Review

Metabolic engineering of cyanobacteria for the synthesis of commodity products

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Through metabolic engineering cyanobacteria can be employed in biotechnology. Combining the capacity for oxygenic photosynthesis and carbon fixation with an engineered metabolic pathway allows carbon-based product formation from CO_2, light, and water directly. Such cyanobacterial 'cell factories' are constructed to produce biofuels, bioplastics, and commodity chemicals. Efforts of metabolic engineers and synthetic biologists allow the modification of the intermediary metabolism at various branching points, expanding the product range. The new biosynthesis routes 'tap' the metabolism ever more efficiently, particularly through the engineering of driving forces and utilization of cofactors generated during the light reactions of photosynthesis, resulting in higher product titers. High rates of carbon rechanneling ultimately allow an almost-complete allocation of fixed carbon to product above biomass.

Cyanobacterial biotechnology

Genetic engineering has made it possible to use photosynthetic microbes – cyanobacteria – to convert CO_2 directly into various chemicals. Accordingly, cyanobacterial biotechnology can contribute to a bio-based economy. In this way metabolic engineering and synthetic biology are used for the construction of cyanobacterial strains that convert CO_2 into chemical commodities, energized by the machinery of oxygenic photosynthesis [1,2].

In microbial biotechnology rational design strategies allow the construction of microbial cell factories. The introduction of an exogenous biosynthetic pathway into a cyanobacterial cell will lead to product formation from CO_2. The new genetic material will encode information to provide a metabolic pathway to convert CO_2-derived carbon-based intermediates into almost any (carbon-based) product of interest, such as a specialty or commodity chemical, a biofuel, or a precursor of a bioplastic [3].

Cyanobacteria can perform oxygenic photosynthesis. At the thylakoid membrane they capture free energy from sunlight and generate ATP and NADPH. Those two high-energy intermediates are utilized for the assimilation of essential nutrients, particularly in the Calvin–Benson–Bassham (CBB) cycle where the ribulose-1,5-bisphosphate carboxylase/oxygenase enzyme (RuBisCO) catalyzes CO_2 fixation for the synthesis of the key building blocks for growth (i.e., the synthesis of new cells) [4]. Through metabolic engineering, this photosynthetic metabolism can be 'hijacked' to synthesize valuable products from CO_2, sunlight, and water, O_2 being the only byproduct. A non-modified cyanobacterial cell is investing fixed carbon and its inherent free energy into growth, survival, and the generation of new biomass. A cyanobacterial 'cell factory' can invest a significant fraction (i.e., >50%; see [5]) of the incoming carbon and energy sources into the synthesis of a carbon-containing product, allowing only the replacement of decaying cells and the maintenance of the surviving cells to be fueled by the native metabolism [6].

This approach was pioneered around the turn of the century. The proof-of-concept studies dealt with the (over)-production of a few compounds: pigments [7], ethylene [8], ethanol [9], and H_2 [10]. Continuing work expanded the product diversity to include isobutyraldehyde and isobutanol [11] and lipids and fatty acids [12,13]. Furthermore, ethanol titers have been increased [14] and the molecular toolbox to perform genetic modifications in the 'photosynthetic chassis' has advanced [15].

Recently, the field of cyanobacterial metabolic engineering has developed faster. The range of carbon-based products that can be derived from intracellular metabolites of cyanobacteria has been expanded to a larger and more diverse range of alcohols, organic acids, sugars, and fatty acids, mostly biofuel and bioplastic precursors [16]. Meanwhile the overproduction of native isoprenoids or the heterologous production of non-native isoprenoids aims at bridging the gap from high-volume-low-price compounds to low-titer-high-price products, with the aim of increasing titers for these too [17]. In addition, the molecular tools to perform classical metabolic engineering and apply synthetic biology have been further developed and evaluated [18].

Here the general aim is to give a broad overview of most of the excreted products for which proof of principle is available to date that can be produced by cyanobacteria from CO_2. We evaluate in detail which native metabolic pathways have been diverted toward product formation

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most successfully. Further, we show production titers and calculate the production rates and the partitioning of the fixed carbon into the product. This allows a timeline-like overview of improvements in the field and which design strategies serve optimization. Essentially we show a 'ranking' of successful versus more challenging product-formation routes (i.e., their respective branching points in the intermediary metabolism).

Targets of metabolic engineering

The proof of principle for cyanobacterial 'direct conversion' (i.e., from CO_2 into product) is available for a wide range of compounds, including biofuels, bioplastics, and commodity products used in the chemical and pharmaceutical industry [1]. Products of interest, which are often excreted, are synthesized from (central) intracellular metabolites. The relevant reactions are catalyzed by heterologous enzymes. The genes coding for these enzymes are introduced through genetic engineering. Cyanobacterial intermediary metabolism makes use of a limited number of central metabolic pathways in addition to a wide variety of anabolic pathways. The CBB cycle and the tricarboxylic acid cycle, connected via glycolysis/gluconeogenesis and the pentose phosphate pathway, are energized by NADPH and ATP. Strikingly, most of the chemical compounds that are overproduced by cyanobacteria are synthesized from a limited number of intermediary metabolites, mostly with the help of fermentative pathways. The most widely utilized intermediary metabolite is pyruvate, the product of the lower part of the glycolytic pathway. Other substrates are acetyl-coenzyme A (CoA) and fructose 6-phosphate (F6P) and, more recently, isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) from the mevalonate pathway. These metabolites are distant from those of the central carbon metabolism and their intracellular concentration is significantly lower than that of, for example, pyruvate [19]. Listing the achieved product titers and their distance from the CO_2 fixation reaction demonstrates the dependency of the respective heterologously expressed production pathways on the starting substrate (Table 1).

If RuBisCO is saturated with substrate, the CBB cycle is driven toward carbon fixation, essentially feeding the metabolism with glyceraldehyde 3-phosphate (GA3P) as basic the building block for cellular biosynthesis (i.e., anabolism). To close the cycle and regenerate the ribulose 1,5-bisphosphate (RuBP), a fraction of the GA3P molecules is converted in several steps to RuBP, driven by phosphoryl transfer from ATP. Wild type *Synechocystis* sp. PCC 6803 (hereafter referred to as PCC 6803) growing in photoautotrophic conditions, shows, for instance, that the highest carbon fluxes are indeed part of the CBB cycle [20]. Determining the number of enzymatically catalyzed steps starting from RuBisCO shows the respective heterologous pathway in relation to its distance from there. The coexpression of preceding steps in the metabolic pathway allows shifting of the branching/tapping point closer to the step of CO_2 fixation in the CBB cycle (Table 1). 'Tapping' metabolism at pyruvate allows the accumulation of large titers of ethanol [21], butanediol [22], L-Lactic acid [23], D-Lactic acid [24], and isobutyraldehyde [11]. Rechanneling carbon from sugar metabolism allows high titers for the formation of sucrose [25], glycerol [26], and mannitol [27]. Products drawn from other pathways of intermediary metabolism show significantly lower titers, like tapping acetyl-CoA (e.g., for the production of 3-hydroxybutyrate [28]) or the isoprenoid biosynthesis pathway.

Interestingly, mannitol and glycerol, originating from F6P and glycerol 3-phosphate, respectively, do not show titers as high as the highest titer reported for sucrose, although they branch off closer to the carbon fixation reaction than the precursor for the biosynthetic pathway toward sucrose [25–27]. In some cyanobacterial strains, sucrose accumulates as compatible solute to provide osmoprotection [29]. Glycerol branches off the synthesis pathway toward the osmoprotectant glycosyl glycerol [26,30]. Also, mannitol may play a role in osmoregulation [27]. Cells, if responding to changed osmolarity (i.e., to acclimate to salt stress), can hence employ different pathways. Nevertheless, these endogenous pathways may vary considerably in their capacity with respect to the rate of synthesis of these polyols. Another factor that may contribute to the high rate of sucrose production [25] is that the export facilitated by the heterologous transporter lowers product inhibition on the enzymes of the biosynthetic pathway and thus allows higher productivity for sucrose despite its distance from the point of CO_2 fixation. The sugar biosynthetic pathway in the wild type organism leads to the synthesis of glycogen, a storage polymer that, in cyanobacteria, can accumulate to levels of more than 50% of the cellular dry weight, depending on the growth conditions [31]. Significantly, cells that lack the capacity for glycogen synthesis and that are starved for nitrogen, a condition that in the wild type organism triggers massive glycogen synthesis, show an overflow of carbon metabolism resulting in excretion of pyruvate and 2-oxoglutarate [32]. This effect can be hijacked for product formation by introducing a pyruvate-utilizing reaction such as lactic acid formation through lactate dehydrogenase (LDH) [33]. Moreover, an alternative heterologous metabolic sink – isobutanol production – can rescue the growth defect of a strain deficient in glycogen formation [34].

Synthetic biology can diminish or eliminate bottlenecks

As mentioned above, pyruvate is the main precursor for most heterologous biosynthetic pathways introduced and evaluated to date, and many compounds derived from pyruvate show high productivity (Table 1). Pyruvate holds a central position in carbon metabolism – also in cyanobacteria – and is separated by only four reactions from the point of CO_2 fixation. Fewer (catalyzed) conversion steps usually means more energy conservation. A central position in intermediary metabolism close to the CBB further suggests a high flux of carbon [20]. The coexpression of a heterologous acetolactate synthase in the biosynthetic routes toward isobutyraldehyde [11] gives rise to increased product formation. Similarly, the coexpression of pyruvate kinase provides a higher flux toward pyruvate such that the overexpressed LDH can form more lactic acid [35]. Effectively, this latter modification results in a heterologous pathway starting from phosphoenolpyruvate instead of from pyruvate. Notably, this has worked only after the LDH had been