have been previously demonstrated for specifically biofuel production analytics [46]. Such integrated bioprocess designs are now increasingly incorporated into pilot-scale demonstrations, marking a maturation of the field beyond pure strain engineering [47].

6. COMPARATIVE ANALYSIS OF CYANOBACTERIAL CHASSIS ORGANISMS

While *S. elongatus* PCC 7942 is arguably the best-characterized cyanobacterial model for biofuel research, it is important to situate it within the broader landscape of photosynthetic cell factories. **Table 3** provides a systematic comparison of the key cyanobacterial chassis organisms, highlighting their relative strengths and limitations for biofuel applications.

Table 3: Comparative overview of key cyanobacterial chassis organisms for biofuel production

Strain	Doubling Time (h)	Photosynthetic Efficiency (%)	Key Biofuel Products	Genetic Toolbox
<i>S. elongatus</i> PCC 7942	~6-8	~5-8	Isobutanol, 2,3-BDO, FFA, ethylene, sucrose	Well-established; CRISPR, RSF1010 vectors
<i>Synechocystis</i> sp. PCC 6803	~12-16	~4-6	Ethanol, fatty acids, isoprene, hydrogen	Well-established; CRISPRi, inducible promoters
<i>S. elongatus</i> UTEX 2973	~2.3-2.7	~8-12	Sucrose, 2,3-BDO, biomass	Developing; high sequence homology with PCC 7942
<i>Synechococcus</i> sp. PCC 7002	~4-6	~5-9	2-methyl-1-butanol, isobutanol, alkanes	Moderate; salinity tolerance
<i>S. elongatus</i> PCC 11801	~3.5	~7-10	Butanol, squalene, pigments	Emerging; stress tolerant
<i>Anabaena/Nostoc</i> sp.	~12-24	~3-5	Hydrogen, bioethanol, PHAs	Limited; nitrogen-fixing capable

Historically, *Synechocystis* sp. PCC 6803 was the first cyanobacteria ever used for a comprehensive genome size analysis and metabolic engineering and has the most complete genome-wide functional classification [48]. However, this organism has a doubling time of approximately 12-16 hours and does not have sufficient transformation efficiency compared to *S. elongatus* PCC 7942, making it difficult for

many researchers to conduct multiple rounds of iterative genetic engineering [49]. Many researchers now use both PCC 7942 and PCC 6803 together - generating constructs in PCC 7942 and then transferring them to UTEX 2973 for improved productivity. *Synechococcus* PCC 7002 is a euryhaline strain that naturally tolerates high salinity and elevated temperatures and therefore is well-suited for outdoor cultivation in coastal areas

and using seawater-grown media [50]. In addition, metabolic engineering of this strain for 2-methyl-1-butanol, isobutanol, and isoprenoid production have led to success in producing competitive titers, while incorporating an NADH-dependent nitrate assimilation pathway is especially promising at rebalancing the intracellular redox environmental state for increased ethanol production [51].

7. PERSISTENT CHALLENGES

7.1 PHOTO INHIBITION AND LIGHT MANAGEMENT

S. elongatus PCC 7942 has recently seen a great deal of success using adaptive laboratory evolution as an approach to obtain high-light resistant strains. Sun *et al.* found hypermutants with deletions in DNA repair genes (*mutS*, *recA*) that produced adaptive mutants containing significant mutations in the upstream non-coding region of *aroK* (shikimate kinase) following repeated subculturing at 45°C and 2,500 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$. The adaptive mutants developed mechanisms to confer thermotolerance and high-light resistance, likely as a result of increased synthesis of antioxidants and photoprotective metabolites [52]. Furthermore, the optimal growth conditions for these evolved strains are substantially less hospitable than those of the parent strain,

thereby increasing the potential for outdoor cultivation with higher productivity.

7.2 CARBON PARTITIONING AND METABOLIC BURDEN

The generations of biofuel molecules with high biosynthetic pathways will, in theory, compete with cellular functions, such as energy storage, biomass generation, and glycogen storage as they all rely on the same resources from carbon fixed during photosynthesis. Due to the workload of constructing large heterologous biosynthetic pathways, i.e., multi-protein enzyme complex encoding the biosynthesis of isobutanol and 2,3-BDO, heterologous production imposes a significant metabolic burden on a cell and could lead to reduced growth rates and ultimately lead to diminished biomass production, as well as negatively impacting the cells' ability to carry out photosynthesis due to secondary effects on NADPH/ATP balance [5]. Two-stage cultivation systems, whereby biomass is produced in the first stage and the desired product in a separate stage using, for example, chemically-inducer-controlled promoters or light-shifted systems, provide some relief to this conflict [40]. Genome-scale modelling (GEM) provide invaluable tools to predict which gene deletions or over-expressions will lead to the greatest biofuel production without adversely

impacting the growth of the organism. The GEM for *S. elongatus* PCC 7942 developed by Santos-Merino identified an objective function using flux scanning based on induced production of ALA (aminolevulinic acid) where increased rate of photo-electron transport would be one of the most advantageous pathways to manipulate, which was subsequently verified in a laboratory experiment [27]. The predictive accuracy of the models will only increase as more transcriptome, proteome and fluxome data are included [22].

7.3 GENETIC STABILITY AND CONTAINMENT

Long-term sustainable production of biofuels from *S. elongatus* PCC 7942 is significantly impacted by genetic instability. Pathways for production which are not naturally present in the organism introduce a metabolic burden to the cell, therefore creating strong selection pressure on the cell to acquire mutations that prevent expression of the transgene. This phenomenon is often termed "evolutionary escapees" [53]. The issue is especially pronounced in continuous culture systems for prolonged periods of time; as there may be only a few of these mutants, but once an escapee acquires a sufficient growth advantage over the producing strain, growth of the producing strain will be

negatively impacted. Some strategies that may help overcome this issue are: placing the essential biosynthetic pathway genes under control of the same promoter as the biosynthetic production pathway (this would create a coupling of growth and production), using multiple neutral site integrations to more evenly distribute the production load, and periodically re-isolating and re-transforming production clones [34]. Current regulatory jurisdictions pertaining to the outdoor cultivation of genetically modified cyanobacteria in open systems are hotly debated. As a result, scientists worldwide are actively developing containment strategies such as synthetic auxotrophies (i.e., dependence on unnatural amino acids) and kill switches that only trigger in the absence of specific inducing agent(s), which will provide fail-safe methods to prevent containment failures or environmental escape of engineered strains [54]. The need for both robustness (long-term genetic stability) and containment (controlled presence in the environment) creates a conflict for system design engineers.

7.4 TECHNO-ECONOMIC AND LIFE CYCLE CONSIDERATIONS

Cyanobacterial biofuel processes are evaluated using technoeconomic analysis (TEA), where the greatest cost contributions

come from cultivation, the supply of CO₂, and the downstream extraction of products [44]. Isobutanol and fatty acid titers currently produced by *S. elongatus* PCC 7942 - while substantial from a scientific perspective - are still multiple orders of magnitude lower than what will be necessary to make cyanobacterial biofuels price-competitive with petroleum-based fuels or even first-generation bioethanol [12]. However, life cycle analysis (LCA) yields a much more favorable result: the use of CO₂ directly (possibly from industrial point sources), sunlight, and non-potable water in the cyanobacterial process results in a significantly lower greenhouse gas footprint when compared to traditional biofuel processes [55].

8. FUTURE DIRECTIONS AND EMERGING OPPORTUNITIES

The future of photosynthetic biofuel research is likely to be shaped by multiple enabling technologies. Advancements in Machine Learning (ML) and Artificial Intelligence (AI) are starting to change our ability to explore the large sequence-structure-function landscapes of enzymes, regulatory elements, and metabolic networks. By applying these techniques to metabolic modelling of *Selenastrum elongatus* PCC 7942 and integrating multiple omics datasets, we expect that the design-build-test-learn

cycle for strain improvement will be significantly shortened [56]. Additionally, the availability of CRISPR-based base editing and prime editing technologies in cyanobacteria may facilitate the introduction of precise single nucleotide changes in the absence of double-strand breaks or the need for exogenous DNA templates, thus allowing for more refined metabolic adjustments than the current methods of genome editing [57]. Thirdly, methods for producing biofuels through co-culturing and synthetic consortia are becoming increasingly popular methods for dividing the metabolic work of producing biofuels. When combining *Selenastrum elongatus* PCC 7942 as a phototrophic producer of sucrose or organic acids with a heterotrophic bacterium or yeast that can convert those substrates into complex biofuels, we may be able to circumvent many of the challenges involved in engineering intricate multi-step pathways into a single photosynthetic host [58]. According to Kratzl *et al.*, the use of a heterotrophic partner can increase the growth rate of cyanobacteria by approximately 80%, as well as enhance their photosynthetic efficiency [59]. While cell-free systems cannot replace whole-cell photosynthesis for sustained, scalable production, they serve as invaluable research tools for decoupled pathway optimization

before reinsertion into the living organism [60].

9. CONCLUSION

The organism *Synechococcus elongatus* PCC 7942 is a well-known model organism in the area of photosynthetic biofuel research. This strain of cyanobacteria is unique in the broad combination of advantageous characteristics such as genetic tractability, well-documented physiology, and a constantly growing synthetic biology toolbox, which have contributed to establishing PCC 7942 as one of the pre-eminent model organisms in this field. Over the last ten years, significant developments and modifications to these characteristics have occurred in the form of increased genetic engineering (CRISPR-based precise genome editing), improved computational techniques for modeling cellular metabolism (genome-scale metabolic modeling), laboratory evolution (adaptive laboratory evolution), and new cultivation methods (photomixotrophic cultivation on lignocellulosic co-substrates). Collectively, these advances in techniques have elevated the levels of achievable titers of biofuels and robustness of strains to levels that would have been deemed impossible just ten years ago. The recent developments in the areas of inducible regulatory systems, riboswitches, and co-culture strategies have

produced new methods for managing the long-standing tension between biomass production and product formation, which has historically limited the use of photosynthetic biofuel platforms. There are still many challenges to address before we will be able to commercially produce biofuels using PCC 7942. Some of these challenges include photoinhibition at high cell densities, instability of transgenes that impose growth burdens on bacteria to produce biofuels, extraction economics of downstream products from the bacteria, and regulatory hurdles associated with the open-environment use of GMOs. The completion of this work will require a continued collaboration of scientists from multiple disciplines. It is likely that the most successful path forward for the commercial production of biofuels will not be through the separate production of only biofuels but via the integrated production of multiple (3–5) high-value products in cyanobacterial biorefineries along with biofuels and the sharing of capital costs across a broader product portfolio. As the global energy transition becomes more imminent, the commercial production of biofuels becomes more actionable. *S. elongatus* PCC 7942, with its direct line from sunlight and CO₂ to fuel molecules, embodies one of the most elegant solutions to the dual challenge of energy

security and climate change. Realizing this potential will require not only continued molecular innovation but also deeper integration with process engineering, systems biology, and technoeconomic analysis to translate laboratory elegance into industrial reality.

ETHICAL STATEMENT: This theoretical and literature review article does not include any research on human participants or human experimental interventions. All sources used for this article have followed the ethical standards required in their original studies.

CONFLICT OF INTEREST STATEMENT: Authors have also declared that there are no conflicts of interest regarding this article.

DECLARATION: We used generative AI or AI-assisted technology, including AI agents or deep research tools.

FUNDING STATEMENT: No funding was received for this research.

ACKNOWLEDGEMENT: The corresponding author is highly grateful to the Dean Prof. (Dr.) R. K. Jain for his constant guidance during this work. The author is extremely grateful to the CEO Prof. (Dr.) Shalya Raj and VC of the University Prof. (Dr. P. K. Sharma for providing knowledge and constant guidance and support during this work.

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