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# On the use of oxygenic photosynthesis for the sustainable production of commodity chemicals

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A sustainable society will have to largely refrain from the use of fossil carbon deposits. In such a regime, renewable electricity can be harvested as a primary source of energy. However, as for the synthesis of carbon-based materials from bulk chemicals, an alternative is required. A sustainable approach towards this is the synthesis of commodity chemicals from CO<sub>2</sub>, water and sunlight. Multiple paths to achieve this have been designed and tested in the domains of chemistry and biology. In the latter, the use of both chemotrophic and phototrophic organisms has been advocated. 'Direct conversion' of CO<sub>2</sub> and H<sub>2</sub>O, catalyzed by an oxyphototroph, has excellent prospects to become the most economically competitive of these transformations, because of the relative ease of scale-up of this process. Significantly, for a wide range of energy and commodity products, a proof of principle via engineering of the corresponding production organism has been provided. In the optimization of a cyanobacterial production organism, a wide range of aspects has to be addressed. Of these, here we will put our focus on: (1) optimizing the (carbon) flux to the desired product; (2) increasing the genetic stability of the producing organism and (3) maximizing its energy conversion efficiency. Significant advances have been made on all these three aspects during the past 2 years and these will be discussed: (1) increasing the carbon partitioning to >50%; (2) aligning product formation with the growth of the cells and (3) expanding the photosynthetically active radiation region for oxygenic photosynthesis.

## Introduction

For an increased sustainability of the future of our society, it will be important to refrain from the use of fossil carbon resources and replace these resources by renewables. For energy purposes, renewable electricity (from e.g.

the sun, the wind, the tides, etc.) will have to play a key role in our future energy supply. However, such decarbonization will be very difficult to achieve for specific parts of the energy sector (like the aviation sector) and the chemicals/plastics industries. Therefore, for these

**Abbreviations** – CRISPRi, CRISPR interference; CRISPR/Cas, Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR Associated Nuclease; FRUITS, Find Reactions Usable In Tapping Side-products; LDH, lactate dehydrogenase; PAR, photosynthetically active radiation; PK, pyruvate kinase; PR, proteorhodopsin; PSI, Photosystem I; PSII, Photosystem II; sgRNA, single guide RNAs; TLA, truncated light-harvesting antenna.

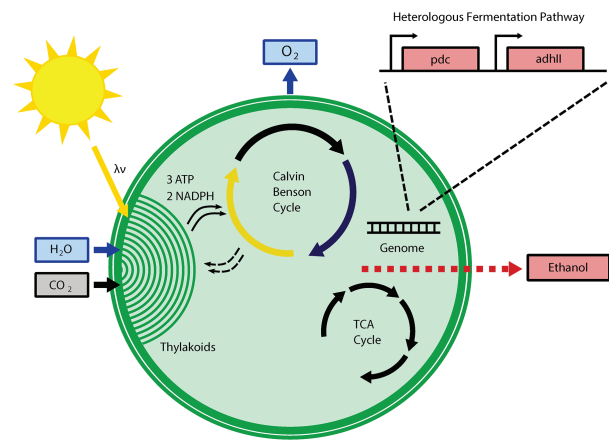
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latter sectors, sustainable (i.e. non-fossil based) alternatives will need to be developed. Physics and chemistry offer various ways to tackle this challenge. Catalysts with excellent specificity and considerable turn-over numbers, have already been developed for electrolysis of water and to a certain extent also for the reduction of CO<sub>2</sub> to more reduced forms of this C(1) compound (Jafari et al. 2016). However, to convert CO<sub>2</sub> with such catalysts into more complex, multiple carbon atoms containing biomolecules, including their enantiomeric specificity, still presents an enormous challenge (Sun et al. 2018).

In the domain of biology this specificity is abundantly encountered. Given the fact that reduction of CO<sub>2</sub> is the key issue in these sustainability applications, in this domain it is specifically oxygenic photosynthesis that offers a convenient pathway to realize this (see e.g. Chen et al. 2016a). This process uses CO<sub>2</sub>, water and sunlight, to reduce CO<sub>2</sub> into a wide range of complex biomolecules. Considering the large scale at which energy and materials will be required, for an ever-increasing world population, it is important that this photosynthesis-based process proceeds with the highest, aerial efficiency possible. It has therefore been proposed that this synthesis is best carried out by cyanobacteria, because they display the most efficient form of oxygenic photosynthesis (see e.g. Janssen et al. 2003, Schuurmans et al. 2015). Furthermore, to simplify product formation, it has been proposed to carry out 'direct conversion', i.e. use metabolically engineered cyanobacteria that are able to convert CO<sub>2</sub> directly into a preferred end-product of a metabolic route (see e.g. Fig. 1; Angermayr et al. 2009, Chen et al. 2016a, 2016b). This approach obviates the need to first synthesize the complex biomolecules that form a living cell, followed by the conversion of this complex mixture of molecules into a preferred product. Significantly, many of the small-molecule target products (like ethanol, *iso*-butanol, lactic acid, geraniol, etc.), rapidly leak out of the production cells, accumulate in the extracellular medium (Savakis et al. 2016), and can be recovered from there. If not, a dedicated sugar transporter can be engineered into the cyanobacterial membranes (Ducat et al. 2012).

During the past 10 years this process of 'direct conversion' has been actively investigated, with the result that now, for a large array of products (>25) proof of principle has been provided that a cyanobacterial production strain can be generated (see e.g. Angermayr et al. 2015, Zhang et al. 2017). The array of compounds ranges from simple, liquid energy carriers, via bulk commodities and specialty chemicals, to food additives and flavor- and pharmaceutical compounds. However, the process from a proof of principle, all the way to an economically



**Fig. 1.** Schematic representation of a cyanobacterial cell factory for ethanol. The incorporation of a heterologous pyruvate decarboxylase (*pdc*) in tandem with an alcohol dehydrogenase II (*adhII*) yields ethanol by redirecting carbon flux away from native metabolic networks. The plasticity of cyanobacteria to introduce, or knock-out/knock-down genes, make them ideal platforms for photosynthesis-driven production of industrial commodities. Figure adapted from Chen et al. 2016a with permission.

competitive production process for a specific target compound, has many hurdles that will have to be overcome.

This contribution will focus on: (1) optimizing the (carbon) flux to a desired product; (2) increasing the genetic stability of production organisms and (3) maximizing the energy conversion efficiency of solar energy available to generate products of interest. Significant advances have been made on all three aspects during the past few years, and here we will discuss: (1) an increase in the carbon partitioning to >50% directly to product; (2) aligning product formation with the growth of the cells, to enhance genetic stability of the latter and (3) expanding the photosynthetically active radiation (PAR) region for oxygenic photosynthesis and increasing photoautotrophic growth rate. Some of the remaining challenges are addressed in the conclusion and perspectives section.

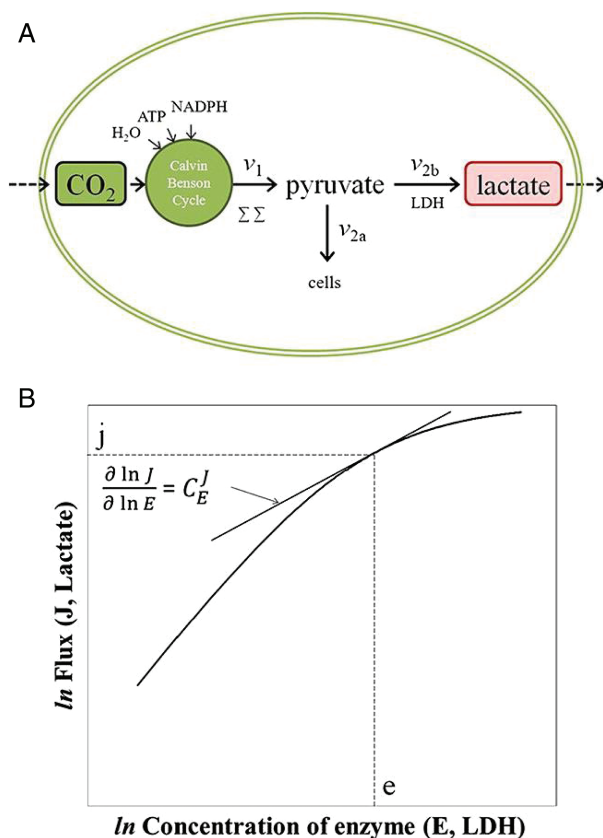
### Optimization of product formation

The endogenous photoautotrophic metabolism of cyanobacteria releases relatively few metabolic intermediates into the extracellular medium, and those that are released, are in low quantity. The only notable exceptions are pyruvate and  $\alpha$ -ketoglutarate, in mutant cells impaired in glycogen synthesis, when exposed to nitrogen limitation (Carrieri et al. 2012, Gründel et al. 2012). Therefore, for the remaining potential products of the approach of 'direct conversion', metabolic engineering is necessary to construct a 'cell factory' for

a specific target product. The simplest way to do this is to 'graft' a heterologous metabolic pathway on the cyanobacterial intermediary metabolism. Most often this will be a fermentation pathway, because these generally have very reduced multi-carbon compounds as their end-product. This grafting could in principle use any of the cyanobacterial metabolic intermediates, but preferably use one of its key intermediates like pyruvate,  $\alpha$ -ketoglutarate, acetyl-CoA or glucose-6-phosphate to aim for high metabolic rates (Angermayr et al. 2015, Zhang et al. 2017).

The next step is to identify one or several suitable enzyme(s) for the heterologous fermentation pathway. High  $k_{cat}$  values, dependency on NADPH rather than NADH as the source of protonated electrons, and insensitivity to inhibition by cyanobacterial metabolic intermediates are important search criteria for these enzymes. Hence, it is likely that a catabolic enzyme will be selected. After proper codon optimization, a procedure to optimize the expression level of the enzymes that form the heterologous pathway for optimal productivity of the cell factory for a target product can be started. This optimization procedure can be carried out with a procedure known as a 'sensitivity analysis', which is an approach well-known from such diverse fields as metabolic engineering, electronic circuit design and the financial sector (Westerhoff and van Dam 1987, Fell 1992). This procedure first identifies the bottle neck(s) in the rate at which a specific process can take place (i.e. in this case the rate of product formation), and secondly modulates the functioning of this bottle neck to the desired level. Full bottle neck functioning of a particular step from a complex system is indicated by unity slope of a log plot of the rate of the overall process, vs the system level of the catalyst of that process (Fig. 2). The slope of this plot ( $\Delta \ln(J)/\Delta \ln(E)$ ) is a quantitative indicator of the degree of the bottle neck function of the enzyme under study. This quantity is also referred to as a metabolic control coefficient.

This approach of carrying out a sensitivity analysis is best illustrated with a cyanobacterial cell factory for D- or L-lactic acid, because for these products it suffices to graft an intermediary metabolism with a fermentation pathway composed of only a single enzyme, a lactate dehydrogenase (LDH), on the metabolic intermediate pyruvate. In our initial work it was only possible to modulate the amount of the 'bottle neck enzyme', LDH, in the model cyanobacterium *Synechocystis* sp. PCC 6803 by varying the strength of the promoter driving its expression. Strain SAA023, carrying the strongest promoter available at the time ( $P_{trc}$ ), achieved a maximal activity in the cells of approximately  $0.5 \text{ mmol g}^{-1} \text{ dry weight (DW) h}^{-1}$  (Angermayr and Hellingwerf 2013),



**Fig. 2.** Sensitivity analysis, or quantitation of the bottle neck function of the LDH enzyme in a lactate producing cyanobacterial cell factory. An overview of metabolic control analysis. (A) Lactate production in cyanobacteria takes into account two separate sets of reactions: (1) reactions leading to the processing of carbon until the production of the metabolite pyruvate, and (2) the conversion of pyruvate to lactate. (B) Method for the calculation of the flux control coefficient of LDH production. The slope of this plot is a quantitative indicator of the degree of the bottle neck function of the LDH, i.e. the control coefficient. Figure retrieved from Angermayr and Hellingwerf (2013), with permission.

which is to be compared with a maximal flux of carbon through the calvin cycle of this organism of approximately  $4 \text{ mmol g}^{-1} \text{ DW h}^{-1}$ . Use of LDH promoters with lower activity led to a log-proportional lowering of the rate of production. Only in follow-up work, when it became possible to increase the gene doses in *ldh* mutants, did a significant decrease of the bottle neck function of this enzyme appear (Fig. 3A). If the logarithmic scales of Fig. 3A are considered, it can be inferred that it would take an unreasonably high amount of cellular resources to take all bottleneck function away from the LDH enzyme. The measured value of the slope of about 0.3 (equivalent to 30% of control) in strain SAW041 may actually be close to the optimal value. The  $v_{max}$  of LDH activity observed in this strain was  $267 \text{ mmol g}^{-1} \text{ DW h}^{-1}$ .

An independent confirmation of the role of LDH in determining the flux of carbon from CO<sub>2</sub> to lactic acid in these engineered *Synechocystis* strains came from follow-up work in which also a modulation of pyruvate kinase (PK) activity was included (Fig. 3). Overexpression of PK in strains in which all bottle neck function in lactic acid production was still in the LDH enzyme (in strains

SYW003 and SYW004) did not lead to a significant increase in the overall rate of lactic acid production. In strain SAW041, a derivative of SYW003 where PK overexpression is coupled to increased *ldh* gene dosage (i.e. LDH expressed in the chromosome and from an expression plasmid), however, it did, consistent with the conclusion that the bottle neck no longer rested on the function of the heterologous LDH in this strain. In this latter strain slightly more than 50% of the incoming CO<sub>2</sub> is directly channeled into the product lactic acid; hence the qualification of such a strain as a ‘cell factory for lactic acid’ (Angermayr et al. 2014). A similarly high carbon partitioning has been achieved for other products as well, be it often based on a more ‘trial and error’ approach. Examples are: sucrose (Ducat et al. 2012), ethanol (Gao et al. 2012, Savakis et al. 2013) and meso-butane-diol (Oliver et al. 2013). Very high rates of product formation can also be achieved by growing the cyanobacteria photoheterotrophically (Matson and Atsumi 2018). A key point to consider there is the economic value of growth substrate vs cell factory product. For other products bottleneck analyses have been carried out, like for instance for isoprene (Gao et al. 2016, Chaves and Melis 2018) and ethanol (Nishiguchi et al. 2019) which have improved the efficiency of the respective strains to product their intended products.

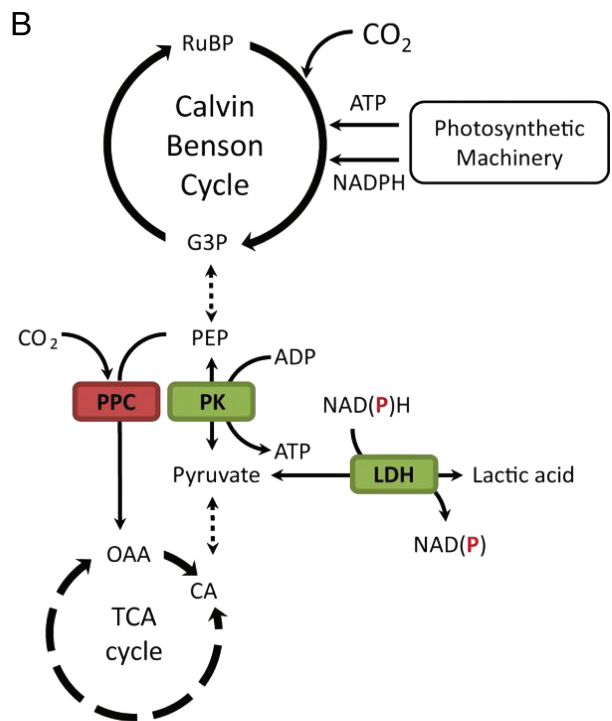
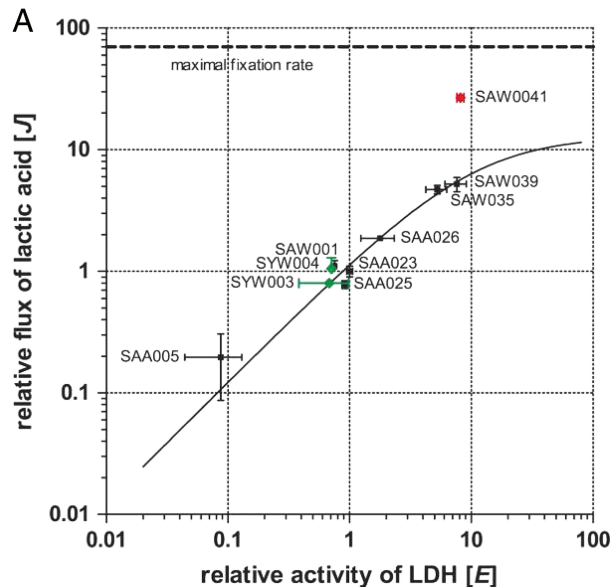


Fig. 3. Legend on next column.

### Consequence of the inherent instability of production strains with high carbon partitioning

The genetic instability of production of cyanobacterial strains engineered for high carbon partitioning has been

**Fig. 3.** Alleviating the bottleneck in a cell factory for lactic acid. (A) Sensitivity analysis for optimal productivity of lactate by fine-tuning expression of the LDH enzyme as the initial bottleneck in lactate production, via the implementation of promoters with variable expression strengths and increasing gene dosage. (B) Modulation of the pyruvate kinase (PK) and phosphoenolpyruvate carboxylase (PPC) to relieve bottleneck of LDH. For further explanation, see main text. *Synechocystis* sp. PCC 6803 strains expressing lactate dehydrogenase (*ldh*/LDH) and/or pyruvate kinase (*pk*) under various promoters (e.g. *PpsbA2*, *Ptrc1*, *Ptrc2*). *Ll*=*Lactococcus lactis* genes; *Ef*=*Enterococcus faecalis* genes; *Ec*=*Escherichia coli*; *Po*=*Plasmodium ovale*; *co*=codon optimized gene for *Synechocystis* sp. PCC 6803; *na*=native gene from organism; *I29R*=isoleucine 29 arginine; *pDF*=plasmid expression: SAA005 *PpsbA2::psbA2:: Ll ldh<sub>na</sub>*; SAA023 *Ptrc1:: Ll ldh<sub>co</sub>*; SAA025 *Ptrc1:: Ll ldh<sub>na</sub>*; SAA026 *Ptrc1:: Ll ldh<sub>co</sub>::ldh<sub>na</sub>*; SAW001 *Ptrc2:: Ll ldh<sub>co</sub>*; SAW035 *Ptrc1:: Po ldh<sub>co</sub> I29R*; SAW039 *Ptrc1:: Ll ldh<sub>co</sub>,pDF\_LDH*; SAW041 *Ptrc2:: Ef pk<sub>co</sub>:: Ptrc2:: Ll ldh<sub>co</sub>, pDF\_LDH*; SYW003 *Ptrc2:: Ef pk<sub>co</sub>:: Ptrc2:: Ll ldh<sub>co</sub>*; SYW004 *Ptrc2:: Ec pk<sub>co</sub>:: Ptrc2:: Ll ldh<sub>co</sub>*. CA, acetyl CoA; G3P, glyceraldehyde 3-phosphate; OAA, oxaloacetate; PEP, phosphoenolpyruvate; PK, pyruvate kinase; PPC, phosphoenolpyruvate carboxylase; RuBP, ribulose 1,5-bisphosphate; TCA, tricarboxylic acid cycle. Figure retrieved from Angermayr et al. (2014), with permission.

described as ‘an elephant in the room’, because it is ‘important, obvious, yet largely ignored’ (Jones 2014). Although scarce, it has been reported for all of the most common cyanobacterial species used in biotechnology: *Synechocystis* sp. PCC 6803 (Ungerer et al. 2012), *Synechococcus elongatus* PCC 7942 (Kusakabe et al. 2013) and *Synechococcus* sp. PCC 7002 (Jacobsen and Frigaard 2014). Furthermore, instability has been observed for different target products, whose synthesis pathways connect to the cyanobacterial metabolic network via different metabolites (Du et al. 2018a). An increased understanding of the burden experienced by cells when engineered to produce a large amount of a target compound is required in order to develop and validate alternative metabolic engineering strategies that will minimize the observed phenotypic instability.

Genetic analyses of mutants that lost the ability to form the product of interest, has thus far been limited to only a few studies (Takahama et al. 2003, Jones 2014, Cassier-Chauvat et al. 2016) that merely analyzed the inserted heterologous genes/operons and, at best, their flanking regions, ignoring the involvement of other loci that might be involved in product formation. Nevertheless, a vast array of mutations that severely impair the functionality of the production pathway have been described in these studies. These mutations can be point mutations, insertions or deletions, affecting enzyme active sites, or leading to the formation of a truncated gene product.

The mutant strains presumably experience the product formation as a burden, relative to their wild-type counterparts. The ‘burden of production’ can manifest itself at many different levels (Bachmann et al. 2017). For instance, the expression of the heterologous proteins could be competing with anabolic processes for RNA polymerases or ribosomes (de Jong et al. 2017). But there could also be competition for the availability of intracellular metabolites (Vickers et al. 2014, Bachmann et al. 2016), disturbance in the native redox balance between (e.g. NADPH, NADH; Zhao et al. 2017), as well as for cellular transport capacity (Zhuang et al. 2011). Or there could be a detrimental effect in cellular homeostasis caused by, for example the accumulation of a metabolite that is toxic to the producing cell (Nicolaou et al. 2010). Resolving the molecular basis of the apparent trade-off between biomass synthesis and product formation is key to the design of stable green cell factories.

Recently, we have tried to further disentangle the contributing factors for lactate production in *Synechocystis* sp. PCC 6803 (Du et al. 2016). We aimed at modulating the rate of lactate production without altering the level

of expression of the heterologous NADH-dependent LDH. This could be done by using a non-metabolizable allosteric activator of the *Lactococcus lactis* LDH, that is commonly used for lactate production in cyanobacteria. This strategy was successful in two respects because: (1) it resulted in a twofold increase in the rate of lactate production, and (2), and more importantly, it revealed the origin of the fitness burden. As the carbon flux through the cyanobacterium was increasingly directed towards product formation, while leaving unchanged the level of LDH expression, the number of non-producing mutants also increased. In other words, this may suggest that phenotypic instability correlates with redirecting carbon flux away from biomass, as evidenced in studies with lactic acid (Angermayr et al. 2012, Borirak et al. 2015) and ethanol (Jones 2014). At first sight this may look very discouraging for the field of ‘direct conversion’, because it most likely will hold true for other products and other production hosts as well. Nevertheless, there are strategies that could significantly lessen, or eliminate, this hurdle.

There are at least two diametrically opposed views on how to try to minimize the genetic instability challenge in production strains for target compounds (Du et al. 2018b). The root of the problem appears to be in the competition between biomass and product formation for metabolic precursors. One approach can be to completely uncouple the synthesis of the product from biomass formation, while the other approach may be the obligatory coupling of product formation to growth of the cells. The strategies are not necessarily mutually exclusive. They could be combined in a single production strain, as long as they are exploited at different times in the production process. The former necessitates the diversion of cellular resources away from biomass formation, therefore resulting in a decrease of the size of the pool of growth-related side products that are the crux of the latter production strategy. The ideal production strain would initially rely on growth-coupled production and later transition to the syphoning of cellular resources, once stationary phase is approached.

Growth-coupled production is not a new idea in biotechnology. It is a strategy widely deployed in Nature in homo-fermentative metabolism by e.g. lactic acid bacteria, acetogens, propionic bacteria etc. (Schlegel 1993). Genetic instability in such organisms is limited to the appearance of mutants that form multiple fermentation products. However, examples of growth-coupled production of heterologous products are much rare. Significantly, a recent *in silico* study of the metabolic network of *Escherichia coli* has shown that a large proportion of the intermediates of central carbon metabolism of this organism could in principle be produced in a

**Table 1.** List of native compounds that can be produced in *Synechocystis* in a growth-coupled fashion based on in silico simulations using FRUITS applied to model iJN678 allowing for a maximum of four gene deletions. Table adapted from Du et al. (2018a).

Native compound	Growth rate(h <sup>-1</sup> )	Yield(mmol g <sup>-1</sup> DW)	Gene deletions required
5-Methylthioadenosine	0.052	0.007	sll0135
Acetate	0.052	0.195	sll0542, sll1299
Mercaptopyruvate	0.034	5.702	sll1027 or sll1502, slr0710, sll1499
5'-Deoxyadenosine	0.052	0.044	sll1185
3,4-Dihydroxy-2-butanone 4-phosphate	0.051	0.732	sll0753, sll0330, sll1556
Adenine	0.052	0.032	sll1430
Adenosine	0.052	0.032	sll1430
S-Adenosyl-L-homocysteine	0.052	0.025	sll1758
Fumarate	0.051	0.848	slr0018
	0.044	3.162	slr0018, slr0458, sll1349
	0.043	3.509	slr0018, slr0458, sll1349, slr1755

growth-coupled model (Klamt and Mahadevan 2015). In cyanobacteria, photoautotrophic organisms, the first attempt to design a strain with growth-coupled production was an in silico study which remains to be validated experimentally (Erdrich et al. 2014). Nevertheless, this study showed that in principle, the product formation can be coupled to growth if it is the last remaining redox sink. These strategies in autotrophs and heterotrophs have in common that they rely on coupling energy generation and/or redox regeneration to product formation. In cyanobacteria, this means that a lot of the intrinsic plasticity of the photosynthetic apparatus is lost, which could impair the robustness of production strains in industrial settings (Nogales et al. 2012, Branco dos Santos et al. 2014). In heterotrophs this is not an issue, because growth-coupled production is linked to catabolic processes.

We recently developed an alternative concept to achieve the coupling of product formation to growth in photoautotrophs (Du et al. 2018b). During growth, anabolic pathways that generate biomass constituents, often release a compound that can be viewed as a side-product of metabolism at a key metabolic step. As life most often evolves in a resource limited environment, Darwinian selection has specialized other enzymes to channel such side products ('spilt carbon') back into intermediary metabolism, so that the fitness of the organism is optimized (Reference). This strict wiring of metabolic networks is most explicitly present in photoautotrophs, which are notorious for not spilling any products unnecessarily (Branco dos Santos et al. 2014). We hypothesized, that if these side-products could be identified, and the reactions that are being used to re-assimilate them can be deleted, we then could design growth-coupled production strains for these compounds. The question is how to identify the pathways that need to be deleted?

For this identification, an algorithm to Find Reactions Usable In Tapping Side-products (FRUITS) has been developed to 'pick' and screen a metabolic network without disturbing the 'tree' and the ability to grow (Du et al. 2018a). In essence, the algorithm takes a genome-scale metabolic model of any desired production host, constrained to mimic any desired production condition, and produces a list of all compounds of which the formation can be stoichiometrically and obligatorily coupled to growth of the cells. Furthermore, it makes quantitative predictions regarding the minimum production rate achievable, by using a maximization of biomass formation as the objective function, from which production-related kinetic parameters, such as carbon partitioning and biomass-specific production rates (Q<sub>p</sub>), can be deduced.

FRUITS has been applied to *Synechocystis* sp. PCC 6803 cultured under photoautotrophic conditions, which resulted in a list of nine different products (Table 1) of which the formation can be coupled to growth by deleting their re-assimilation pathway(s), limited to a maximum of four gene deletions within the pathway. Acetate was chosen as a target compound to validate this approach experimentally. It is important to note that through this metabolic engineering strategy, acetate production in *Synechocystis* sp. PCC 6803 is a side-product of anabolism, and not an end-product of catabolism, as in many fermentative organisms. In this study, we not only constructed the first photoautotrophic strain with growth-coupled production, but more significantly, we could also show that this strategy indeed leads to a stable production of acetate. The latter aspect was demonstrated by the continuous cultivation of the production strain at its maximal specific growth rate, in photobioreactors operated in the turbidostat mode and with a strong selection against product formation (Bryson and Szybalski 1952).

## Minimizing the consequences of the inherent genetic instability

### General considerations

In the previous section we have seen that highly productive ‘cell factories’ are subjected to strong negative Darwinian selection, and thus will ultimately be outcompeted by mutant strains with lower productivity (Angermayr et al. 2012, Jones 2014). To circumvent this outcome, a variety of alternative strategies are being explored to minimize the consequences of this inherent cyanobacterial genetic instability. The best known of these are: (1) employing inducible, growth-phase dependent genetic systems (Yao et al. 2016); (2) creating an obligatory coupling of product formation to growth of the cells (Feist et al. 2010); (3) making use of ‘overflow metabolism’ (Cano et al. 2018); (4) targeted repression of essential genes (Yao et al. 2016) and (5) creating ‘synthetic addiction’ (Rugbjerg et al. 2018).

The last three of these approaches have in common that the production phase is characterized by a very low growth rate. This reduces the competitive disadvantage of the extreme resource allocation towards the product in the ‘cell factories’, relative to spontaneous non-producing mutants. In chemotrophic bacteria, low growth rates ( $\mu$ ) are often paralleled by low rates of product formation, because the lower  $\mu$  is often a consequence of a lower thermodynamic driving force of the catabolic reactions. For the cyanobacteria this should not be a problem, because as long as a sufficient flux of photons is available, an ample amount of NADPH and ATP will be provided by the reactions taking place around the thylakoid membranes.

Nevertheless, even in the best cell factories, the common observation is that in batch cultures, in the stationary phase in conditions in which still an abundant amount of light and CO<sub>2</sub> is available to the cells, product formation stops rather rapidly (Lips et al. 2018). The mechanistic explanation for this observation is not known at the moment, but it is likely that this is because of a regulatory down-shift of metabolism. The molecular basis of this down-shift remains to be elucidated.

### Overflow metabolism

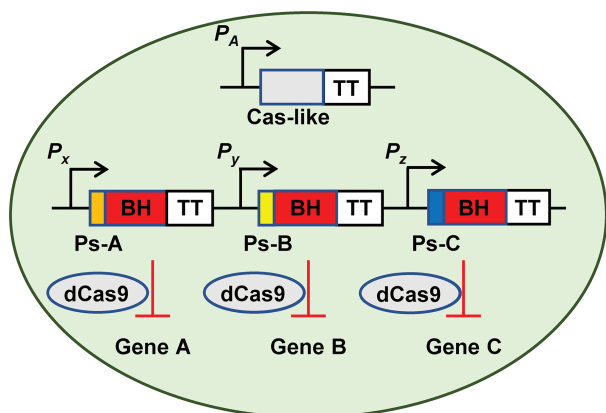
The term ‘overflow metabolism’ was coined for a type of metabolism displayed by chemo-heterotrophic bacteria when these organisms were subjected to specific nutrient limitations. Such a limitation reduces growth rate, but the corresponding rate of intermediary metabolism depends strongly on the nature of the limiting nutrient. It is relevant to explore whether overflow metabolism can also be used in cyanobacterial

cell factories to minimize the fitness burden during product formation. A well-known example of overflow metabolism in cyanobacteria occurs during glycogen storage, in a conserved part of the interconnected carbon-, nitrogen- and photosynthetic metabolic network (Gründel et al. 2012, Jackson et al. 2015, Cano et al. 2018). With excess light and CO<sub>2</sub>, available carbon is preferentially channeled into this carbon storage polymer. However, in glycogen-deficient mutants ( $\Delta$ glgC), cells convert excess carbon into organic acids such as pyruvate and  $\alpha$ -ketoglutarate which are then secreted out of, or leaking from the cells (Carrieri et al. 2015). Besides carbon and other energy sinks such as glycogen and poly-hydroxy-butyric acid (Carpine et al. 2017), cyanobacteria also contain nitrogen and phosphorus sinks in the form of cyanophycin and phycobiliproteins, and polyphosphate, respectively (Kromkamp 1987). However, overflow metabolism based on limitation of these latter nutrients, nor of the minerals required for growth of cyanobacteria, such as K<sup>+</sup>, Mg<sup>++</sup>, etc., have not been reported yet.

### Use of multiplex CRISPRi/dCas9 for commodity production with increased genetic stability

As explained above, application of the FRUITS algorithm may require the introduction of many mutations in a specific production strain. Traditional methods for generating cyanobacterial production strains are labor-intensive and time-consuming; their construction often requires multiple genetic manipulations via natural transformation, electroporation and/or conjugation, e.g. knock-ins, knock-outs, knock-downs etc. (Ruffing et al. 2016, Carroll et al. 2018). In recent years the CRISPR/Cas (Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR Associated Nuclease) system has revolutionized gene editing in eukaryotes and prokaryotes (Hsu et al. 2014). Its application in cyanobacteria soon followed suit, with knock-down and knock-out systems developed for relevant cyanobacterial hosts such as *Synechococcus* sp. PCC 7002 (Gordon et al. 2016), *Synechococcus elongatus* PCC 7942 (Huang et al. 2016), and the to date fastest-growing cyanobacterium *S. elongatus* UTEX 2973 (Wendt et al. 2016). The power of the CRISPR-Cas system could be exacerbated by its coupling to tight regulatory, and reversible, expression platforms that can discard production during phases that are detrimental to growth – and then be switched back on. Most importantly, CRISPR/Cas is not limited to a single target thanks to the small size (approximately 20 bp) required of the protospacer in the guiding RNA that hybridizes with the target DNA. This allows multiple protospacers to be arrayed with ease





**Fig. 4.** Overview of the CRISPR-Cas Knock-Out/ Knock-Down Approach in prokaryotes. CRISPR-Cas systems allow for the multiplex manipulation of genes including knock-outs, knock-downs, the upregulation of genes etc. The CRISPRi/dCas9 was the first multiplex system used in cyanobacteria. BH, Cas9/dCas9 binding handle; dCas9 D10A/H840A Cas9; P<sub>n</sub> (n = A, X, Y or Z), promoter; Ps-N (N = A, B or C), Protospacer; TT, transcriptional terminator. The green oval represents a cyanobacterial cell.

in a single expression vector, and accordingly modify multiple genes in one engineering step (Fig. 4).

Multiplex CRISPR/Cas9 technology significantly reduces the time required to alter multiple genes in a metabolic pathway. Yao et al. (2016) reported the effectiveness of CRISPR/Cas9 in *Synechocystis* sp. PCC 6803 to silence genes necessary to produce the carbon storage compounds polyhydroxybutyrate. These authors also demonstrated the first example of multiplexed repression of various aldehyde reductases and dehydrogenases in a cyanobacterium. This was achieved by using the CRISPR interference system (CRISPRi), that contains a nuclease-deficient D10A/H840A Cas9 variant (dCas9), which is capable of silencing the expression of the targeted gene by preventing RNA polymerase binding or RNA elongation (Qi et al. 2013).

Multiplex CRISPRi/dCas9 provides an opportunity to tune the expression of multiple gene targets to globally affect one or more metabolic pathways during discrete periods, if coupled to a tight titratable expression system. In the example of Yao et al. (2016), four putative aldehyde reductases and dehydrogenases from the genome of *Synechocystis* sp. PCC 6803 were targeted (slr0942, slr0990, slr0091 and slr1192). Their reading frames were verified in silico to contain an NADPH-binding motif and share homology with known reductases/dehydrogenases from cyanobacteria and chemotrophic bacteria. The results of this report lead to the conclusion that Multiplex CRISPR/Cas9 or CRISPRi/dCas9 can be achieved by tandem organization of multiple single guide RNAs (sgRNA), containing their own promoter (a Cas9/dCas9

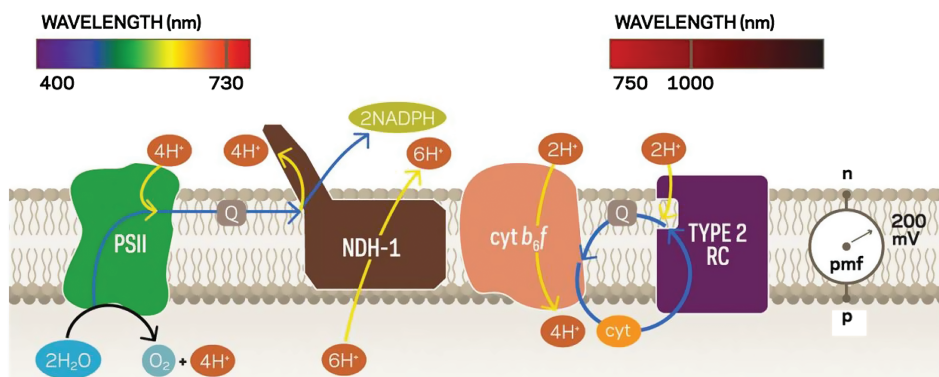
binding handle), a protospacer and a transcriptional terminator. A lower expression level of the corresponding enzymes should generate a larger aldehyde pool, that could lead to increased production of hydrocarbons (i.e. alkanes, via the decarbonylation of fatty acids; Rodriguez and Atsumi 2014). mRNA quantification after constitutive repression of the aforementioned aldehyde reductases with CRISPRi/dCas9 yielded a greater than 10-fold repression of all targets, with the exception of slr0091 which only exhibited twofold repression. The latter was attributed to inefficient abrogation of RNA polymerase elongation, because of an unknown promoter; CRISPRi/dCas9-directed repression is less effective the greater the distance is between the sgRNA and the site of initiation of transcription (Yao et al. 2016).

### Improving the 'chassis strain'

#### Maximizing the efficiency of the conversion of solar energy

The most basic way to increase the efficiency of the conversion of solar energy into bioproducts is to expand the range of the spectrum of electromagnetic radiation that can be used for oxygenic photosynthesis. This can be achieved by expanding this spectrum into the far-red- or even infrared region, as oxygenic photosynthesis as we know it, is largely limited to the use of photons with a wavelength ranging from 350 to 700 nm, the so-called PAR. The PAR contains only about half of the total number of available photons from the sun that reach the surface of the earth (Blankenship et al. 2011). It is relevant to note that some cyanobacteria exist that do function with light of wavelengths >700 nm, making use of chlorophyll d (Chl *d*; Manning and Strain 1943, Ains et al. 2014, Gan et al. 2014) and Chl *f* (Chen et al. 2010), which capture photons in the range of 700–720 and 700–740 nm, respectively. Significantly, for bacteriochlorophyll (Scheer 2012), variants are known that utilize light with a wavelength of up to 1100 nm. Interestingly, even photons of the latter wavelength have enough energy to drive photophosphorylation and phototrophic growth.

For the Chl *d*-utilizing cyanobacterium *Acaryochloris marina*, it has been shown that it can indeed display a higher photosynthetic efficiency than a comparable Chl *a*-utilizing cyanobacterium (Gloag et al. 2007, Chen and Blankenship 2011). Furthermore, a recent publication reported the successful heterologous synthesis of Chl *f* in the model cyanobacterium *Synechococcus* sp. PCC 7002 (Ho et al. 2016). However, the low level of production of the exogenous chlorophyll presumably prevented the emergence of a corresponding phenotypic trait in energy metabolism.



**Fig. 5.** Membrane-embedded light-driven electron transfer systems in the thylakoid membrane of an engineered oxyphototroph with an extended region of photo-synthetically active radiation. Photosystem II (PSII) transfers electrons from water, via the quinone pool, to reduce NADP<sup>+</sup>. The BChl *b*-containing type 2 reaction center (RC) uses light up to 1100 nm and functions with the cytochrome (cyt) *b<sub>6</sub>f* complex to generate a proton-motive force (PMF) via a cyclic electron transfer. Figure retrieved from Ort et al. (2015), with permission.

In a landmark paper on photosynthetic efficiency (Blankenship et al. 2011), a comparison of ‘artificial’ and natural photosynthesis was made. Together with a review of options to increase the efficiency of light energy conversion in oxygenic photosynthesis, the use of the entire solar spectrum is proposed, through exploiting cyanobacteria that are engineered to express bacteriochlorophyll-based reaction centers. By lowering the free energy gap between photosystems, the entire solar emission spectrum reaching the earth could then be used effectively. However, this approach has the disadvantage that it strongly reduces the amount of free energy available to drive electron transfer between the two photosystems, and hence may impair linear electron flow.

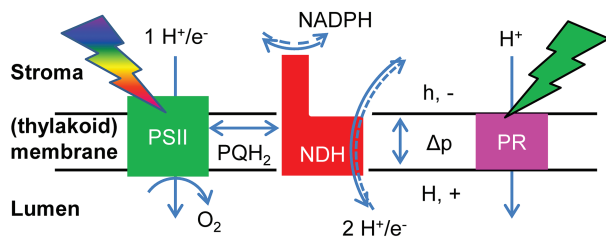
In a recent update of this discussion paper (Ort et al. 2015) it is proposed to cut electron transfer through the Z-scheme into two halves, which then should lead to: (1) linear electron flow via Photosystem II (PSII) plus NDH-1 for water-driven reduction of NADPH, and (2) replacement of Photosystem I (PSI) by an infra-red absorbing system for proton pumping, similar to a cyclic electron transfer chain as it exists in purple-non-sulfur bacteria (Fig. 5). Such engineering may lead to the exploitation of the complete solar radiation spectrum for oxygenic photosynthesis. However, it may be demanding, even though ‘pink plasmids’ exist, which contain one or more super-operons, encoding all components necessary for such a cyclic electron transfer system (Bauer et al. 1991). Among those are the ones that only function in the presence of oxygen (Petersen et al. 2012).

A simplification of this approach is to express a proteorhodopsin (PR) that absorbs light with a wavelength beyond 700 nm in a PSI deletion strain. Such a near-infra-red light driven retinal-based proton pump can also drive proton translocation at physiological levels of the proton motive force, even more so because

bacterial rhodopsins pump only a single proton per photon (Govorunova et al. 2017). Purple bacteria like *Rhodospseudomonas viridis* on the other hand, absorb photons with a wavelength close to 1000 nm and provide the organism with enough free energy to translocate two protons over their cytoplasmic membrane per photon absorbed (Deisenhofer and Michel 1989).

In addition to that, far-red absorbing variants of Proteorhodopsin have been described, that have retained considerable capacity of proton pumping (Ganapathy et al. 2017). In parallel, it was shown that expression of a PR in *Synechocystis* sp. PCC 6803 does contribute to harvesting light energy for the host organism, as evidenced by an increased growth rate (Chen et al. 2016a, 2016b). To demonstrate this more clearly, we expressed Proteorhodopsin also in a PSI-deletion strain of the organism (Chen 2017). Indeed, in this background the stimulatory effect of energy harvesting by the retina-based proton pump can clearly be demonstrated. However, this stimulatory effect could only be observed under conditions that would allow photoheterotrophic growth of the organism, and not when the growth medium would only allow photoautotrophic growth. The latter holds for a PR absorbing in the visible light, as well as for variants absorbing in the far-red part of the visible spectrum.

Of note is the fact that for photo-autotrophic growth, the splitting of the linear electron-transfer system of oxygenic photosynthesis will require the functioning of an alternative linear electron transfer chain to form NADPH with electrons liberated from water, composed of PSII and NDH-1 only (Ort et al. 2015). This will require considerable activity of NDH in the direction of NADP<sup>+</sup> reduction, driven by the free energy of the proton gradient across the thylakoid membrane (Fig. 6). This activity is well known from NADH dehydrogenases from purple bacteria (as ‘reversed electron transfer’). A possible explanation for the inability to observe stimulation of



**Fig. 6.** Schematic representation of a strain with an expanded PAR region via the use of a retinal-based photosynthesis. Solid arrows indicate preferential direction of electron/proton transfer. Dotted lines represent alternative directions for electron/proton transfer. In a photosystem I (PSI) deletion background strain, far-red absorbing variants of proteorhodopsins (PR) that retain significant capacity for proton pumping could be employed to increase photosynthetic efficiency by expanding the range of wavelengths are absorbed. Concomitantly, linear electron flow would be maintained through use of PSII and NDH for reduction of NADPH. For further explanation, see main text.

photoautotrophic growth by light absorbed via a PR, may be in the inability of NDH-1 of *Synechocystis* to carry out this reaction at a sufficient rate.

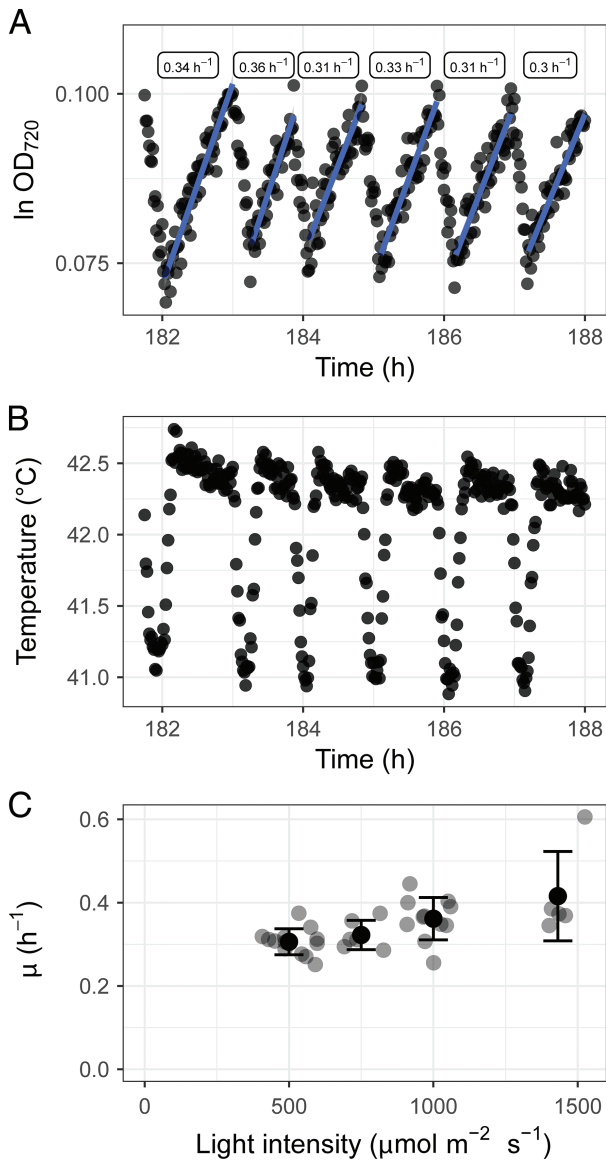
In addition to expanding the spectrum of electromagnetic radiation that can be used for oxygenic photosynthesis, it has been demonstrated that the concept of a truncated light-harvesting antenna (TLA) can improve photosynthetic productivity under conditions of high irradiance and cell densities. Although initially controversial as a concept, because of the apparent decrease in biomass correlating with a truncated light-harvesting antenna (Page et al. 2012, Liberton et al. 2013), multiple studies have now confirmed this concept (Shin et al. 2016, Kirst et al. 2017). Kirst et al. (2014) developed a phycocyanin-less and shorter phycobilisome antenna in *Synechocystis* sp. PCC 6803, which is capable of roughly 57% higher biomass productivity than the wild-type strain in conditions emulating bright sunlight (approximately 2000  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) and densities of 0.5  $\text{g l}^{-1}$ . TLA stipulates the decrease of wasting excitation energy via non-photosynthetic quenching, by generating cells unable to ‘over-absorb’ electromagnetic radiation at a rate that would normally exceed the rate of photosynthesis. These cultures enable higher transmittance of electromagnetic radiation to more cells within a high-density culture thus increasing photosynthetic productivity. In microalgae, the effects of TLA have been observed by both the truncation of phycobilisomal structures (Nakajima and Ueda 1999) and Chl antenna size in green algae (Polle et al. 2003). Although TLA does have disadvantages for low to ‘standard’ light conditions of growth, often employed for wild-type strains (Page et al. 2012, Liberton et al. 2013), in industry it could prove beneficial to enhance the sunlight to biomass/product energy conversion.

### On the limits of the photoautotrophic growth rate

Sustainability applications with cyanobacteria generally suffer from a comparatively slow growth rate of the production organism, as compared to classical chemoheterotrophic cell factories such as *E. coli* and *Saccharomyces cerevisiae* (Oliver and Atsumi 2014). Therefore, an exciting development is the characterization of *Synechococcus elongatus* UTEX 2973 (Yu et al. 2015), which appears to have a superior growth rate as compared to many other cyanobacteria. In a recent publication the authors reported its maximal growth rate to be 0.46  $\text{h}^{-1}$ , quoted as a doubling time of 1.5 h (Ungerer et al. 2018). Unfortunately, these latter numbers are a bit ambiguous because the experiment from which they were derived, is described to be performed at 42°C in the text of the paper and at 38°C in the legend of the relevant figure (Fig. 1C). Furthermore, the characterization was performed with a PSI MC-1000 multicultivator, without a clearly defined light source, beyond that ‘cool white’ LEDs were employed.

Maximal growth rate in cyanobacteria is dependent on the optimal distribution of resources over the various cellular functions, like observed in all other living organisms as well. As a first approximation it increases hyperbolically with light intensity. However, this stimulatory effect by actinic illumination is counteracted by a series of complicated damage-causing processes, jointly referred to as ‘photoinhibition’. These latter processes can often be approximated with a quadratic dependence on light intensity. The ‘efficiency’ of this photoinhibition process is dependent on the wavelength of the actinic light: the longer the wavelength, the higher the intensity at which half-maximal photoinhibition occurs. As a result, the light-intensity dependence of the growth rate of cyanobacteria generally has a parabolic shape.

The balance between photosynthesis and photoinhibition may also give rise to the observation that a higher maximal growth rate can be rather achieved with near-red- than with white light. For the model cyanobacterium *Synechocystis* sp. PCC 6803 the optimal wavelength of the actinic light for maximal growth rate was 635 nm (van Alphen et al. 2018). We therefore wanted to test light-intensity dependence of the growth rate of *Synechococcus elongatus* UTEX 2973 with LEDs emitting maximally at 635 nm, to test whether the 0.46  $\text{h}^{-1}$  really represents the maximal growth rate of this organism under true steady state conditions. For this, we selected turbidostat conditions in BG-11 medium at 42°C and low cell density ( $\text{OD}_{730} < 0.1$ ; Materials and methods in Appendix S1, Supporting Information). The initial results show that up to an incident actinic intensity of



**Fig. 7.** Fast growth of *Synechococcus elongatus* UTEX 2973 in turbidostat culture. (A) Time-dependent  $OD_{730}$  dynamics in a PSI-100 turbidostat, run at 42°C. (B) Temperature dynamics measured inside the turbidostat vessel. (C) Variation of growth rate with the incident intensity of 635 nm LED illumination, derived from linear-log fits of the  $OD_{730}$  [see insets in (A)].

1400  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (the highest intensity achievable in the current set-up) growth rate of the organism increases with light intensity up to values higher than 0.4  $\text{h}^{-1}$  (Fig. 7).

These results make it clear that it still remains to be decided what the maximal growth rate of *Synechococcus elongatus* UTEX 2973 is. For this, conditions will have to be further optimized, not only with respect to the illumination regime, especially the light intensity, but

also with regard to the temperature control, because the turbidostat regime requires a more stringent temperature control (Fig. 7C).

## Conclusions

Two decades ago, the first report of genetic engineering to obtain a cyanobacterial cell factory was published in the scientific literature (Deng and Coleman 1999). It took about one decade before this approach really took off, but since then, this field has developed remarkably quickly, producing proof-of-principles for a wide range of compounds, and significant progress towards its scale-up (Angermayr et al. 2015). This increased interest in cyanobacteria has shed light on, and increased our understanding of many aspects regarding their molecular physiology and genetics, which may begin to unveil more general principles of biology (Klotz et al. 2016). A better fundamental understanding of cyanobacteria is of paramount importance to devise optimized metabolic engineering strategies (Branco dos Santos et al. 2014). Very recent contributions to the field on understanding resource re-allocation and sensitivity analysis of central metabolism during photoautotrophic growth may prove to be very useful in future engineering efforts (Jahn et al. 2018, Janasch et al. 2018). It may turn out to be essential to better take into account the cyanobacterial physiological flexibility with respect to the ratio of the rate of ATP over NADPH production in such modeling studies.

Additionally, future developments should center on the identification of suitable titratable and reversible expression systems, adequate for each cyanobacterial model organism that can modulate the Cas, or Cas-like nuclease and the sgRNA arrays (Ungerer and Pakrasi 2016). Multiplex CRISPR/Cas9 and CRISPRi/dCas9 provide avenues for knock-out and knock-down of multiple genes in a single experiment, respectively, and could also be modified to include knock-ins and single point mutations (Behler et al. 2018). To our knowledge, there is no available work in cyanobacteria that has employed the CRISPR-Cas technology to upregulate gene expression by coupling an RNA polymerase activating element, or other enhancers of expression, to the dCas9 enzyme (Dong et al. 2018). Moreover, a multiplex system based on the CRISPR-Cas technology could be engineered for cyanobacteria in which each sgRNA can have a discrete purpose (e.g. markerless knock-in, knock-out etc.) to globally fine tune, or re-program a metabolic pathway towards the optimal production of a commodity.

Considering the rapid progress of the field and its current heading, we expect that 'direct conversion' of  $\text{CO}_2$  by cyanobacteria will increasingly become an important

alternative to counteract one of the major global challenges of our time: the use of fossil carbon.

## Author contributions

This review was written on the template of the lecture presented by K.J.H. at ePS-1 in Uppsala (2018). The results presented in Fig. 7 were obtained by A.A.P. and H.P.H. All authors contributed to the writing, with major contributions from A.A.P., F.B.d.S. and K.J.H. All authors commented on and approved the manuscript.

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## Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Appendix S1.** Materials and methods pertaining to growth of *Synechococcus elongatus* UTEX 2973 in turbidostat mode.