Cyanobacteria as whole-cell factories: Current status and future prospectives

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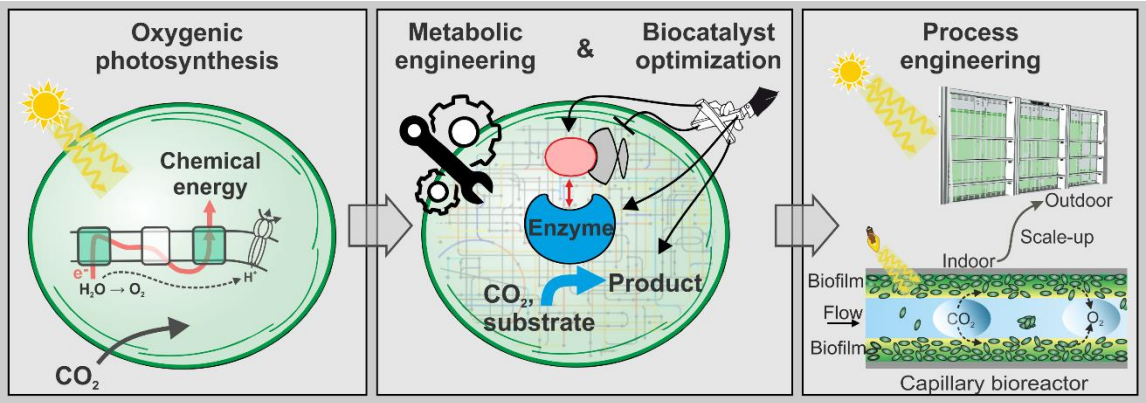
1 Abstract:

Cyanobacteria as phototrophic microorganisms bear great potential to produce chemicals from sustainable resources such as light and CO_2. Most studies focus on either strain engineering or tackling metabolic constraints. Recently gained knowledge on internal electron and carbon fluxes and their regulation provides new opportunities to efficiently channel cellular resources towards product formation. Concomitantly, novel photobioreactor concepts are developed to ensure sufficient light supply. This review summarizes the newest developments in the field of cyanobacterial engineering to finally establish photosynthesis-based production processes. A holistic approach tackling genetic, metabolic, and biochemical engineering in parallel is considered essential to turn their application into an ecoefficient and economically feasible option for a future green bioeconomy.



Graphical abstract

2 Highlights

Concepts making direct use of light energy to drive biotransformation reactions

Regulatory proteins as tools to control energy and carbon fluxes in cyanobacteria

Capillary-based bioreactor concepts to improve light utilization efficiency

3 Introduction:

Cyanobacteria bear great potential to function as cell factories for producing chemicals using sunlight as sole energy source via oxygenic photosynthesis [1, 2]. These organisms use light to oxidize water and provide electrons to run an autotrophic metabolism based on CO_2 fixation. Compared to terrestrial plants, the CO_2 fixation efficiency of cyanobacteria is 10–50% higher [3, 4], giving them a high potential for carbon sequestration and the ecoefficient production of fuels and chemicals. Whereas eukaryotic microalgae bear a similar photosynthetic potential, cyanobacteria profit from a lower degree of structural complexity, which simplifies strain and metabolic engineering. Currently, cyanobacteria are engineered to produce diverse compounds ranging from short-chain hydrocarbons and low-molecular synthons to complex bioactive compounds of nutritional or pharmaceutical interest [2]. However, compared to heterotrophic production strains, achieved rates, yields, and titers are typically lower and often below the needs of a commercial deployment. In this regard, energy allocation, i.e., light supply, constitutes a major constraint raising the question: does sunlight provide enough energy and is its photosynthetic exploitation efficient enough to sustainably drive production? Albeit sunlight is an abundant energy source, it is area-wise rather “diluted”. In plants or microalgae, the maximum theoretical efficiency of the photosynthetic apparatus to convert light energy into chemical energy is 26%, but overall photosynthetic CO_2 fixation reaches a real maximum of only 1-3% [5]. Thus, the development of efficient photosynthesis-based production processes possibly requires an enhancement of photosynthetic efficiency in addition to the implementation of productive pathways and their efficient coupling to native metabolism. Thereby, genetic tools and knowledge on regulatory networks are required to control pathway operation as well as electron and carbon fluxes towards products. Besides strain design, reaction/reactor engineering is crucial to attain high active biomass concentrations and resilience to temperature and light intensity variations.

Against this background, recently developed strategies to improve productivities and yields in cyanobacteria are reviewed. For the production of chemicals, one can distinguish electron- and CO_2-based product formation. The former only uses light-driven water oxidation to fuel redox-intensive bioconversions, whereas the latter also requires diversion of CO_2-derived carbon (**Fig. 1**). Both approaches demand specific strategies. Overall, we aim to answer the following questions: What chemicals can be produced in cyanobacteria and with what efficiency? What are currently the best production strains? What are the limitations of/in cyanobacterial cells? What are promising metabolic, regulatory, and biochemical engineering targets?

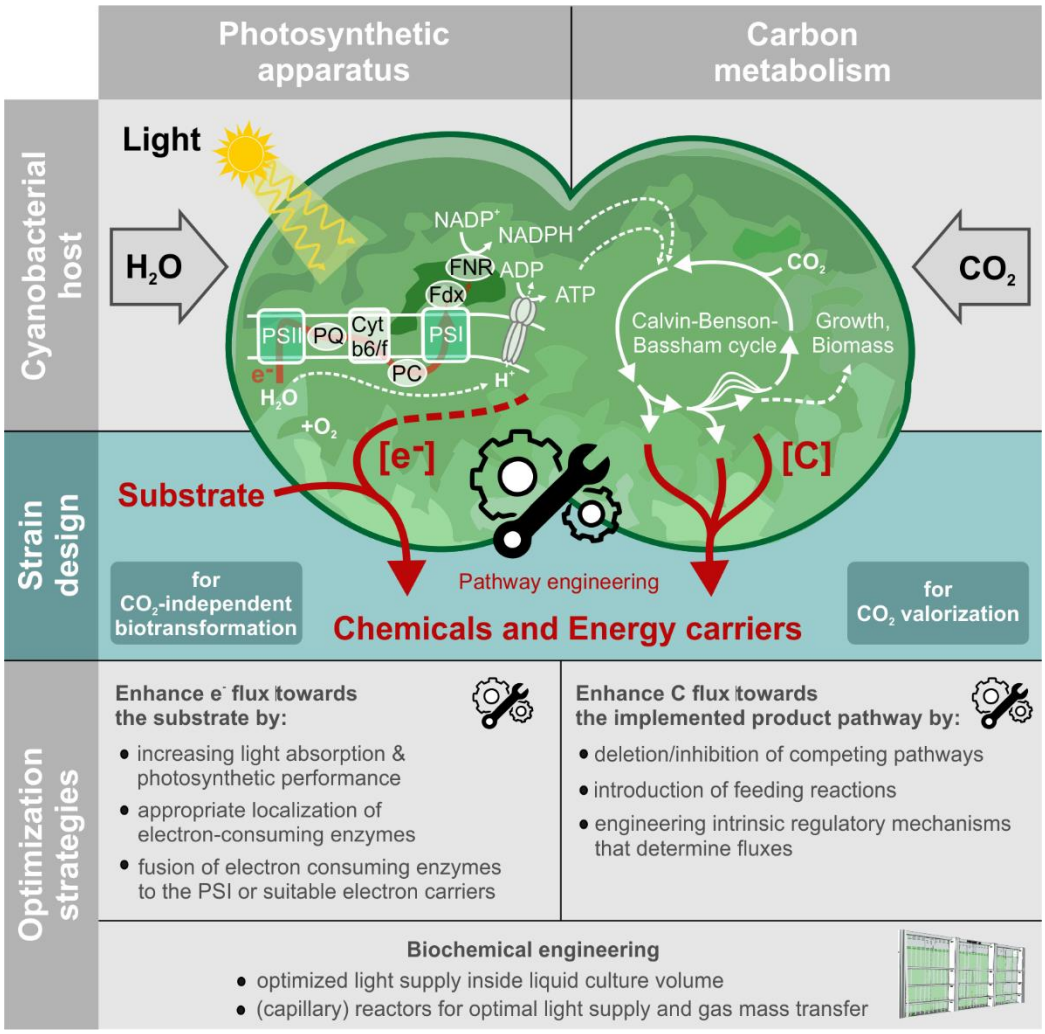


Figure 1: Engineering of cyanobacteria for light-driven production of chemicals and energy carriers. The design of a cyanobacterial biocatalyst involves the introduction, deletion, or upregulation of genes to enable either biotransformation or the direct conversion of CO_2 into chemicals. In both cases, electrons obtained from light-driven water oxidation are the driving force. In addition to mere synthetic pathway operation, further optimizations on the metabolic as well as biochemical engineering side are required to establish a biotechnological process based on oxygenic photosynthesis.

3.1 Production of chemicals in cyanobacteria - current status and limitations

Fast growth is considered a central selection criterium for cyanobacterial production strains and heavily depends on the conditions applied in terms of light and CO_2 availability and medium composition [6]. While widely used models like *Synechocystis* sp. PCC 6803 (*Synechocystis*, 4.3 h doubling time) show moderate growth rates, faster growth has been reported for several *Synechococcus elongatus* strains, namely PCC 11801, 11802, and 11901 and UTEX 2973, the latter reaching a minimal doubling time of 1.5 h. In-depth studies on the molecular basis of fast autotrophic growth revealed that it correlates with increased production of ATP and NADPH, high CO_2 uptake rates, and elevated transcription of genes for precursor biosynthesis and protein translation [6]. Model-guided pathway design and host strain engineering based on such findings combined with advanced synthetic biology tools and high-cell-density (HCD) cultivation /

bioprocessing technologies will be central to improve performance parameters (**Fig. 1**). Thus, an integrated, multidisciplinary approach is required.

As an example for CO_2-based formation of high-volume products, the combined production of isobutanol and 3-methyl-1-butanol has been tackled. NADPH-dependent and NADH-evolving production of these two alcohols in *Synechococcus* sp. PCC 7002 was improved by introducing NADH-dependent nitrate assimilation as additional NADH sink [7]. Another example is ethylene, whose production from CO_2 profited from a deeper understanding of associated metabolic limitations [8]. However, titers achieved for these two products remained rather low (~300 mg L^{-1}). Further optimization may be achieved by a truly integrated approach. For lactate production, CRISPRi-mediated cycling between growth and production phases has been presented as an interesting strategy to increase product titer (to 1 g L^{-1}) as well as process stability [9]. Sucrose represents another well-investigated potential high-volume product. Its synthesis and intracellular accumulation could be improved in several species by manipulating gene expression for two key enzymes [6, 10]. Efficient sucrose export was achieved by introducing a heterologous sucrose permease [10]. Highest product titer (8 g L^{-1}) and productivity (1.9 g L^{-1} day^{-1}) were reached with UTEX 2973 under salt stress conditions, with a cell dry weight (CDW)-based sucrose yield of 3.1 g g_{CDW}^{-1} [10]. A life cycle assessment (LCA) to investigate the environmental impact and limitations of cyanobacteria-based butanol production revealed a high cumulative energy demand in all tested scenarios, indicating that significant metabolic engineering towards a carbon partitioning of > 90% as well as improved light utilization are necessary to displace fossil fuels or even first and second generation bioethanol [11].

For higher-value products, cyanobacterial process commercialization is closer or even already has happened, with pigment production as a prominent example [12, 13]. Phycobiliprotein production by *Anabaena variabilis* CCC421 was enhanced via medium engineering to a product titer of 408.5 mg L^{-1}, demonstrating the potential to efficiently produce a specific protein in cyanobacteria [13]. Further, many studies have tackled lipid and fatty acid production in cyanobacteria with a special focus on the dietary omega-3, polyunsaturated acids with yields up to 100 mg g_{CDW}^{-1} [14]. To better exploit cyanobacterial cell factories for high-volume product formation, combined production of ethylene together with carotenoids was targeted [15]. For this purpose, *Synechococcus* sp. PCC 7002 was engineered and applied in a 100 L air-lift photobioreactor, enabling ethylene and biomass productivities of 2.5 mL L^{-1} h^{-1} and 0.3 g_{CDW} L^{-1} d^{-1}, respectively, and a carotenoid titer of 64.4 mg L^{-1}.

Bioconversions exclusively driven by the light reaction independently from CO_2 fixation (**Fig. 1**) represent an interesting novel approach to make efficient use of natural photosynthesis [16-18]. Biocatalytic redox reactions depend on efficient and sustainable redox cofactor supply, which can be established via photosynthetic water oxidation – primarily in the form of reduced ferredoxin (Fd) and NADPH – making use of abundant, cheap, land-sparing, and thus highly sustainable resources. Fd-dependent cytochrome P450 monooxygenases (CYP450s) have been applied, with specific activities up to 40 U g_{CDW}^{-1} achieved on bioreactor scale [19]. Further, Bayer-Villiger monooxygenases (BVMOs) and reductases have been applied with specific activities up to 150 U g_{CDW}^{-1} [18, 20, 21]. Oxygenase catalysis thereby also profits from O_2 derived from water oxidation, a substrate typically limiting the performance of heterotrophic microbes [17, 19]. Another main product targeted via light reaction coupling is H_2, with efficient electron transfer and the O_2 sensitivity of H_2 producing enzymes as major challenges [22]. Recent advances include hydrogenases-photosystem I (PSI) fusion and the introduction of an O_2-tolerant hydrogenase into *Synechocystis* [23-25]. The combination of these approaches and/or the generation of a low O_2

environment via technical measures for *in situ* gas removal constitute promising strategies to achieve efficient photosynthetic H_2 production.

Electron drainage via biotransformation reactions may cause an ATP/NADPH imbalance, (possibly) affecting both the biocatalytic reaction and host metabolism [26]. Changes in electron demand have for example been shown to involve changes in PS I photochemistry [27]. Further, light stress constitutes a major limitation in nature. The photosynthetic apparatus is evolutionarily optimized to handle over-excitation rather than to maximize light usage efficiency [28]. The introduction of heterologous pathways relying on CO_2-fixation and/or reducing power indicated that they can act as a photosynthetic sink and partially substitute photoprotective mechanisms, while potentially conserving otherwise lost energy within useful products [26, 29]. In the following, novel promising approaches to improve photosynthesis-based product formation are reviewed in more depth focusing on photosynthetic efficiency, regulatory network engineering, and biochemical process engineering.

3.2 Improving cyanobacterial production by tackling photosynthetic efficiency and energy channeling

To prevent losses in energy conversion, the photosynthetic light reactions need to be improved. Exemplary studies targeting antenna systems and other photosynthetic proteins as well as the content and ratio of photosynthetic pigments have been reviewed recently [30]. To enhance photosynthetic efficiency, two major strategies can be defined: (i) expanding the absorbed light spectrum, especially towards infrared (IR) light, and (ii) reducing the energy loss associated with photoinhibition or photodamage. Photosynthetic organisms developed multiple mechanisms to protect themselves from excessive light exposure at the expense of photosynthetic efficiency. The latter can be improved either via efficient rerouting of electrons to productive enzymes or by eliminating such protection mechanisms like flavodiiron proteins consuming up 20% of the electrons originating from PSII [31, 32]. Spontaneous electron rerouting to a CYP450 in *Synechococcus* sp. PCC 7002 has been reported to result in reduced expression of natural electron dissipation pathways [33]. Further, catalytic CYP450 performance could be fostered by deleting the respiratory cytochrome c oxidase.

Direct coupling of desired redox reactions to the photosynthetic apparatus is another approach to increase productivity as recently demonstrated and mentioned above for hydrogenase-based H_2 production [22]. Other examples are CYP450s anchored with a linker to the thylakoid membrane [34]. These approaches follow the hypothesis that reducing the distance to the photosynthetic apparatus gives attached enzymes an advantage over competing intrinsic pathways, like the Calvin-Benson-Bassham (CBB) cycle or nitrate assimilation. As an alternative, the fusion of a CYP450 to a flavodoxin-like electron carrier, with a redox potential suitable for electron transfer to the CYP450 but not to other native electron sinks, has been shown to enable a nearly 25-fold improvement in *in-vivo* electron transfer to the CYP450 on a per protein basis [34]. Furthermore, modulation of the ferredoxin-$NADP^+$ reductase (FNR) could enhance the electron flow into the desired pathway [35]. For the production of chemicals from CO_2, CBB cycle operation must also be efficient. Several studies tried to enhance the CBB cycle and streamline carbon flow towards the desired product [36]. For this purpose, determination of bottlenecks via genome scale modeling and fluxomics as well as detailed knowledge of product-forming enzymes are crucial.

Finally, cell shading and light scattering effects obviate light penetration at high cell densities, leading to low and/or highly fluctuating light conditions. These can be mitigated by reducing the light absorption per cell, e.g., via the reduction of antenna systems, which simultaneously reduces light stress [37]. Strain engineering towards high light resistance also can improve productivity, as

it has been achieved by adaptive laboratory evolution [38], adapting *Synechocystis* to cope with high light on account of light-harvesting capacity. Additionally, screening for and analyzing fast-growing cyanobacteria with high photosynthetic activity can disclose strategies to improve photosynthesis in workhorse strains like *Synechocystis* [39].

3.3 Improving product yields by rerouting metabolic fluxes via intrinsic regulatory mechanisms

Although we are only at the beginning of a full understanding of the regulation of cyanobacterial metabolism, recent research has opened the window towards a more comprehensive view on fundamental control principles. Obviously, small regulatory proteins of less than 100 amino acids play a crucial role [40]. For instance, the CBB cycle is mainly controlled by CP12 that interacts with and inhibits glyceraldehyde-3-phosphate dehydrogenase and phosphoribulokinase, when light availability is not sufficient [41, 42]. Adequate assembly and hence activity of ribulose-1,5-bisphosphate carboxylase-oxygenase (RuBisCO) requires the small chaperone RbcX [43]. Efflux from the CBB cycle is then mainly controlled at the phosphoglycerate-mutase reaction via interaction with the recently discovered PirC protein [44]. Further examples of such regulatory protein-protein interactions have been revealed for the control of the TCA cycle [45], nitrogen assimilation [46], the synthesis of key amino acids [47], ATP synthesis [48], and the photosynthetic apparatus itself [49].

Beside synthetic biology tools to introduce pathway genes and trigger their expression [50], strategies to redirect carbon flux towards specific routes have come into focus, e.g., the blockage of competing pathways or storage compound synthesis. This is usually achieved by enzyme/pathway deletion, whereby the permanent loss of certain functions often is a major disadvantage, e.g., under (fluctuating or harsh) outdoor conditions, especially in the long-term. Intrinsic regulators punctually addressing metabolic reactions pose interesting alternative engineering targets to fine tune metabolic fluxes towards products. For example, deletion of a transcriptional repressor of the glucosylgycerol phosphate synthase gene in *Synechocystis* increased glucosylglyerol yields [51]. This also has been demonstrated for enzyme effectors such as CP12 or PirC. CP12 deletion, for example, resulted in higher production rates for 2,3-butanediol in *S. elongatus* PCC 7942 [52] or bisabolene and limonene in *Synechocystis* [53]. Similarly, PirC deletion in *Synechocystis* overexpressing genes for the plastic alternative polyhydroxybutyrate (PHB) resulted in increased production yields of up to 0.81 g g_{CDW}^{-1} [54]. This impressively demonstrates the power of regulatory engineering as a rather new metabolic engineering concept for cyanobacteria. Further understanding of these control principles may allow a transfer to other pathways and the design of synthetic effectors to control fluxes through any enzyme of interest.

3.4 Biochemical engineering to improve cyanobacteria-based process performance

One of the critical bottlenecks for phototrophic bioprocesses is the lack of suitable photobioreactors (PBRs) allowing cost-effective HCD cultivation. The light-energy dependence resulted in diverse reactor concepts, including tubular, flat-panel, and vertical-column photobioreactors, to maximize light supply while satisfying constraints such as nutrient and CO_2 supply, O_2 removal, temperature and pH regulation, and scalability. Recent publications address available photobioreactor concepts and modeling tools for optimizing reactor performance [55, 56]. Illumination via the reactor surface and light sources distributed inside liquid cultures constitute two general light-supply strategies. Light sources inside liquid cultures can distribute light more uniformly and enable higher light utilization efficiencies [57], as investigated for the reduction of 2-methylmaleimide to (R)-2-methylsuccinimide by *Synechocystis* [20]. Thereby,

suspended wireless light emitters enabled more than two-fold improvement in specific activity compared to surface-illuminated systems. However, the specific activity dropped by 61% upon an increase in cell density from 0.48 to 2.4 g_{CDW} L^{-1}, restricting HCD cultivation. Additionally, technological complexity, scaling issues, and energy demands (LED) can limit application.

Tubular photobioreactors featuring external illumination are recognized as mature cultivation systems for commercial applications [58]. Typically, tubes with 10 to 60 mm inner diameter offer a surface area to volume ratio (SA/V) of over 100 m^2 m^{-3} but poor light penetration depth and limited gas transfer, i.e., CO_2 supply and removal of otherwise inhibitory O_2 [59]. Recently, different geometric configurations, such as capillary reactors or tree-like structures, have been investigated to maximize the SA/V ratio. For example, capillary reactors with inner diameters of 3 mm provide a high SA/V ratio of 1333 m^2 m^{-3}, require low light penetration depth, and offer the option of slug flow mode operation with gas slugs enabling high gas mass transfer [60, 61]. Importantly, such systems enable cultivation and operation of immobilized cells, e.g., making use of the native ability of microbes to immobilize as biofilms. In such a system, dual-species phototrophic biofilm cultivation resulted in a high cell density (above 30 g L_{CDW}^{-1}) of phototrophic microorganisms [61, 62]. Such capillary biofilm reactor operation enabled continuous cyclohexane oxidation with a volumetric productivity of 0.2 g L^{-1} h^{-1}. Critical aspects for such systems include mass transfer and possible cell heterogeneities in the biofilm [63].

Often, outdoor cultivation is targeted for commercial application. Thus, challenges and limitations arising from the variation in sunlight intensity (including the day-night cycle), adequate CO_2 availability, temperature variations, and contamination issues must be considered. Based on simulations, a V-shaped photobioreactor has been proposed for low-latitude locations, giving 1.4 times higher biomass productivities than flat horizontal PBRs [64]. Such modelling approaches augur well to accelerate the design and scale-up of PBRs for efficient outdoor operation [55, 56, 64].

4 Conclusions

Recently gained understanding of cyanobacterial metabolism and responses to environmental and production-related perturbations, new tools for strain engineering, and novel photobioreator concepts substantially advanced cyanobacterial photobiotechnology over the last years and are considered essential to achieve high efficiencies [27]. Concepts making direct use of light energy to drive biotransformation reactions repesent an interesting option. However, efficient operation of and energy transfer from the photosynthetic apparatus, efficient channeling of energy and carbon towards product formation, and the development of bioreactor concepts enabling high cell density cultivation still constitute major challenges for cyanobacterial bioprocessing. To this end, basic research on cellular regulation of electron and carbon fluxes under natural conditions (including fluctuating light or light stress) is required. Small regulatory proteins were identified as promising tools to control energy and carbon fluxes in cyanobacteria. This and other developments in systems biology (omics tools, metabolic models, and respective predictions) and synthetic biology will form the basis for efficiently implementing synthetic pathways in production strains with a controllable metabolism. Further, reactor systems efficiently operating under outdoor conditions need to be developed with capillary biofilm reactors constituting a promising option. Given that sustainable biotechnology demands a holistic approach, pathway implementation, the control of competing pathways, photosynthetic efficiency, and development of advanced bioreactor systems have to be tackled in an integrated parallel way, which can be guided by life cycle assessment. Overall, such holistic approaches augur well for cyanobacterial solutions based on sunlight and water, contributing to a sustainable bioeconomy.

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