

## Energy biotechnology with cyanobacteria

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The world's future energy demand calls for a sustainable alternative for the use of fossil fuels, to restrict further global warming. Harvesting solar energy via photosynthesis is one of Nature's remarkable achievements. Existing technologies exploit this process for energy 'production' via processing of, for example, part of plant biomass into ethanol, and of algal biomass into biodiesel. Fortifying photosynthetic organisms with the ability to produce biofuels *directly* would bypass the need to synthesize all the complex chemicals of 'biomass'. A promising way to achieve this is to redirect cyanobacterial intermediary metabolism by channeling (Calvin cycle) intermediates into fermentative metabolic pathways. This review describes this approach via the biosynthesis of fermentation end products, like alcohols and hydrogen, driven by solar energy, from water (and CO<sub>2</sub>).

### Addresses

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### Introduction

In the October 2008 issue of Current Opinion in Biotechnology a number of reviews were devoted to illustrate how today's biotechnology may contribute to provide sustainable energy supplies, to fight global warming and pollution, and play a major role in food and health issues [1<sup>\*</sup>]. Impressive progress has been made with respect to metabolic engineering of heterotrophic microorganisms like *Escherichia coli* [2<sup>\*</sup>] and Clostridia [3<sup>\*</sup>], and the same is true for the employment of photosynthetic (micro)organisms as a platform for the production of energy-rich compounds [4,5<sup>\*\*</sup>]. Photosynthetic life forms have attracted enormous interest as vehicles to capture light energy and subsequently convert that into the free energy of organic compounds, using water as the ultimate

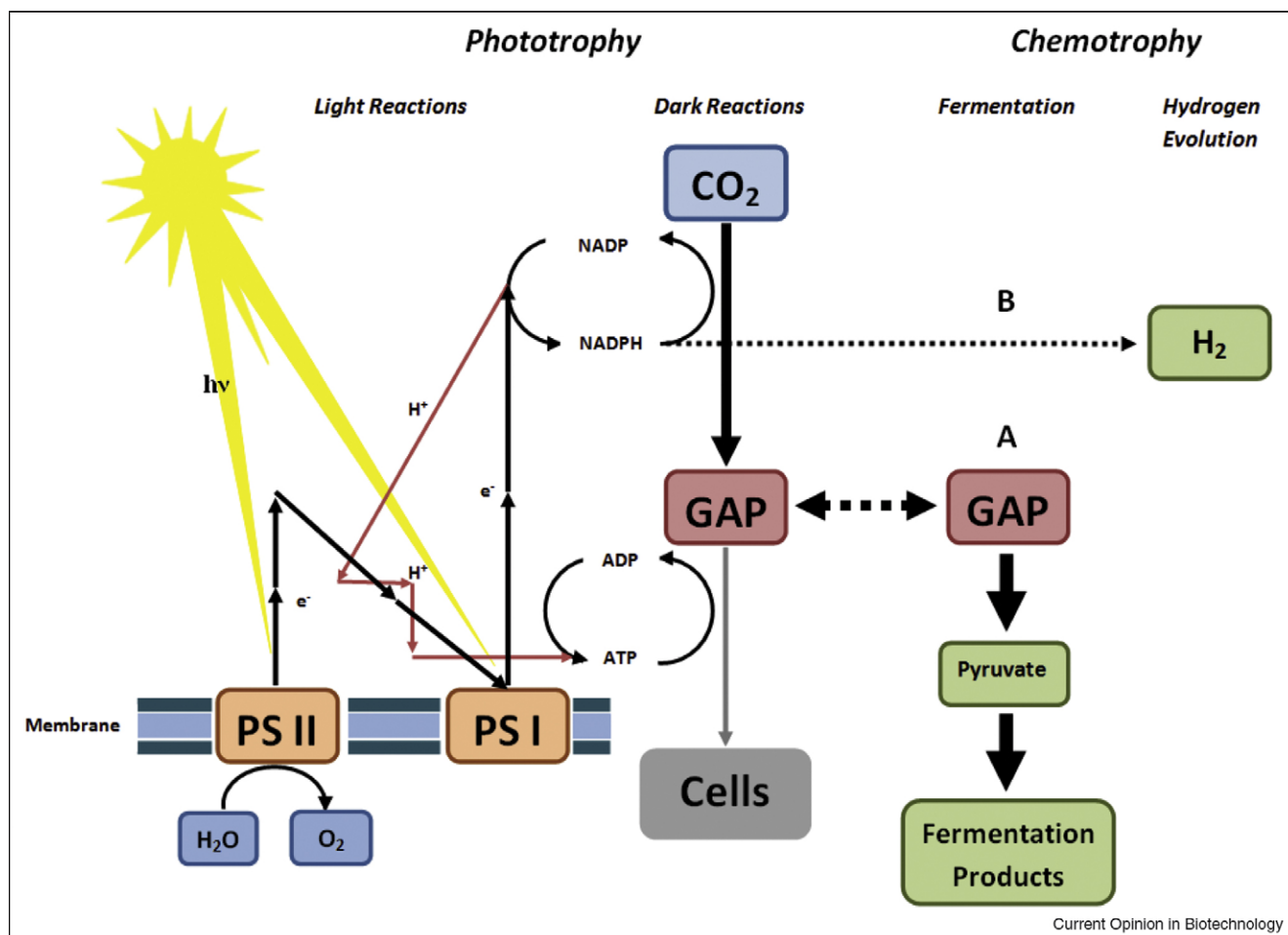
electron donor. Currently, two major technologies are employed with phototrophic organisms: first, plant-based biofuel production via fermentation of its sugar content to ethanol and, to a much lesser extent, second, algae-derived biodiesel production through lipid extraction of biomass from large-scale cultures. The pros and cons of these first-generation and second-generation biofuel production platforms have been discussed extensively in political, economic as well as technological context and will not be reviewed here. Suffice it to say that what transpires through all publications on this issue is the consensus that in view of the many adverse effects of the use of fossil fuels and the negative effects of first-generation and second-generation technologies [6,7], the scientific community should continue to seek for even more sustainable forms of biofuel production.

A technology that would employ engineered microorganisms as catalytic units, producing biofuels extracellularly in an essentially continuous process, would not suffer from the many disadvantages encountered with the current technologies. Compared to heterotrophic fermentative platforms that start from high energy substrates like sugars, the use of a production organism that feeds on solar energy, water, and CO<sub>2</sub>, would yield an enormous gain in energetic efficiency of the overall process. Here, we propose that cyanobacteria, that is oxygenic photosynthetic prokaryotes, are the cell factories of choice for such applications, as they are easily cultivable with little nutritional demands for the production of organic biofuels and of hydrogen. Both applications will be discussed separately below.

### Photanol

Both first-generation and second-generation biofuel production technologies consist essentially of two phases. First, solar energy drives synthesis of highly complex molecules (cell constituents) and structures (cells and plants) from CO<sub>2</sub>. In the second phase a (small) fraction of these complex products are converted to small molecules (like ethanol) in the case of plants, or are extracted and converted chemically to fatty acid-methyl esters in the case of algae. Thus, much of the light energy is lost in nonfermentable waste (but see e.g. [8]) and both processes contain numerous steps, each with its typical efficiency, which leads to low overall efficiencies. Second, both processes are limited by the capacity of the phototrophs to intracellularly store the substrates to be fermented or extracted. A third disadvantage is the fact that these technologies cannot be carried out in a continuous process design.

Figure 1



Schematic representation of the metabolism underlying 'photofermentation', based on the introduction of a fermentation pathway or a hydrogen evolution pathway (i.e. a hydrogenase) from a chemotrophic organism into a cyanobacterium. Coupling between the endogenous metabolism of the phototrophic organism and the (heterologously encoded) pathways may occur through central metabolites like glyceraldehyde-3-phosphate or NADPH (and ATP).

Nevertheless, it is possible to envisage a technology that minimizes the number of steps from starting material ( $H_2O$ ,  $CO_2$ , and solar energy) to end product (biofuel), and does so in a catalytic process, when a photosynthetic organism is endowed the capacity to convert (a) metabolic intermediate(s) directly into a biofuel product, via the addition of a heterologous fermentative metabolic pathway to the phototroph. When scrutinizing the metabolic pathways presented in Figure 1, as they occur in photosynthetic bacteria on the one hand and in heterotrophic species on the other, striking similarities are seen: most importantly there is the central position of glyceraldehyde-3-phosphate (GAP). In photoautotrophs this is a key metabolite in the Calvin cycle, as a substrate for biosynthesis (i.e. anabolism). In chemoheterotrophs, in contrast, it serves as the major intermediate in numerous anaerobic catabolic pathways, like homolactic, solventogenic, and ethanolic fermentation. The essence of the Photanol

concept (patent filed [9<sup>••</sup>]) is then, using the methods of synthetic biology [10], to design and construct a metabolic network that merges phototrophic and fermentative metabolism into a new network that uses  $H_2O$ ,  $CO_2$ , and solar energy as input and has a fermentation product as output (and oxygen as the inevitable by-product of water oxidation):



Photanol thus employs Nature's mechanisms of capturing solar energy to convert this energy into the reducing power of fermentation end products by highly efficient pathways of fermentative metabolism.

Most importantly, this type of metabolism, which we refer to as 'photofermentation', involves a minimal number of steps in the conversion of  $CO_2$  to biofuel, by

Table 1

## Calculation of the maximal yield of photofermentative ethanol production per unit of surface area.

Parameter		Units	
Irradiation	~1300	$\mu\text{mol photons/m}^2 \text{ s}$	[53]
Per 40 m $\times$ 100 m (~1 acre)	5.2	mol photons/acre s	
Number of sunhours/day	10	hours	
Annual irradiation per acre	4E+07	$\mu\text{mol photons}$	
Photosynthetically active radiation	50%	%	[54]
Photons per: $2\text{CO}_2 \rightarrow \text{C}_2\text{H}_5\text{OH}$	36	number	[55]
Annual production of ethanol	3.6E+04	l/acre year	

bypassing the formation of the complex set of molecules of biomass. Therefore, the theoretical efficiency of biofuel production, expressed as liter of biofuel produced per unit of surface area per year can be significantly increased as compared to first-generation and second-generation biofuel production processes. Calculation (Table 1) shows that with a photosynthetic organism that is metabolically engineered to convert  $\text{CO}_2$  to ethanol, a production of  $\sim 2 \times 10^4$  l/acre/year would be obtained, if it is assumed that in the process all incident photons would be captured and their energy converted to NADPH. With respect to ethanol formation, indeed experimental evidence has been presented showing that in the cyanobacteria *Synechococcus* sp. strain PCC 7942 [11], and *Synechocystis* sp. PCC 6803 [12\*\*] ethanol formation takes place when these organisms are made to express two genes that code for pyruvate decarboxylase and alcohol dehydrogenase II, respectively.

The Photanol approach is not limited to ethanol, but rather allows for a range of products, essentially as broad as the range of products from glycolysis-based fermentations found in nature. Thus, a collection of cyanobacterial strains can be envisaged, obtained by introducing the proper fermentation cassette through the application of molecular genetic engineering, that each will perform as a fermenting photoautotroph when provided with  $\text{CO}_2$  and light, that is as an organism carrying out photofermentation.

Diverting the carbon flow from GAP by genetic modification to some product, away from the natural flow toward cell synthesis, will usually lower the organisms' fitness. The living cell contains complex regulatory networks that function in concert to tune metabolic activity to the environmental conditions, and to respond appropriately when conditions change. Therefore, the physiological effects of genetically introducing a metabolic pathway into a microbe are extremely difficult to predict and it should be realized that the introduction of (a set of) genes is only the very first step. Controllable and stable expression demands thorough understanding of the organism's genetic, regulatory, and metabolic makeup. Only then one can interfere successfully with cellular activities toward optimal biofuel formation. The information

necessary relates to growth demands, adaptive mechanisms, physiology, and regulation at all omics levels. Obviously then, the choice of organism to use as a production platform in a Photanol approach should be guided by our level of knowledge of the organism.

### *Synechocystis* sp. PCC 6803

To date, of all cyanobacteria, *Synechocystis* sp. PCC 6803 is the best-characterized species and, given its robust growth characteristics, therefore the organism of choice for applications such as outlined above. *Synechocystis* is a naturally transformable organism [13], genetically well characterized, and with its genome sequenced [14] and annotated, being publicly available at CyanoBase [15]. Detailed studies on techniques for genetic manipulation of this species are available [16,17] (for a review see [18,19]). Research on the regulation of gene expression in response to the nutritional status of the environment led to the understanding of adaptive responses at quite some detail [20–24]. At the turn of the millennium the first complete genome-broad transcript studies of *Synechocystis* appeared. The aims of these transcript analyses range from comparison of cells grown at high-light and low-light intensities [25], to the determination of the regulation of gene expression by low temperatures [26], with many aspects in between.

Not unmentioned should be the impact that *Synechocystis* has had on the elucidation of the molecular mechanisms of photosynthesis. The accessibility of this organism at the molecular genetic and biochemical level has made this organism the organism of choice in many seminal studies (e.g. [27,28]). Meanwhile, also the molecular mechanisms that underlie light sensing [29\*] and other adaptive responses [30] are beginning to be elucidated.

Finally, it should be mentioned that *Synechocystis* is physiologically well characterized. It is a relatively fast growing (minimal doubling time seven to eight hours) cyanobacterium, with no specific nutritional demands. Thus, it can grow fully photoautotrophically, mixotrophically, and chemoheterotrophically (provided they are exposed to a short period of (blue)light [31]). The activities of the two photosystems and the Calvin cycle enzymes are subject to various regulatory networks, that

respond to environmental parameters such as light intensity [32<sup>•</sup>], color [33], and periodicity [34], nutrient availability [35] and intracellular parameters like the redox and energy state [36] as signal input.

In conclusion, a firm body of information on *Synechocystis* sp. PCC 6803 is available to be called upon for engineering purposes. For example, with the data available on the regulation of gene expression, genetic designs related to promoter activities that result in controlled expression of heterologous metabolic pathway enzymes under given experimental conditions can easily be envisaged.

## Hydrogen production

Hydrogen as a fermentation end product warrants a dedicated discussion. This is because cyanobacteria are able to diverge the electrons emerging from the two primary reactions of oxygenic photosynthesis directly into the production of H<sub>2</sub>, making them attractive microorganisms for a renewable production of H<sub>2</sub> from solar energy and water [37<sup>••</sup>]. Again, minimal and inexpensive growth media can be used for the cultivation of cyanobacteria, and, theoretically, the overall energy conversion efficiency may become high. Cyanobacteria can produce H<sub>2</sub> through three main routes: first, H<sub>2</sub>-production directly from the native bidirectional hydrogenase; second, H<sub>2</sub>-production from a native nitrogenase; and third, H<sub>2</sub>-production from an introduced hydrogenase [38<sup>•</sup>].

In *Synechocystis*, like in other unicellular and filamentous, non-N<sub>2</sub>-fixing cyanobacterial strains, a single pentameric NiFe-hydrogenase can either produce or oxidize H<sub>2</sub> at a significant rate. The biological function of this bidirectional hydrogenase is not fully understood, but three main functions have been suggested; to remove excess reducing equivalents during either anaerobic fermentation (maybe the main function) or photoautotrophic growth, or to deliver electrons to the respiratory electron transport chain by the oxidation of H<sub>2</sub>.

Since the activity of the bidirectional hydrogenase is not directly dependent on ATP, use of this enzyme may be energetically more efficient than the use of nitrogenase. Nevertheless, the hydrogenase enzyme may be inhibited by the oxygen generated at photosystem II, and since it operates close to chemical equilibrium, H<sub>2</sub>-production is inhibited above a certain H<sub>2</sub> partial pressure. Therefore, continuous removal of both O<sub>2</sub> and H<sub>2</sub> is necessary to obtain appreciable turnover rates. Furthermore, also accumulation of ATP might impede its turnover, since ATP is produced by photosynthetic electron transport, but not consumed by the hydrogenase.

Ananyev *et al.* [39<sup>•</sup>] recently demonstrated that in the hypercarbonate-requiring filamentous cyanobacterium *Arthrospira* (*Spirulina*) *maxima* anaerobic H<sub>2</sub>-production in the dark occurs in two temporal phases, involving two

distinct metabolic processes. In this organism H<sub>2</sub> evolution represents a major pathway for energy (ATP) production during fermentation, by regenerating NAD<sup>+</sup> essential for the glycolytic degradation of glycogen, and the catabolism of other substrates. Moreover, the removal of nitrate during fermentative H<sub>2</sub> evolution produces an immediate and large stimulation of the rate of H<sub>2</sub> evolution, as nitrate is a competing substrate for the consumption of NAD(P)H. Environmental and nutritional conditions that increase anaerobic ATP production and the intracellular reduction potential (NADH/NAD<sup>+</sup> ratio) are key variables for increased H<sub>2</sub> evolution.

Many cyanobacteria can fix N<sub>2</sub>. In the filamentous strains that contain heterocysts, N<sub>2</sub>-fixation occurs in an anaerobic environment, achieved by separating N<sub>2</sub>-fixation and oxygenic photosynthesis through the inactivation of PSII in the heterocysts. The nitrogenase requires the input of at least two molecules of ATP per pair of electrons, which makes the overall efficiency for H<sub>2</sub>-production rather low. Cyanobacterial nitrogenases typically contain molybdenum (Mo) and iron (Fe) in the active site, when sufficient amounts of Mo are available. Under Mo-deprived conditions the Mo-nitrogenase is replaced by an alternative, vanadium-containing (V)-nitrogenase, and if V is limited, some microorganisms can synthesize a third alternative, a Fe-nitrogenase. Depending on the type of nitrogenase (Mo, V, or Fe) present, different amounts of reducing equivalents are allocated for N<sub>2</sub>-fixation and H<sub>2</sub>-production, see [38<sup>•</sup>]. When the cell uses the V-nitrogenase, for instance, it only expends half as many electrons per H<sub>2</sub> evolved, compared to when the Mo enzyme is used. Consequently, the alternative nitrogenases may be better H<sub>2</sub> producers compared to the more common Mo-nitrogenases. The evolved H<sub>2</sub> is rapidly consumed by an uptake hydrogenase, a NiFe-enzyme present in N<sub>2</sub>-fixing cyanobacteria. Since any produced H<sub>2</sub> is reoxidized by the uptake hydrogenase, no net production is detected.

The first obvious step in the design of a H<sub>2</sub> evolving N<sub>2</sub>-fixing cyanobacterial strain is to engineer a mutant without the capacity to recycle H<sub>2</sub>. Such mutants show light-dependent nitrogenase-based H<sub>2</sub> evolution with relatively higher H<sub>2</sub>-production rates with increasing light intensity, demonstrating that the photosynthetic energy flow may be directed toward H<sub>2</sub>-production rather than N<sub>2</sub>-fixation during such conditions [40].

The development of synthetic biology opens up new possibilities for the construction of efficient H<sub>2</sub> evolving cyanobacterial strains. An obstacle in biological H<sub>2</sub>-production is the oxygen sensitivity of the H<sub>2</sub> evolving enzymes. Some attempts have already been made to introduce less oxygen sensitive hydrogenases into cyanobacteria. An elegant strategy for the creation of more efficient H<sub>2</sub> producers would therefore be the expression

of more efficient noncyanobacterial hydrogenases, for example FeFe-hydrogenases with higher turnover rates, in the anaerobic environment of the heterocysts of a filamentous cyanobacterium. Since protons are abundant within the cell, the main limitation for H<sub>2</sub>-production is the rate of supply of reducing equivalents. The primary electron donors for the H<sub>2</sub> producing enzymes are ferredoxin (nitrogenase and FeFe-hydrogenases) and NAD(P)H (NiFe-hydrogenases). However, these reductants are also used by competing pathways like respiration. Therefore, it will be important to direct the electron flow toward the H<sub>2</sub> producing enzymes and away from competing pathways. As the cyanobacterial bidirectional hydrogenase evolves H<sub>2</sub> at relatively high levels of NAD(P)H, the construction of mutants with blocked electron transfer in selected key pathways may be a promising route to increased H<sub>2</sub>-production. Answers to questions as to how much of the maturation system needs to be introduced and translated into functional units, and what kind of regulation is needed, will be crucial for success.

## Prospects

With the body of knowledge available, large-scale application of *Synechocystis* strains, metabolically reprogrammed according to the Photanol concept or for hydrogen production, as outlined above, seems a challenging but feasible approach. Here, we will not discuss technical aspects of large-scale culturing of unicellular photosynthetic organisms but it should be noted that major progress is being made in this field with respect to cost reduction, stability, and efficiency improvements in effective light capturing, see for example [41,42\*,43]. An interesting development in this respect is the application of new emerging LED-technologies as an additional, low-energy artificial supply of light with optimal properties for photosynthesis [44]. Another important development is the work to increase photosynthetic efficiency in mass cultures through the modulation of antenna size [45\*].

Given the complexity of biological systems, one may expect serious hurdles to encounter when it comes to designing optimization strategies for robust cellular performance. The metabolic flexibility and adaptive potential of prokaryotes is the mere cause of their successful applications, but these properties at the same time should be well understood to keep control over performance. This calls for a systems biological approach where all levels of cellular organization are integrated to describe quantitatively the input–output relationships of the organism as a whole. As exemplified above, genome-broad transcriptome analyses can be, and are, carried out with *Synechocystis*, and recently the first metabolomic data have been published (e.g. [46\*]). With the primary metabolic network being well resolved, with the data obtained by metabolic flux

analysis [47,48] and with increased information on regulatory systems becoming available, *in silico* modeling of the organisms' performance will be a strong tool soon. For example, a controlled flux distribution from GAP to biomass and the programmed product must be maintained in order to ensure a continuous process without the loss of viability. At the same time, maximization of production formation must be guaranteed. An example of the usefulness of metabolic flux balancing [49,50] to quantify flux distributions for a given condition has been recently provided by Fu [12\*\*] when simulating ethanol production by an engineered strain of *Synechocystis*.

Growing understanding of *Synechocystis*' molecular biology, including gene manipulation, regulation of transcription, and translation, etc., will improve the synthetic biology of the organism. One should realize, however, that often the heterologous gene products are supposed to be properly functioning under cellular conditions they are not optimized for. Most prominently this may be the case with fermentative enzymes that have been evolved in (absolute) anaerobic conditions (e.g. in the Clostridia) when expressed in an oxygenic phototroph. Thus, the well-characterized fermentative pathway for butanol production by these genera contains enzymes that are sensitive to oxygen [51\*]. To take this hurdle, alternative donor organisms, which thrive in less strictly anaerobic habitats, may be sought for in Nature. Indeed, in the case of butanol formation, such alternatives can be found among the lactic acid bacteria. However, current developments in the field of directed evolution may be another route leading to modified enzymes that maintain their catalytic function under production conditions.

Finally, one should be aware that metabolically engineered organisms are forced to produce a compound that may lead to autoinhibition of growth. Fermentative organisms are often endowed with a relatively high resistance to their own end products [52]. A host strain like *Synechocystis* may not be fit to withstand high concentrations of such products. Understanding resistance mechanisms and increasing cellular resistance, for example by applying selective pressure to enhance the growth of mutants that are already slightly resistant, engineering, and heterologous expression of new resistance systems, or continuous removal of the fermentation products are possible solutions for this issue.

Obviously, hurdles are to be taken but these should be seen as the great scientific challenges in biotechnology. With the current state of molecular biology and synthetic biology techniques, combined with powerful modeling techniques, the approaches described here could well contribute to a new road to safe, clean fuel production methods that could be open to all.



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