Engineering cyanobacteria for direct solar chemical and fuel production from CO_2

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Cyanobacteria, prokaryotic microorganisms with basically the same oxygenic photosynthesis as higher plants, are becoming excellent green cell factories for sustainable generation of renewable chemicals and fuels from solar energy and carbon dioxide. In the presentation I will visualize the concept green cell factories by introducing and discussing two examples: (i) engineering cyanobacteria to produce the important bulk chemical and potential blend-in biofuel butanol from sunlight and carbon dioxide, so called photosynthetic butanol, and (ii) generation of a functional semisynthetic [FeFe]-hydrogenase linking to the native metabolism in living cells of the unicellular cyanobacterium Synechocystis PCC 6803.

1. Introduction

Cyanobacteria, prokaryotic microorganisms with oxygenic photosynthesis, evolved on earth billions of years ago. They convert solar energy, carbon dioxide and water into chemical energy while releasing O_2 into the atmosphere. In addition, some strains are able to convert N_2 into ammonia. Compared to other oxygenic photosynthetic organisms, cyanobacteria possess the highest solar energy capturing efficiency with corresponding adequate carbon dioxide concentrating mechanisms and carbon dioxide fixation. With their modest nutrient requirements and ability to grow on non-arable land and thereby not compete with e.g. food or fodder production, cyanobacteria have emerged as potential green cell factories for sustainable generation of truly carbon neutral, or even carbon negative, renewable chemicals and fuels. The rapid progress in synthetic biology and genetic engineering have made it possible to custom design microorganisms including cyanobacteria. This may include deletion of native and addition of new capacities as well as modified and optimized metabolic flow towards desired product(s). Until today, cyanobacteria have been engineered, as proof-of-concept, to synthesize numerous non-native products.

2. Engineering cyanobacteria to produce photosynthetic butanol

Cyanobacteria do not produce butanol naturally. However, a combination of synthetic biology and system biology strategies have been employed to enable photosynthetic butanol production, i.e. butanol produced from solar energy and carbon dioxide, in a limited number of model cyanobacterial strains.

Butanol is a four-carbon alcohol (C_4H_9OH) occurring in four structural isoforms: 1-butanol, 2-butanol, isobutanol and *tert*-butanol. It is mainly used as solvent, intermediate in chemical synthesis, and as fuel. The global market for this important bulk chemical and excellent blend-in fuel is very large, projected to reach USD 5.6 billion by 2022. Most butanol is presently as produced from fossil resources, fossil butanol. Additionally, there are biological routes for renewable butanol production, mainly to produce 1-butanol and isobutanol. Existing bio-based 1-butanol is produced from starch, sugar, or cellulose such as wheat, beet, corn and wood. Products of these fermentation processes additionally include acetone and ethanol. It is the understanding that the existing, and further developed, bio-butanol market will grow significantly in the near future. Photosynthetic butanol directly from solar energy and carbon dioxide will be the most sustainable and carbon dioxide neutral production of this important bulk chemical and fuel.

Adding *kivd* and *adh* on a self-replicating plasmid into cells of *Synechocystis* PCC 6803 (*Synechocystis*) introduced the ability to form isobutanol in the cyanobacterial cells in a direct process from solar energy and carbon dioxide [1]. Kivd was then identified as the bottleneck for isobutanol production. Single modifications of selected amino acids in the active center of the enzyme, based on knowledge-based modelling, resulted in significantly improved versions of Kivd with higher catalytic activities in the cells [2]. Using the best isobutanol producing strain in long-term experiments (as detailed below) resulted in a maximal cumulative titer of 911 mg photosynthetic isobutanol per L with a maximum rate of 43.6 mg photosynthetic isobutanol per L and day [3].

By extensive work, we designed and constructed cyanobacteria that form significant levels of photosynthetic 1-butanol in a direct process [4]. Based on wild-type cells of *Synechocystis*, we introduced the ability to produce 1-butanol from acetyl-CoA and pyruvate, a pathway consisting of six individual genes introduced into three different locations in the genome. In addition, we increased the flow of carbon (C) from the Calvin-Benson-Bassham cycle to the product by introducing the enzyme phosphoketolase and by removing the ability to form the storage product acetate - all with the aim to increase the amount of 1-butanol being formed. Our systematic work generated many different genetically modified cyanobacterial strains with progressively higher 1-butanol production, from wild-type cells via strain BOH1 to the final

strain BOH78 with the highest photosynthetic 1-butanol production, Figure 1 [4].

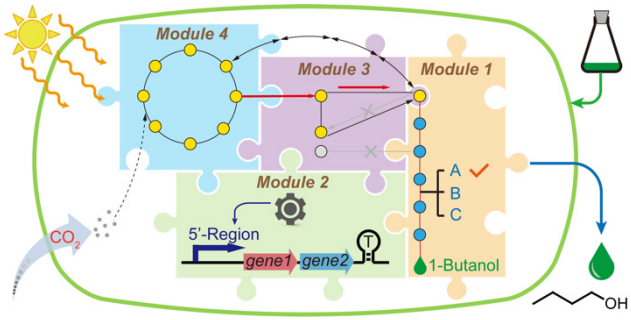

Fig. 1: Systematic modular engineering of the unicellular cyanobacterium *Synechocystis* PCC 6803 resulted in cells with significant levels and rates of photosynthetic 1-butanol production from solar energy and carbon dioxide [4]

When strain BOH78 was grown on smaller scale (25 mL) in long-term trials (at least 1 month) with daily manual adjustments of pH and cell culture removal (removing 2.5 mL (10%) of cell culture replaced by 2.5 mL of new medium) every second, respectively each day, the maximum amount of 1-butanol in the cell culture was 1.86 and 2.13 grams of photosynthetic 1-butanol per L (*in-bottle* titer), respectively. If we add the produced amount of 1-butanol that was removed every second, or each day, the total production became 3.0 and 4.8 grams photosynthetic 1-butanol per L (*cumulative* titer), respectively, with a maximum rate of 302 mg photosynthetic 1-butanol per L and day [4]. This was, within the European Photofuel consortium, increased to a rate of 600 mg photosynthetic 1-butanol per L and day with a carbon partitioning of 60%, i.e. 60% of the fixed carbon ends up in the secreted product butanol (instead of as biomass) [5]. This is a very high production of a solar fuel/chemical in a direct process from solar energy, carbon dioxide and water – it holds great promise for the future.

3. Generation of a functional semisynthetic [FeFe]-hydrogenase in cyanobacteria

Almost all cyanobacteria contain an inherent capacity to produce hydrogen gas catalyzed by a nitrogenase and/or a bi-directional hydrogenase. Wild type (WT) cells of *Synechocystis* harbor a single, bidirectional, oxygen sensitive [NiFe]-hydrogenase encoded by the *hox*-genes. Hydrogen production occurs both under dark, fermentative conditions and, in a short burst, during the transition from darkness to light. In darkness, the hydrogen production is dependent on external or internal fermentation substrate(s). In light, the native hydrogenase can utilize electrons from PSII-catalyzed water splitting and/or fermentative sources, although it is rapidly inactivated by oxygen generated by PSII. However, neither nitrogenase nor [NiFe] hydrogenase can be considered Nature's premier H_2 production catalysts. Indeed, the [FeFe]-hydrogenases (HydA), found in some eukaryotes and in prokaryotes outside the cyanobacteria phylum, are known to have catalytic activities that vastly outperform any hydrogen producing enzyme naturally occurring in cyanobacteria. Moreover, the [FeFe]-hydrogenases are also much less prone to catalyse the oxidation of H_2 to protons and electrons. Thus, the introduction of such an [FeFe] hydrogenase is expected to result in significant enhancement in H_2 production from the cyanobacterial host. An heterologous expression of HydA is complicated by the requirement for efficient co-expression of its dedicated maturation machinery.

We expressed the [FeFe]-hydrogenase HydA1 from the green algae *Chlamydomonas reinhardtii* (CrHydA1) in WT and a hydrogenase deficient mutant (Δ*hoxYH*) of *Synechocystis* PCC 6803 (*Synechocystis* WT and *Synechocystis* Δ*hox*, respectively) using a broad host range shuttle vector [6]. To reliably express the *hydA1*-gene in the cyanobacterial host strains, we used the $Ptrc_{core}$ promoter together with a bicistronic design adapter. The strain *Synechocystis* Δ*hox* offers a unique opportunity to examine the activity of a heterologous hydrogenase without interference from any native hydrogen metabolism. A functional semisynthetic [FeFe]-hydrogenase was formed by adding a synthetic [2Fe] subcluster mimic $[Fe_2(adt)(CN)_2(CO)_4]^{2-}$ (complex 1, adt: $-SCH_2NHCH_2S-$), the active site of the [FeFe]-hydrogenase, to the cell cultures, Figure 2 [6].

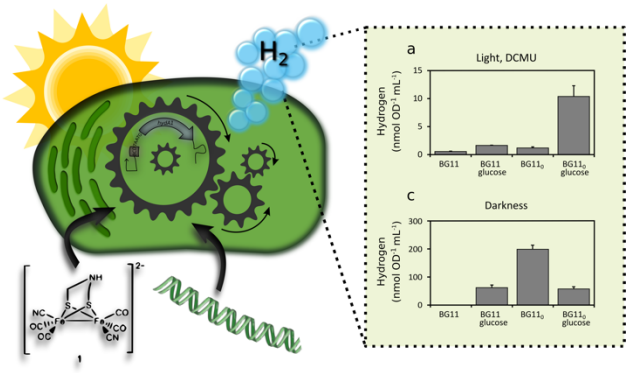

Fig 2: A functional, semi-synthetic and heterologously expressed [FeFe]-hydrogenase is generated in *Synechocystis*, resulting in cells with sustained hydrogen production [6]

The activated hydrogenase evolved hydrogen both in light and in darkness with an activity directly linked to the metabolic status of the cell. This was the first example of synthetic activation of a metalloenzyme within any photosynthetic microorganism.

In following work, we extended the work of artificial activation to two newly discovered [FeFe]-hydrogenases, *Sm* HydA from *Solobacterium moorei* and *Tam* HydS from *Thermoanaerobacter mathranii*, in cells of *Synechocystis* [7]. We demonstrated *in vivo* H_2-evolution activity from both enzymes after synthetic activation by the diiron subcluster mimic. *Synechocystis* cells carrying the activated *Sm* HydA exhibits clear H_2 production under different environmental conditions, and the semisynthetic enzyme remains stable *in vivo* for several days [7].

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