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Review article

CyanoFactory, a European consortium to develop technologies needed to advance cyanobacteria as chassis for production of chemicals and fuels

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ABSTRACT

CyanoFactory, *Design, construction and demonstration of solar biofuel production using novel (photo)synthetic cell factories*, was an R&D project developed in response to the European Commission FP7-ENERGY-2012-1 call "Future Emerging Technologies" and the need for significant advances in both new science and technologies to convert solar energy into a fuel. CyanoFactory was an example of "purpose driven" research and development with identified scientific goals and creation of new technologies. The present overview highlights significant outcomes of the project, three years after its successful completion.

The scientific progress of CyanoFactory involved: (i) development of a ToolBox for cyanobacterial synthetic biology; (ii) construction of DataWarehouse/Bioinformatics web-based capacities and functions; (iii) improvement of chassis growth, functionality and robustness; (iv) introduction of custom designed genetic constructs into cyanobacteria, (v) improvement of photosynthetic efficiency towards hydrogen production; (vi) biosafety mechanisms; (vii) analyses of the designed cyanobacterial cells to identify bottlenecks with suggestions on

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further improvements; (viii) metabolic modelling of engineered cells; (ix) development of an efficient laboratory scale photobioreactor unit; and (x) the assembly and experimental performance assessment of a larger (1350 L) outdoor flat panel photobioreactor system during two seasons.

CyanoFactory - Custom design and purpose construction of microbial cells for the production of desired products using synthetic biology – aimed to go beyond conventional paths to pursue innovative and high impact goals. CyanoFactory brought together ten leading European partners (universities, research organizations and enterprises) with a common goal – to develop the future technologies in *Synthetic biology* and *Advanced photobioreactors*.

1. Introduction

CyanoFactory was a European research consortium with a global concept, whose aim was to custom design, purpose construct and use engineered cyanobacterial cells for the production of a product. It involved highly cross-disciplinary complimentary competences spanning many scientific disciplines, and connected academia, engineering, entrepreneurship and industry. The background was a growing interest to engineer cyanobacteria to produce chemicals, including fuels, in direct processes [1]. This overview presents and discusses achievements in the European CyanoFactory Consortium three years after its successful completion.

2. ToolBox for cyanobacterial synthetic biology - reliable transcription and translation of heterologous genes in cyanobacteria

The rapid advancement in molecular biology during the last decades, together with the drastic drop in cost of DNA sequencing and synthesis, have allowed the development of advanced and efficient tools for engineering and customization of microbial genomes. Most astonishing are the advancements in genetic engineering of the most common model organisms, like *Escherichia coli* (*E. coli*), where libraries of well characterized genetic devices and parts enable quick and reliable construction of recombinant strains with desired properties. In cyanobacteria, the knowledge and availability of genetic tools and standardized biological parts are far behind in comparison to traditional model organisms. In recent years, advancements in this research field have started to enhance our ability to modify and engineer cyanobacterial genomes in a predictable and reliable way.

A key feature for successful modification or redesign of any cellular machinery or function in any biological system is the tuning of expression level of a given gene. Much attention was, therefore, given to the characterization and development of promoter regions, and an increasing number of promoters of different strengths, both constitutive and inducible, are available for the more commonly used cyanobacterial strains. Up to this date, the larger part of heterologous expressions attempts in cyanobacteria have used endogenous promoters. A number of interesting chemicals have been successfully produced in cyanobacteria, using overexpression from these natively occurring promoters [2]. Due to their driving of high transcription levels, promoter regions related to light harvesting complex and photosystems, such as *Pcpc* and *PpsbA*, have been widely used. Improved variants of the *Pcpc* in *Synechocystis* PCC 6803 (henceforth *Synechocystis* 6803) have been shown to enable very high yields of heterologously expressed proteins [3,4]. Although often resulting in high levels of expression, the light regulated nature of these promoters can make them unsuitable for usage under differing and low light conditions.

To address the lack of well characterized promoters with more modest expression levels, a library of constitutive promoters based on *Pcpc* from *Synechocystis* 6803 was developed for the closely related *Synechococcus* PCC 7002 [5]. By truncation and randomized mutagenesis, a wide variety of promoters with different strengths were developed and shown to span three orders of magnitude in expression levels

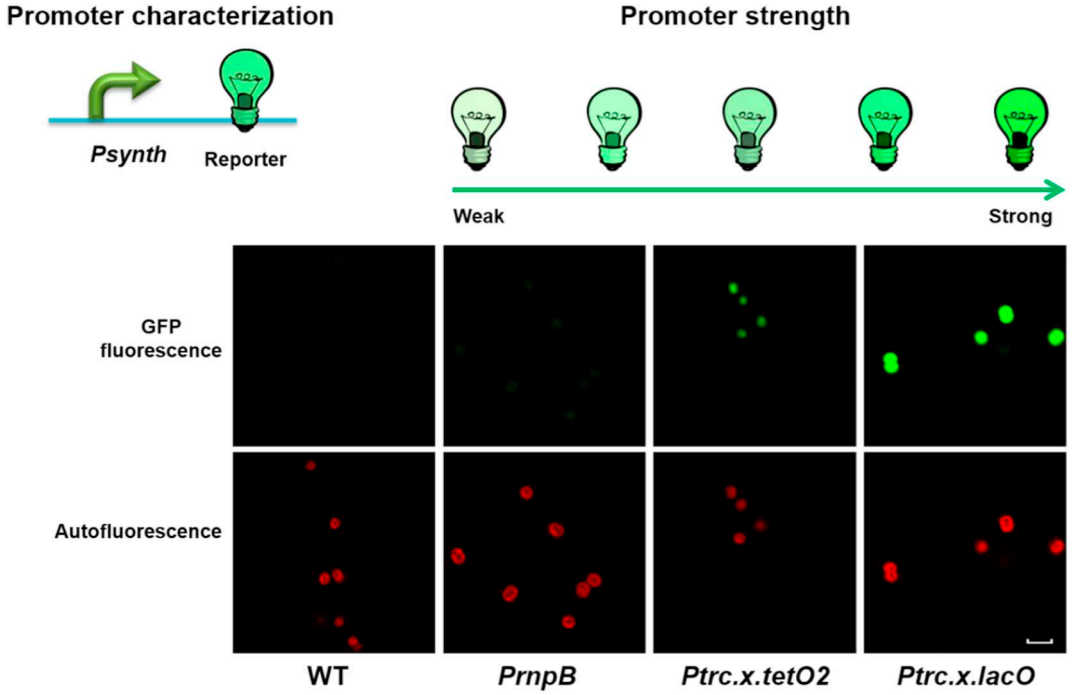

Fig. 1. Confocal micrographs of *Synechocystis* wild-type (WT) cells and *Synechocystis* mutant cells harboring the *PrnpB*, *Ptrc.x.tetO2* or *Ptrc.x.lacO* promoters assembled with a GFP generator. GFP fluorescence is depicted in the top row and the autofluorescence is depicted in the bottom row. Scale bar, 5 µm.

using a yellow fluorescent protein (YFP) reporter. Moreover, the developed promoters are reported to be decoupled from the light regulation that hampers the original promoter sequence.

Other endogenous promoters used for synthetic biology approaches in cyanobacteria include a number of micronutrient and metal induced promoters. These promoters are favorable in many applications due to their inducible nature and wide dynamic range. Apart from endogenous promoters, modified inducible promoter systems from *E. coli* have been successfully introduced and employed in cyanobacteria. Although well studied inducible promoters, such as *Plac* and *Ptet* from *E. coli* have proven to function poorly in cyanobacteria [6], later efforts to adapt non-native induction systems to cyanobacterial hosts have yielded promising results. A TetR-regulated promoter system has been developed for cyanobacteria, displaying a wide induction range [3]. Efforts also have been made to introduce isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible transcription initiation systems. Two lac operated promoters, *PtrcO1* and *PtrcO2*, were introduced and tested in *Synechocystis* 6803 [6]. Both exhibited high activity in the cyanobacterial host, although *Ptrc* was not well repressed by LacI, and *PtrcO2* could not be effectively induced by IPTG. By using a combination of the endogenous *Pcpc* from *Synechocystis* 6803 and *E. coli* promoter libraries, Markley et al. developed a library of IPTG inducible promoters in *Synechococcus* PCC 7002, where the top performer exhibited a 48-fold increase in expression level of the reporter gene upon induction [5]. In addition, a set of synthetic promoters whose design was based on the well characterized *PpsbA2*, *Ptrc*, *PtacI* and *PT7* (some including operator sequences for regulatory proteins) were developed. For the characterization of the promoters in *Synechocystis* PCC 6803, the devices were assembled to include a *gfp* reporter, and the *Synechocystis* 6803 *PrnpB* was used as a reference [6]. The results showed a range of functional synthetic promoters with strengths varying from 0.13- to 41-fold compared to *PrnpB* [7]. Fig. 1 shows confocal micrographs of *Synechocystis* mutants exhibiting differential *gfp* expression depending on the promoter used. These regulatory elements can be used for the construction of synthetic devices/circuits for introduction into the photoautotrophic chassis.

A predictable and tunable initiation of transcription is not the only component of well-controlled gene expression. The initiation of translation via the attachment of the ribosome to the ribosome binding site (RBS) of the mRNA is also key in expression regulation. Most examples of heterologous gene expression in cyanobacteria have reported the usage of native RBS sequences, commonly the one associated with the promoter used for expression. In 2011, Heidorn et al. developed RBS* for synthetic biology approaches in cyanobacteria, which widely outperformed three commonly used RBSs in terms of expression level of a reporter gene in *Synechocystis* 6803 [8]. Also, an 11-member RBS library with a 30-fold range in expression levels has been developed for and analyzed in *Synechococcus* PCC 7002 [5].

The fact that quantitative expression levels from a given transcription and translation initiation unit can differ greatly depending on the

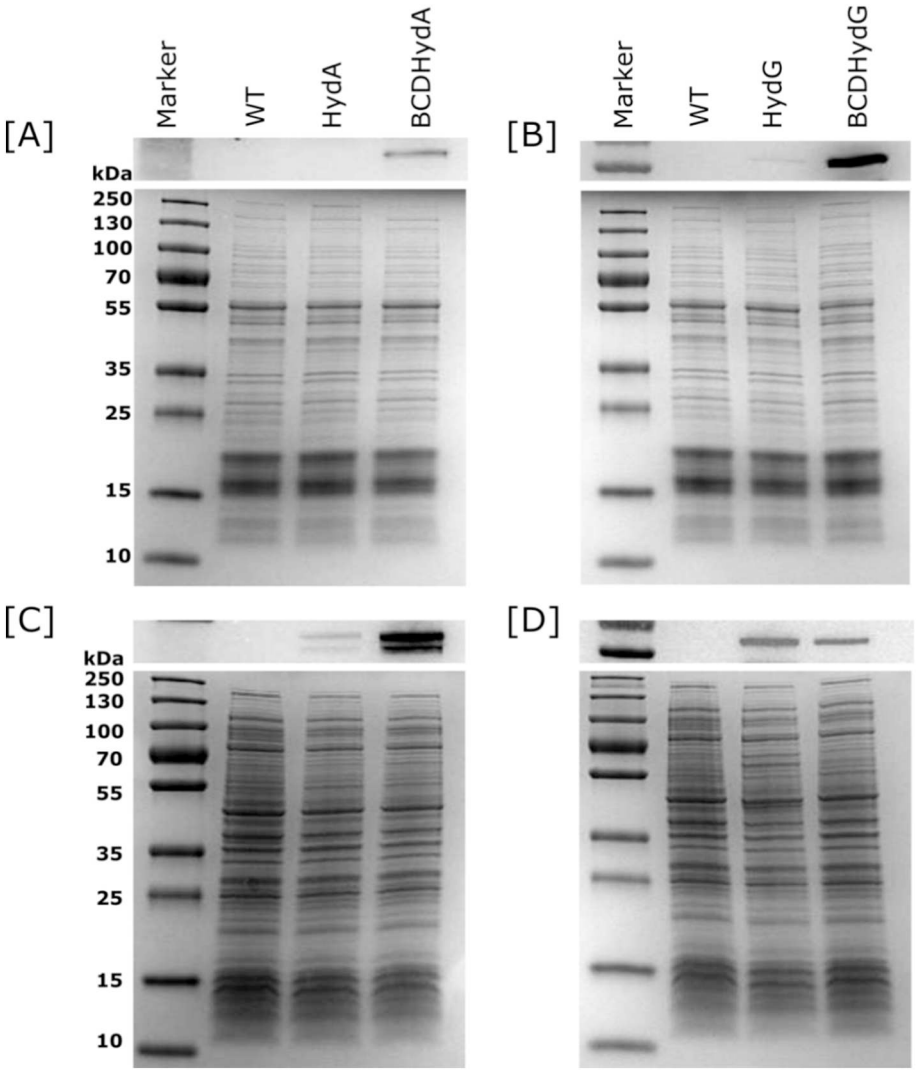

Fig. 2. SDS-PAGE and Western immunoblots analysis of crude protein extracts from *Synechocystis* (A, B) and *E. coli* DH5 α (C, D) wild type and engineered strains using HydA antibodies (A, C) and flag tag antibodies (B, D). Proteins from *Synechocystis* were isolated from 25 mL mid-log phase cultures (O.D. = 1.0 ± 0.2) as described in Heidorn et al. [8]. The protein concentrations were determined using colorimetric Bradford protein assay (Bio-Rad Laboratories) and 10 µg total proteins were loaded in each well. For *E. coli* protein extraction, pre-cultures were grown overnight at 37 °C, 200 rpm shaking, in LB media containing 50 µg/mL kanamycin, and used to inoculate 20 mL of LB media supplemented with antibiotics and 0.5 mM IPTG. Cultures were grown aerobically at 37 °C for 3 h and thereafter 1 mL of culture was harvested and re-suspended in water as per the O.D. 18 µL of total proteins were loaded in each well. The proteins were separated on precast (any kD) acrylamide gels (Bio-Rad) run at 200 V. Separated polypeptides were transferred to PVDF membranes (Bio-Rad), probed with (A, C) rabbit-anti-HydA IgG (Agrisera AS09 514) as per the manufacturer's instructions before visualization using goat-anti-rabbit IgG-HRP conjugate (Bio-Rad) and (B, D) with mouse-anti-flag IgG (Sigma F3165) as per the manufacturer's instructions before visualization using goat-anti-mouse IgG-HRP conjugate (Bio-Rad). The secondary antibodies were used at a 1:5000 dilution for 1 h. Detection was performed using Immuno-Star HRP Substrate (Bio-Rad), and recorded using a ChemiDoc XRS system (Bio-Rad).

genetic context, has presented an obstacle for further advancement towards tunable and predictable gene expression [9]. This phenomenon is speculated to result from different mRNAs forming different secondary structures in and around the 5′-untranslated region (5′-UTR), hiding the RBS in varying and unpredictable ways [10]. It has been shown in *E. coli* that this unpredictability can be largely overcome by the usage of insulator sequences, like the bicistronic design (BCD) described by Mutalik et al., where the RBS of the gene of interest (GOI) is included in a short upstream ORF [10]. This design permits temporary melting of RNA secondary structures in the junction between the 5′UTR and the GOI by the translation of the leader peptide, ensuring availability of the RBS for translation initiation and providing a more reliable gene expression [10]. We used BCD-insulation to enable expression of two synthetic constructs in *Synechocystis* 6803, which yielded very low protein levels when expressed with a traditional monocistronic design assembled from standardized biological parts.

The *PtrcO1*-RBS* and *PtrcO1*-B0034 (BBa_B0034, Registry of Standard Biological Parts) expression units have been shown to function well in unicellular cyanobacterium *Synechocystis* 6803 when employed upstream of a reporter gene [8]. However, when using these rational synthetic biology designs to express other heterologous genes in *Synechocystis* 6803, we observed considerable variation in expression levels from the same transcription and translation initiation unit in the presence of different GOIs. We cloned *PtrcO1*-RBS* upstream the codon-optimized *hydA* from *Chlamydomonas reinhardtii* and *PtrcO1*-B0034 upstream of the codon-optimized, flag-tagged, *hydEFG* operon from *Clostridium acetobutalicum,* and transformed these constructs into both *E. coli* strain DH5α (henceforth *E. coli*) and *Synechocystis* 6803, using the self-replicating broad host shuttle vector pPMQAK1 [6]. Corresponding constructs with translation initiation elements replaced with the BCD2-sequence (only the first element when constructing the operon) [10] were also developed and transformed in a similar fashion. SDS-PAGE and Western immunoblot analyses of extracted proteins from *Synechocystis* 6803 revealed expression from the *hydA*-gene only in the BCD construct (Fig. 2A). Expression of the flag tagged HydG protein from the *hydEFG* operon was barely detectable from the monocistronic construct, but strongly evident from the BCD counterpart (Fig. 2B). Similar Western blot analyses of the *E. coli* strains revealed protein expression from all constructs, monocistronic and bicistronic alike (Fig. 2C and D), but with notably higher expression levels from BCD-*hydA* than from the corresponding monocistronic construct. Transcription analysis by reverse-transcriptase polymerase chain reaction (RT-PCR) and gel electrophoresis confirmed transcript presence in all *Synechocystis* 6803 constructs (Fig. 3) and revealed no visible differences in transcript levels between the monocistronic and bicistronic designs.

Our results highlight the unpredictability of using biological parts in different genetic contexts, especially in cyanobacteria. Even though the monocistronic expression designs used in our investigation had proven to be functional for heterologous expression in *Synechocystis* 6803 [6], the change of GOI totally changed the performance of the promoter/RBS combinations, resulting in our case in protein levels below satisfactory for biological applications. The presence of transcripts from the monocistronic expression constructs in *Synechocystis* 6803 (Fig. 2), together with the very low or undetectable levels of protein (Fig. 2A and B) led us to conclude that inefficient translation may be causing the remarkably low expression levels compared to corresponding levels in *E. coli*. It is evident from our results that employment of a BCD-adapter in cyanobacteria can aid to express heterologous proteins in satisfactory amounts, in a genetic context where a traditional synthetic biology design fails to do so. In the case of the *hydEFG* operon in *Synechocystis* PCC 6803, the insertion of a BCD-adapter between the promoter and the first gene of the operon (*hydE*) enabled translation of the last gene (*hydG*) (Fig. 3B). This result suggests that one BCD-adaptor following the promoter is enough to enable efficient translation of the whole operon. Indeed, the presence of both HydE and HydF was later confirmed by proteomic analysis (data not shown). We speculate that the distance between the stop codons of *hydE* and *hydG* and the RBSs of the following genes (11 bp in our construct) is short enough for the ribosome to disrupt possible secondary RNA structures in the following RBS:GOI junctions.

Using the developed synthetic biology tools, a synthetic in vivo activation of a heterologously expressed [FeFe]-hydrogenase in the unicellular cyanobacterium *Synechocystis* PCC 6803 was recently demonstrated [11]. The non-native, semi-synthetic enzyme not only functioned in the cells but also linked to the native metabolism where the activated hydrogenase evolved hydrogen both in light and in darkness.

3. Improvement of chassis growth, functionality and robustness

Aiming at the stable integration of synthetic devices into the *Synechocystis* 6803 chromosome, several genomic neutral sites were identified. Disruption mutants in those *loci* were generated and extensively characterized in terms of fitness, transcription and proteomics, validating the neutrality and functionality of these sites. The constructed integrative vectors include BioBrick-compatible multiple cloning sites flanked by transcription terminators, constituting robust and insulated cloning interfaces. The mutants and vectors generated are available and can be used for synthetic biology approaches (Fig. 4; [12], European Patent Granted EP3106521).

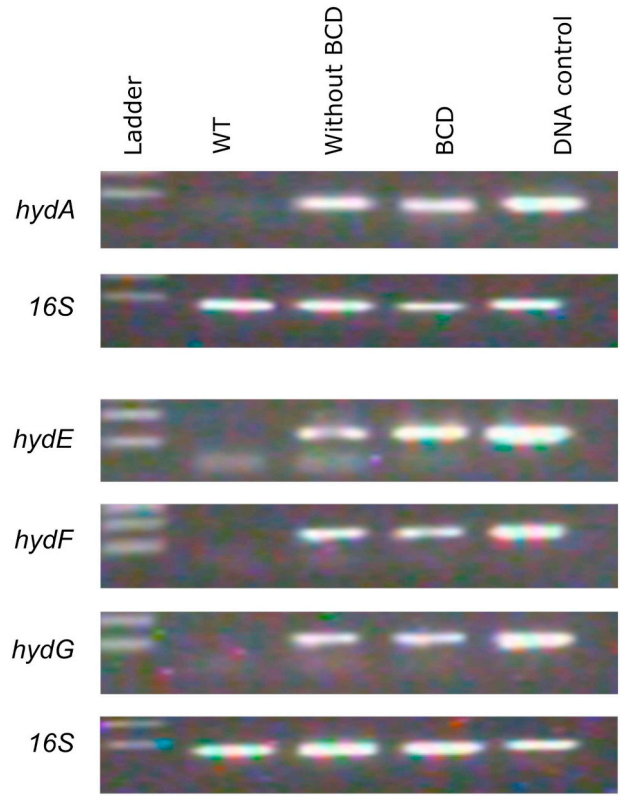

Fig. 3. RNA isolated from 10 mL early log-phase *Synechocystis* PCC 6803 wild type and engineered strains (O.D. = 0.4 ± 0.2) as described in Heidorn et al. [8]. 50 ng of plasmid or genomic DNA were used as control. cDNA was prepared as per the manufacturer's instructions (Quanta BIOSCIENCES). 100 ng of the first-strand reaction was used for PCR amplification (28 cycles). PCR amplicons (20 cycles) of 16S rRNA were used as loading control. The primer pair sequences (5′ to 3′) used in the study were as follows: RTHydA-F: gcaaacaaagtgaagctgatcg, RT HydA-R: ctcccatgggattatcccattc; RTMatE-F: cactgtagagaaaatgaaatatatcg, RTMatE-R: gcatcaatttctttaaagaacaaaat; RTMatF-F: ctattccctcttattagagaaaaag, RTMatF-R: cgtaattcaaattccttcgtaac; RTMatG-F: caagacatgggtcataaacg, RTMatG-R: gcttccgtatgatacgcat; RT16S-F: cacactgggactgagacac RT16S-R: ctgctggcacggagttag.

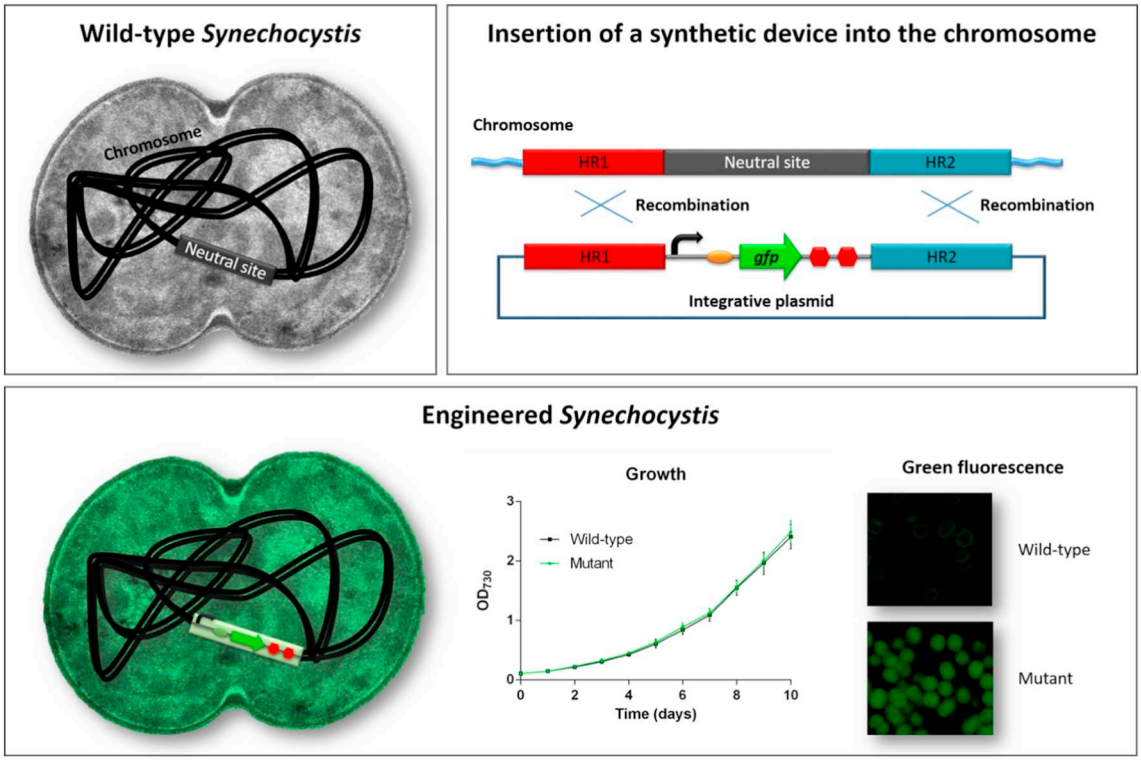

Fig. 4. Schematic representation of the methodology employed by Pinto et al. [12] to validate *Synechocystis* genomic neutral sites. The identified potential genomic neutral site (upper left panel) was modified by the insertion of a synthetic device containing a GFP generator (upper right panel). The neutrality of the putative neutral site was further validated assessing cell fitness, while functionality of the inserted device was analyzed by confocal microscopy (bottom panel).

With the aim of improving chassis robustness, a set of targets (candidate genes/pathways) was identified leading to the improvement strategies focusing on heat shock response proteins (HSP) and compatible solutes (CS) synthesis. Several devices were constructed using synthetic promoters developed within CyanoFactory and implemented in *Synechocystis* 6803. Some of the generated mutants exhibited a remarkable increase in the transcription of genes encoding the enzymes responsible for e.g. glucosylglycerol production - *ggpS* and *ggpP* - even without NaCl supplementation. The increase in compatible solute production is being evaluated by NMR.

Furthermore, the chassis functionality and robustness were also assessed by inactivating *slr1270* which encodes a TolC homologue. TolC is an outer membrane protein associated to biomolecule secretion, including proteins and *endo*- and/or exogenous metabolites. Our results show that the TolC-like Slr1270 bestows a marked physiological fitness to *Synechocystis* 6803. Moreover, our work presents a valuable model for studying outer membrane vesicle (OMV) formation and release [13]. In the future, OMVs and optimized secretion system(s) can be used as tools to increase the functionality of a chassis based on *Synechocystis* 6803.

For modulation of the chassis intracellular oxygen concentration, important when introducing synthetic modules encoding O_2-sensitive

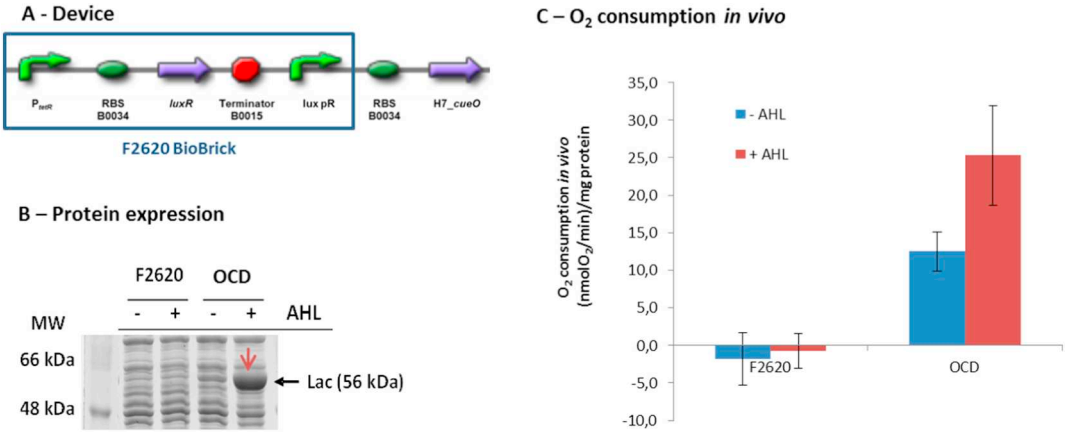

Fig. 5. Characterization of *E. coli* Top10 harboring the laccase-based OCD. (A) Specifications of the synthetic device. (B) Analysis of protein expression by SDS-PAGE. Protein extracts were obtained from *E. coli* Top10 containing the F2620 BioBrick or the OCD, in presence (+) or absence (−) of the inducer - AHL. The band corresponding to the laccase is highlighted by a red arrow. (C) O_2 consumption measurements in vivo. Cultures from *E. coli* Top10 containing the F2620 or the OCD, in presence (+) or absence (−) of AHL were used; and respiration rates were determined polarographically for suspensions with a final OD_{600} = 1, 2 or 4 (1 mL working volume) using a Clark-type O_2 electrode. Data are shown as mean ± SD, n = 3. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

enzymes (such as hydrogenases), several Oxygen Consuming Devices (OCD) were designed and synthesized. The OCDs are comprised of the *cueO* encoding *E. coli*'s native laccase and its variants (with improved activity), preceded by the F2620 BioBrick, which allows inducible expression of the device by adding acyl-homoserine lactone. These devices were characterized in vitro in *E. coli* (protein crude extracts). Assessment of the specific laccase activity and oxygen consumption rate revealed that the OCDs are functional in all conditions tested. Moreover, characterization of the OCDs in vivo using *E. coli* cells confirmed the consumption of O_2 in cells that harbored the devices [14] (Fig. 5).

Genetic stability of the constructs is an important issue. However, this was not specifically addressed in the CyanoFactory. In a different project a H_2-evolving cyanobacterial strain (genetically engineered by deleting the uptake hydrogenase) in an automatic 5 L photobioreactor (3 L cell culture) was examined in up to 150 h experiments [15]. No antibiotic pressure was used and no genetic instability was observed. In addition, one of the partners of CyanoFactory examined and published the stability when using self-replicating plasmids [7]. The conclusion was that at least 90% of the cells retained the plasmid when cultivated for 16 days without selective pressure. However, for genetic stability a genome integration, instead of using a self-replicating plasmid, should be used.

4. Improving electron flow towards H_2 production

For efficient production of hydrogen, cyanobacterial metabolism of electron acquisition and distribution has to be engineered. Key players of the optimization process are Photosystem II (PSII), Ferredoxin-NADP-Oxido-Reductase (FNR), and ATP synthase (Fig. 6).

For an increase in the supply of reduction equivalents, the starting-point for the optimization is the water splitting process. Importantly, we have shown that the introduction of electrons into the photosynthetic electron transport chain is significantly increased in phycobilisome mutants with reduced light harvesting antennas [16]. Besides increasing the amount of PSII per cell, higher cell densities in photobioreactors are also obtained, which further increase the space-time yield of the process. While the positive impact of a truncated phycobilisome structure on linear electron transport was observed in the Olive mutant, reports on lower performance of phycocyanin deletion mutants were published by other investigations [17].

As the Olive mutant with a truncated phycobilisome as its main characteristic had been generated by chemically induced random mutagenesis in 1989 and had an unclear genomic background, the complete genome of the Olive mutant was analyzed by next-generation sequencing. The results of the sequencing activity display a frame-shift within the *cpcB* gene. While this leads to a loss of function on the protein level and explains the truncation of the light-harvesting antenna, on RNA and DNA levels the gene remains virtually unchanged. This might suggest a role of regulatory RNAs, which are thought to play an important role in the transcriptional regulation of the light-harvesting apparatus, to explain the differences in the phenotype of the rational phycobilisome truncation mutants and the Olive mutant.

In addition to truncation of the light-harvesting antenna, partial decoupling of the ATP synthase was identified as a route for increasing photosynthetic electron transport. For a further increase in electron transport rate, the deletion of the C-terminal domain of ATP synthase subunit ε, which was shown to accelerate photosynthetic electron transport [18], was introduced into the Olive mutant. The resulting Olive-ΔCε mutant displayed a fourfold increased electron transport rate in comparison with the wild type. However, only 25% of the electrons produced in the Olive-ΔCε mutant are transferred into the cyanobacterial metabolism, while 75% are transferred to O_2 in the Mehler reaction catalyzed by the flavodiiron protein Flv1 and Flv3. If these electrons can be redirected to a hydrogenase, a future H_2-production would not be in competition with the demands of the metabolism.

Re-routing of the reduction equivalents is a key step for the integration of the hydrogenase into the cyanobacterial metabolism, the flux of reduction equivalents has to be redesigned. In the focus of this design process is the Ferredoxin-NADP-Oxido-Reductase (FNR), as it transfers the main share of reduction equivalent towards carbon fixation. Based on the structural information [19], rational design variants of the FNR with reduced ferredoxin affinity were created and screened for their activity by two independent in vitro assays (Fig. 7, Table 1) – a cytochrome *c* reduction assay and light-activated proflavin assay with a direct competition of hydrogenase and FNR for reduced ferredoxin.

Based on the cytochrome *c* reduction assay, FNR variants K78D, K75A-K78D and K75D-K78D show a very low cytochrome reduction rate compared to the wild-type protein. This low electron transfer efficiency is also confirmed by the competition experiment. Forty to 60% of the electrons are transferred to hydrogenase when FNR variants K78D, K75A-K78D and K75D-K78D compete with the [FeFe] hydrogenase from *Chlamydomonas reinhardtii,* while only 5% are used for hydrogen production when the wild-type FNR is used for the experiment. An additional improvement was achieved by the allocation of specific interaction residues by NMR analysis. This approach led to a combined construction of Fd- and FNR-mutants, which enabled an about 18-fold enhanced electron flow from Fd to HydA1 in in vitro assays [20]. The negative impact of these mutations on the Fd-FNR electron transport was dominated by the FNR variants (up to 42%), but also Fd-variants contributed up to 23%, and surprisingly had in parallel a direct positive impact on the Fd-HydA1 electron transport (up to 23%). This is an excellent basis for the construction of a hydrogen-producing design cell and the study of photosynthetic efficiency-optimization with cyanobacteria.

5. A synthetic biology approach to biosafety of genetically modified cyanobacteria

Currently, cyanobacterial biotechnology is building on wild-type strains that were at one time isolated from nature. Genetic modifications towards improving the performance of these strains are focused on introduction of new biosynthetic pathways, while biosafety issues tend to be put aside. However, especially in synthetic biology, there has been a pronounced concern from the public as well as scientists about biosafety. Therefore, we decided to implement a biosafety device into *Synechocystis* 6803 that would present a biological barrier against the spread of genetically modified *Synechocystis* cells into the environment.

In principle, there are many different approaches for improving biosafety of genetically modified microorganisms, as reviewed specifically for synthetic biology elsewhere [21]. For our needs, an active containment strategy seemed the best option. It relies on an introduced

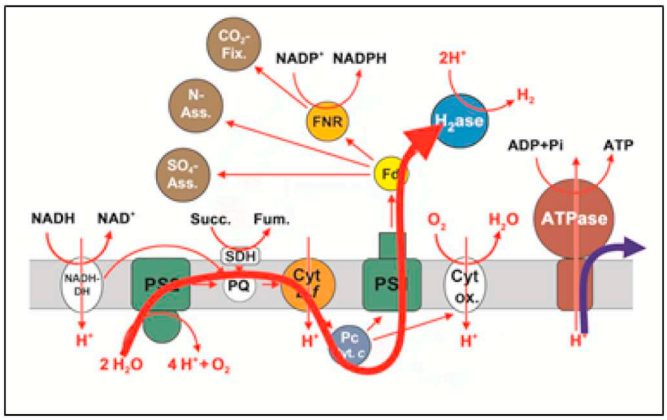

Fig. 6. Schematic outline of an optimized electron flow from water splitting in Photosystem (PS) 2 via PS 1 towards the hydrogenase for photosynthetic hydrogen production.

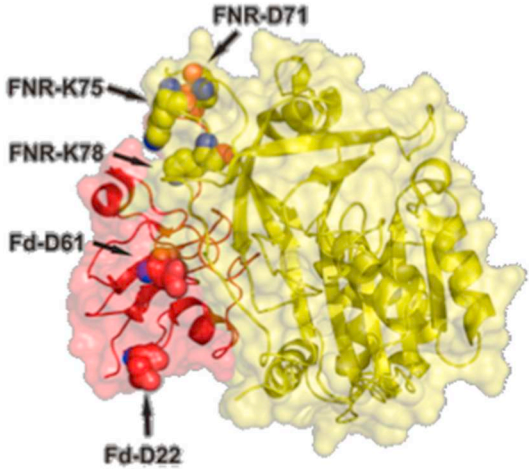

Fig. 7. Structure of the FNR:Fd complex. The sites targeted for reducing the complex stability are indicated, identified proteins followed by their respective amino acid and its position within the structure.

Table 1
List of FNR and Fd variants ordered by their decreasing affinities.

FNR variants	Fd variants
$FNR_{L/S}$	Fd
FNR_S-D71K	Fd-D22A
FNR_S-K75A	Fd-D61A
FNR_S-K75D	Fd-D22A-D61A
FNR_S-K78A	
FNR_S-K78D	
FNR_S-K78A-K78D	
FNR_S-K75D-K78D	

kill-switch device that enables controlled killing of only those engineered microorganisms that carry the device. These types of devices had been implemented previously in some bacterial species, but only recently have we implemented them in cyanobacteria [22].

For the cyanobacterial kill-switch we envisaged that it should be based as much as possible on the cyanobacteria's own genetic elements (to minimize transgene use), should be induced specifically by using low molecular weight compounds that are easily available, could be used in large-scale experiments, and should trigger cell death from within the cell, leaving other organisms in the environment unaffected as much as possible.

Two approaches were examined, one based on a toxin/antitoxin system (TAS) and the other on a nuclease/nuclease inhibitor system (NIS). Both are intrinsic cyanobacterial regulatory systems with specific physiological roles in coping with stress (TAS) and serving for nutritional purposes or sometimes as bacteriocides (NIS). In the first approach, we constructed two recombinant strains by rewiring two related TAS pairs originating from *Synechocystis* 6803, reviewed by Kopfmann et al. [23], and reintroducing the resulting two circuits into the originating strains. Toxin/antitoxin pairs Ssr1114/Slr0664 and Slr6101/Slr6100 are originally encoded as single operons in which protein products act as transcription regulators. For the construction of biosafety devices (Fig. 8), the toxin- and antitoxin-encoding units were placed under separate promoters, which were either constitutive or inducible. In the second approach, we took advantage of the non-specific nuclease NucA and its cognate inhibitor NuiA, originating from the filamentous cyanobacterium *Anabaena* sp. PCC 7120 [24]. The options selected for reorganization of wild-type operons are presented in Fig. 8.

In our work, we limited the choice of promoters to those that respond to metal ions, bearing in mind that the cost of inducer compounds should be kept low in large-scale biotechnological applications. Since heavy metals are actually quite toxic to cyanobacteria, we searched for metal inducers that are active in subtoxic concentrations, allowing for selective killing of only those cyanobacteria that carry synthetic biology suicide switches. All the kill-switches tested contained a constitutive and an inducible promoter, regardless of the type of effector/inhibitor pair and the approach (Fig. 8B–D). Of the known

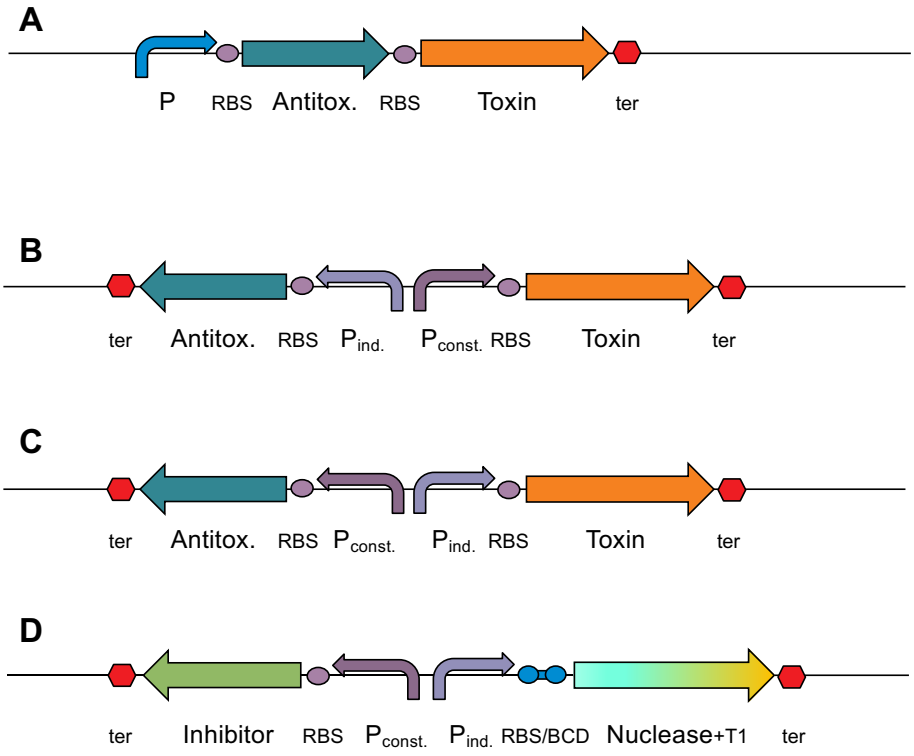

Fig. 8. The native TAS (A) and engineered effector/inhibitor device design for controlled cell death (B–D); toxin/antitoxin-based devices (B, C) and the optimized nuclease/inhibitor-based device (D). (A) The native arrangement of the toxin and antitoxin genes under the control of a single common promoter. (B) The engineered arrangement that could be used for induced expression of the antitoxin in the bioreactor. Upon spillage, inducer would no longer be available, resulting in toxin activity and possibly cell death. (C) Alternative engineered arrangement that could be used for induction of the toxin gene at the spill site upon leakage, thus saving costs of continually adding inducer in the bioreactor under regular growth conditions. (D) A variant of (C) with the controlled expression of nuclease/inhibitor; this configuration showed best results in induced cell killing. Abbreviations: Antitox., antitoxin-coding region; BCD, bicistronic design (a specific transition between the RBS and the start codon that promotes reliable translation initiation); P, wild-type promoter; $P_{ind.}$, inducible promoter; $P_{const.}$, constitutive promoter; RBS, ribosome binding site; ter, transcription terminator, T1: degradation tag.

inducible promoters, we tested the copper- or zinc-inducible *copB* and *copM* promoters, the zinc-inducible *smtA* promoter and the nickel-inducible *nrsB* promoter, all from *Synechocystis* 6803. The constitutive promoters that we tested were *rnpB* (RNaseP subunit B), and *rbcL1A* (RuBisCO large subunit), promoters from the same strain. Due to high copper toxicity, best results were obtained by zinc induction.

Since there is limited data available on promoter behaviour in *Synechocystis* 6803, several optimized constructs had to be developed to achieve the necessary fine-tuning of devices that resulted in efficient induced cell killing. Promoter combinations were investigated with both a nuclease/nuclease inhibitor system (NIS) and a toxin/antitoxin system (TAS) in different combinations with device construction, see Table 2.

With the *copM* and *nrsB* promoters, full-length and shortened promoter versions were tested. Additional contribution towards improved device behaviour was achieved by replacing the native RBS with a synthetic one, as well as by introduction of a BCD arrangement [10], the aforementioned specific insulator sequence that facilitates reliable translation initiation of random downstream genes. Additionally, fusion of degradation tags to the C-terminus of the toxic effectors helped curtail and thereby optimize effector cellular concentration - in many cases this was the only way to clone the toxin genes due to the high toxicity for the heterologous *E. coli* host.

Although in perspective, the suicide device would likely be best positioned on the chromosome, we completed all the experiments with the previously developed broad-host expression plasmid pPMQAK [6]. This enabled us to test the constructs faster, since segregation of clones after genomic incorporation of the constructs could be avoided.

The effectiveness of the genetic constructs in *Synechocystis* 6803 was monitored by RT-PCR to assess effector expression in the presence of inducer (and expression leakiness in inducer absence), and by screening the growth rates and viability of recombinant cyanobacterial strains. We found out that TAS-based devices had either no effect on cell growth or caused only a bacteriostatic effect. Since a bactericidal effect would be preferred, we sought other combinations of genetic elements. Only one of these proved to be potentially useful for application in synthetic biology and biotechnology. It consisted of a bicistronic arrangement downstream of the prolonged *copM* promoter, which was linked to the NucA nuclease coding region. From the coding region, the nuclease signal peptide had been removed, while a C-terminal T1 degradation tag had been added. The NuiA inhibitor coding region was joined to the *rnpB* promoter fused to a synthetic ribosome binding site (Fig. 7D). The presence of the inhibitor in the kill-switch helped ensure that any unwanted leakage of the toxic effector was neutralized in the cell.

Cyanobacteria with the best-performing device repeatedly displayed cell killing in culture when induced by 12–14 μM Zn^{2+} ions, which was visible by naked eye and later confirmed by cell viability assay (the MTT test), growth curve analysis and RT-PCR. We believe that this construct could be further optimized for robustness and tested at higher cell densities and under simulated outdoor conditions. Finally, we would like to note that the nuclease-based suicide device that we developed and tested is not only a cell-killing synthetic biology circuit, but also a potential shredder of (recombinant) DNA. This way, the risk of spread of transgenes into the environment is further reduced.

6. Analyses of the purpose designed cyanobacterial cells to identify bottlenecks and suggest further improvements

During CyanoFactory, a number of the challenges affecting the development of *Synechocystis* as a synthetic biology and biotechnology production platform were assessed; primarily with proteomics, but also with transcriptomics and ^{13}C metabolic flux analysis. The aim of this systems-level approach was two-fold; first it provided insight into the system of responses the cell produces to the genetic and environmental factors studied in the project; and second it enabled monitoring of the flow of metabolites through the cell to give an impression of reaction rates. Both of these outputs enabled rational design choices to be made based on the intracellular response of the cell, when combined with an in-silico model of the organism. In addition to the use of these techniques for assessing the system, a number of key developments of the methods were also conducted over the course of the project, to improve the quality of the data and to facilitate its upgrade into knowledge.

Proteomics is the study of the protein complement within a system, where a population of cells is (usually) measured quantitatively at a single point in time to give a snap-shot impression of the protein-level cellular response [25]. While the whole proteome and its various interactions and post-translational modifications are poorly understood, comparing an engineered system to a control condition enables us to identify unexpected or off-target responses to a design (and potential 'bottlenecks'). This technique is incredibly powerful, and is gaining

Table 2

Promoter-nuclease		Promoter-nuclease inhibitor		Switch activity
$copM_{195\text{-}BCD}$	*nucA-T1*	$rnpB_{RBS}$	*nuiA*	complex autodestruction upon Zn^{2+}-induction
$copM_{72}$	*nucA-T1*	$rnpB_{RBS}$	*nuiA*	marked autotoxicity upon Zn^{2+}-induction
copM	*nucA-T2*	$rnpB_{RBS}$	*nuiA*	only mutated clones survive
$rnpB_{RBS}$	*nucA*	*copB*	*nuiA*	only mutated clones survive
$rnpB_{RBS}$	*nucA-T2*	*copB*	*nuiA*	cyanobacterial transformation failed
$nrsB_{native}$	*nucA-T1*	*rbcL1A*	*nuiA*	weak autotoxicity
$nrsB_{RBS}$	*nucA-T2*	*rbcL1A*	*nuiA*	weak autotoxicity
$nrsB_{BCD}$	*nucA-T1*	$rnpB_{RBS}$	*nuiA*	weak autotoxicity
$nrsB_{native\text{-}short}$	*nucA-T1*	*rbcL1A*	*nuiA*	weak autotoxicity
$nrsB_{native\text{-}short}$	*nucA-T2*	*rbcL1A*	*nuiA*	weak autotoxicity
rbcL1A	*nucA-T1*	$nrsB_{native\text{-}short}$	*nuiA*	metal-ion dependent toxicity *not* observed
rbcL1A	*nucA-T2*	$nrsB_{native\text{-}short}$	*nuiA*	metal-ion dependent toxicity *not* observed

Promote-toxin		Promoter-antitoxin		Switch activity
smtA	*slr0664*	$rnpB_{RBS}$	*ssr1114*	only mutated clones survive
$rnpB_{RBS}$	*slr0664*	*smtA*	*ssr1114*	weak autotoxicity (Zn^{2+} or Cu^{2+} withdrawal)
$rnpB_{RBS}$	*slr0664*	*copM*	*ssr1114*	weak autotoxicity (Zn^{2+} or Cu^{2+} withdrawal)
copB	*slr0664*	$rnpB_{RBS}$	*ssr1114*	weak autotoxicity (Zn^{2+} or Cu^{2+} withdrawal)
$rnpB_{RBS}$	*slr0664*	$rnpB_{RBS}$	*ssr1114*	metal-ion dependent toxicity *not* observed

popularity for assessment of industrially relevant organisms and processes. Groups working to develop multi-omics approaches are starting to bridge the gap between the most powerful proteomic techniques and production strain analysis in, for example, *E. coli* [26].

Prior to method development, the existing literature relating to *Synechocystis* 6803 and the leading-edge developments in proteomics for production strains were analyzed for trends and gaps [27]. A number of improvements to the physical and analytical methodology were identified, including the extraction method used for collecting proteins, quality assessment techniques, the grouping (e.g., gene ontology, metabolic mapping, and clustering of cellular functions) and analysis of the large datasets. *Synechocystis* 6803 is strongly placed as a flagship organism for systems-level design of photosynthetic synthetic biology, as it was the third living system to have its genome sequenced and the first photosynthetic organism to be sequenced [28,29].

The latest studies using the most advanced techniques and machinery enable just over 60% of the predicted proteins in the proteome to be identified and quantified rapidly and with confidence. Due to the complex membrane structure, one of the major hurdles to a more complete analysis is reproducible cell lysis and protein collection. A literature analysis, with experimental verification, demonstrated that a combination of sonication and bead-beating steps for protein extraction provided the most effective extraction technique. It provided more reliable results than liquid nitrogen cracking in a mortar with pestle, which gave variable output and was highly operator dependent, and avoided issues found with pressure-related and freeze-thaw methodologies – both of which *Synechocystis* 6803 was found to be resistant to. Notably, for a less hardy organism these techniques could lead to protein damage and reduced data quality.

As a photosynthetic microorganism, one of the major constituents of the *Synechocystis* 6803 cell is a thylakoid membrane system loaded with light-harvesting proteins. For context, these complexes account for close to 60% of the total soluble cellular protein fraction and over 20% of the organism dry weight. Indeed, these high abundance proteins found in *Synechocystis* cause issues for analysis as they form the majority of the sample. They effectively reduce what is referred to as the 'dynamic range' or the difference in concentration between the most abundant and least abundant proteins – the result is repeated identification and quantification of these proteins by the mass spectrometer to the exclusion of many lower abundance proteins. This can be mitigated to a certain extent by using liquid chromatography to separate and concentrate peptides during analysis, giving better quality data. It was found that using a hypercarb HPLC separation yielded a more effective separation of peptides than either strong cation exchange (SCX) or HILIC. In addition, it was found that the wavelengths of light absorbed by the light-responsive proteins caused problems with typical spectrophotometric analyses used for protein quantification, such as in the Bradford assay, where repeat analyses on the same sample had widely varying results. Alternative techniques are required to minimize this issue [30].

To further improve the organism for future synthetic biology developments, a range of different investigations were carried out. Firstly, an analysis was performed on a series of neutral integration sites within the *Synechocystis* genome. A number of key sites were found to generate no unexpected background protein expression within *Synechocystis* and as a result are suitable for stable integration of synthetic constructs [12]. These sites were tested with an antibacterial resistance cassette, which was shown to affect expression of only a single protein of unknown function. Secondly, to better understand the flow of carbon through the cell when comparing an antenna mutant (Olive) with the wild type cells, a transient ^{13}C labelling experiment was performed with a corresponding quantitative proteomic analysis. A third major experimental program was undertaken to assess the organism in a pilot-scale environment. A quantitative proteomic assessment of *Synechocystis* found that whilst the background proteomic state appeared to vary significantly between repeated runs in our 1350 L scale PBR system, systematic changes were observed through the passage of the day.

7. Metabolic modelling of the engineered cells

Rational design of living organisms for biotechnological purposes is a challenging interdisciplinary effort which can be supported by in silico analysis. Modelling strategies serve as a basis for unravelling the underlying mechanisms responsible for cell behavior and allow theoretical assessment of environmental and genetic variations, whereas laboratory experiments are often expensive, time demanding or shed light on just a specific process of the system. Engineering cyanobacteria for producing metabolites of interest, such as hydrogen, entails an overall knowledge of metabolism but also a detailed comprehension of photosynthesis, in particular. Additionally, the application at industrial-scale requires a specific search for optimal growth conditions in designed photobioreactor frameworks.

In order to understand the biochemical processes occurring within a *Synechocystis* cell, metabolic models of the strains of interest are needed. The departing point to build an accurate, up-to-date, genome-scale metabolic model of *Synechocystis* 6803 was a model previously developed, iSyn811 [31,32]. More than 200 reactions were added and/or updated leading to significant improvements in simulations, such as greater plasticity, better accuracy at representing electron consuming pathways and a more realistic quantification of the cell's energy needs, which yield more precise metabolic characterization of the strains and enhance the predictability of the model. The improved model can be considered as our "reference" for wild type *Synechocystis* 6803. However, other wild type or mutant strains have also shown interesting properties, therefore, derivative models are required to study them. We have developed a pipeline that aims at the elucidation of how differences in sequence might affect the metabolic function of these strains, allowing the construction of models for the new strains based on the original one.

These metabolic models can serve to guide strain design by assessing how different modifications in media or genomes can improve growth or productivity of desired metabolites. In that sense, a substrate study was performed to determine the best sources of inorganic nitrogen and sulfur in terms of hydrogen production. In the case of nitrogen, the results obtained from the simulations qualitatively matched previous experimental observations [33,34], while regarding sulfur, this is, to our knowledge, the first work in which different sources have been tested to improve hydrogen production. Altogether, the results of this study showed that by choosing the appropriate source among these inorganic substrates, maximum optimal H_2 production can be substantially increased (Fig. 9). As a further application, genetic changes were analyzed in order to choose the best option between some candidates for the synthesis of compatible solutes. The results of this study showed that in terms of metabolic cost, glycine betaine is most promising since a greater production with respect to the other tested compatible solutes can be achieved in silico at equal cell growth rates when using BG-11, a common growth medium for cyanobacteria. Both case studies exemplify how, by means of metabolic simulations, support can be given to experimentalists in the selection of the best environmental and genetic conditions enabling enhanced H_2 production.

Although classic metabolic simulations appropriately solve some biotechnological problems, nowadays with the advent of high-throughput technologies, the field of systems biology has amassed plentiful omics data that can be combined with metabolic models to heighten the predictive capabilities of computational simulations and their plasticity when dealing with perturbed conditions. After carefully considering different options in terms of available information and computational cost, we finally decided to implement a previously described methodology, IOMA [35], in which proteomics, metabolomics and kinetic parameters, are integrated into metabolic simulations. This scheme allows to qualitatively improve the simulations' response to

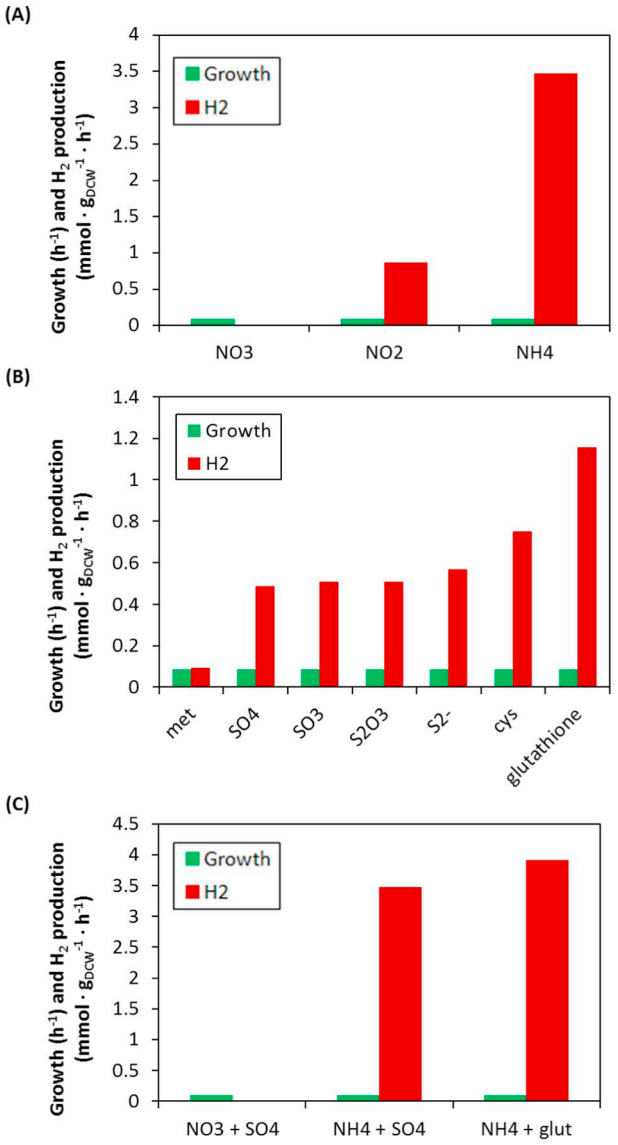

Fig. 9. Results of the simulation of hydrogen production at a given growth rate with different sources of inorganic nutrients obtained by using the *Synechocystis* 6803 metabolic model. Simulations were carried out under phototrophic conditions, with fixed amount of photons and CO_2, at a fixed growth ratio (normal growth for this carbon uptake), and allowing the input of different (A) nitrogen sources: nitrate (NO_3^-), nitrite (NO_2^-) and ammonia (NH_4^+); (B) sulfur sources: methionine (met), sulphate (SO_4), sulphite (SO_3^-), thiosulphate (S_2O_3), sulphide (S_2^-), cysteine (cys) and glutathione; and (C) combination of nitrogen and sulfur sources leading at best maximum optimal hydrogen production.

environmental changes, depending on the quality and amount of available experimental information. A further development, META-MODE, is an algorithm that performs multi-objective optimizations of a genome-scale metabolic model allowing the use of biologically-relevant non-linear constraints and that, with the inclusion of experimental flux data, permits researchers to have realistic metabolic simulations without having to impose constraints that are tiresome to find and even sometimes cryptic to understand. With META-MODE, simulations can be driven to maximize growth without stating any biomass equation, so that different biomass compositions will be obtained depending on the conditions. Our final goal is to incorporate different algorithms into META-MODE, leading to a comprehensive tool prepared for different omics integration that performs advanced metabolic simulations.

A further goal within CyanoFactory was the construction of a kinetic model of photosynthesis, as this process is highly dynamic, regulated and drives the main energetic pools in autotrophic conditions. Such in silico tool provides the opportunity to estimate electron fluxes and their dynamics in the transport chain by using several sets of data including reaction stoichiometry, flux rates, kinetic and thermodynamic constraints. It allows to calculate the time dependent response of the photosynthetic electron chain to varying light or ambient conditions, allowing a better understanding of several processes, such as respiration, carbon fixation or hydrogen production. One interesting result of the model was the description of the thermodynamic mechanism by which, under normal conditions, NADPH acts as an electron acceptor rather than a donor, explaining why it is not possible to sustainably generate such gas in the wild-type strain in normal conditions. Besides, oxygen at cytoplasmic concentrations blocks the hydrogenase activity and moreover, inhibitory levels of oxygen can be reached within seconds after the onset of light. Interestingly, these conditions were numerically confirmed by the model giving not just a qualitatively well-known description of the phenomenon but also shedding light on the process by means of quantitative predictions expressed in physical units.

Another objective was the creating a photobioreactor scale model that is able to predict physiological properties of photosynthetic cultures. Cells grown in such reactors experience complex events: irregular trajectories, inhomogeneity of dissolved carbon and salt concentrations, shear and light stress, among others. However, if cells are grown with an appropriate culture medium and are mixed in a homogeneous and random manner, it can be assumed that they are constantly moving from the reactor surface to the interior part of the vessel. Further, in dense cultures light is not normally leading to photoinhibition but is a limiting variable. In such cases, which are typical for large-scale photobioreactor facilities, it is reasonable to assume that there are two main factors to consider for optimal photosynthesis yields: light as energy input and CO_2 as carbon source. Additionally, in most of the studies performed on photosynthesis research, the photosynthetically active radiation (PAR) value is indicated as reference input for the experiment. Though this is a practical way to show the influence of light on the studied biological mechanism, it does not allow a proper benchmarking with analogous experiments. This is due to the fact that not just the total incident light intensity but also the lamp emission characteristics, the cell concentration, the shape, the depth of the reactor and its previous photoacclimation [36] affect the real irradiance inside the culture vessel and henceforth photosynthesis, too. In addition, all photosynthetic processes are directly regulated by certain wavelengths: pigment light absorption, non-photochemical quenching or state transitions among other mechanisms are controlled by the spectral composition of light and consequently treating light as a simple value is not sufficient for unravelling the underlying mechanisms that control photosynthetic responses. For all these reasons, it was decided to apply the Inherent Optical Properties methodology to predict light quantity and quality in the cell suspension. The model [37], using experimental optical properties validated with literature data of wild-type and Olive strain, delivers reliable results on PAR and spectral light attenuation at different cell densities and LED lamps of several colors. It was later updated with fractional calculus to improve prediction at very depleted light conditions [38]. Finally, the light model was coupled with CO_2 gas-liquid mass transfer and cyanobacterial carbon fixation and concentration mechanisms to build up a simplified PBR model that predicts growth with light and carbon as main variables.

Future in silico work will seek the integration of detailed photosynthesis mechanisms as input to genome-scale metabolic models in order to further restrict the solution space towards more realistic metabolic landscapes. Specifically, the effect of light on cells, the so called photoacclimation processes, not yet included in most modelling approaches could be covered in a culture level approach. The final aim is of this analysis is to predict the physiological evolution of main metabolic reporters, understand the resource allocation at any moment of

the day and hence suggest operation guidelines for improving overall culture performance.

8. Production of flat panel photobioreactors for an industrial standard

One key factor for a successful solar biofuel production is the development of an economically feasible PBR production system. Much attention has been given to closed PBRs. Although the setup costs of PBRs are significantly higher than for open ponds, they provide several advantages such as reduced water and CO_2, consumption as well as maintaining unialgal cultures [39]. However, cost-effective closed PBR designs for algal mass cultivation are rather scarce [40–43], with the major limiting factors being light availability and interphase mass transfer [44]. Major costs drivers for PBR construction are material, manufacturing expenses and personnel costs [45]. Thus, the focus was put on developing a low-cost PBR and an online control system to automate the cultivation process.

A flat panel PBR design was chosen since it allows a better exposure to light. Further, 18 different polymers were tested to check material compatibility. Scarce literature can be found regarding material biocompatibility for PBR construction. To determine the biocompatibility of each compound, the optical density (OD) of the samples was determined after 100 h, 250 h and 400 h of operation. The results showed that OD increased in all tested samples. Since polymers do not seem to harm algae growth ([46,47]) we used such type of material to construct the vessel. In this regard, most of the reactor parts can be produced by milling, which enables lower construction costs in comparison to handmade or other saleable versions. The reactors are equipped with industrial standard connectors (G1/8″, G1/4″, Luer Lock) to ensure adaptability for various applications.

To automate the process of cultivation, an online control system was developed. The programme was designed to be freely adjustable to various application setups. Signals can be read by and sent to different ports of the operating computer. Two PCI-Cards for digital in- and output were installed. Thus, different parameters can be measured and stored during the cultivation and several actuators (valves, pumps, etc) are activated depending on the culture parameters or user settings. Such process monitoring enables an online control of the reactor for a user-defined period of time. One advantage of this control system is the possibility of a continuous cultivation without the need of a manual adjustment of parameters such as the pH or the medium flow. In this way, the culture can be kept under constant conditions (e.g., constant growth rate) which in turn promotes experimental reproducibility.

To further reduce the setup costs for cultivation in PBRs, a new chemical reactor sterilization process was developed using organic acid to neutralize the peroxide solution used to that purpose. After the chemical sterilization, another neutralizing solution is added, which remains in the reactor. This process is especially suitable for large-scale reactors since no further rinsing processes or additional sterilized water is required. The nutrient solution and culture medium are then directly added to the solution. This process is environmentally less harmful than the previously one, which leverages an ethylenediaminetetraacetic acid (EDTA) solution.

Besides, water scarcity is gaining in significance due to climate change and population growth and, therefore, ensuring cost-effective and resource saving solutions are crucial in treatment and reutilisation of the produced wastewater containing microalgae cells. Thus, the establishment of a simple and low-cost process to separate cyanobacterial biomass from the medium is necessary. Different methods for the separation of the clear phase and the biomass were tested. Those include precipitation, electrolysis and ultrasound processes. While electrolysis and the ultrasonic method failed to fully segregate the cells from the aqueous phase, some precipitation agents led to a complete separation of both components. Yet, no separation appeared regardless of the amount and type of employed agent in high salt content solutions. Hence, precipitation can be considered as a simple and affordable separation strategy for non-salty solutions. Additionally, in order to operate a closed loop system, it was essential to test if the separated clear phase led to an undesired precipitation when reintroduced into the PBR. However, no precipitation reaction occurred after the recirculation of the chemically sterilized clear phase. Likewise, the second recirculation test after one week showed no further coagulation process. Another aspect that needs further research is the nutrient consumption. In order to reduce operation costs, it is fundamental to investigate which growth medium elements need to be replenished after operation, precipitation and filtration. Our test showed that potassium and phosphate have to be added after recirculation with almost no change in iron and zinc after the operation.

9. Assembly and performance assessment of a larger prototype photobioreactor system

A novel flat panel and large-scale PBR was designed and satisfactorily tested for future industrial biotechnological applications. The reactor performance was assessed with a culture of *Synechocystis* PCC 6803, which is a promising candidate for production of biofuels [48,49]. Indeed, this outdoor PBR reached a light dilution factor close to four, thus, reducing the light saturation effect and improving energy utilization. Moreover, the first attempt to scale up hydrogen production by *Synechocystis* PCC 6803 cultures using an indirect light driven process, is reported.

The PBR design was the result of a cooperation between CNR-ISE and M2M Engineering (Naples, IT) who also constructed the PBR. It was

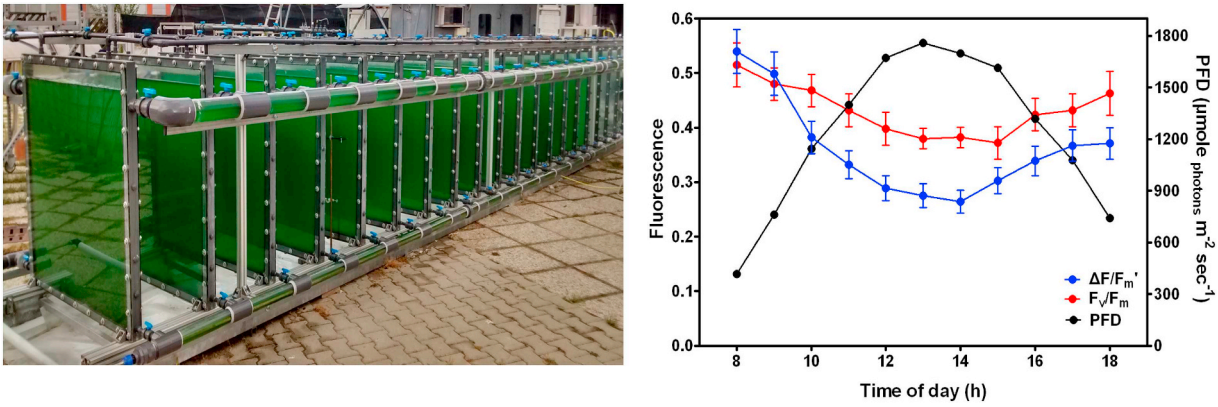

Fig. 10. The flat panel PhotoBioReactor (PBR) system (maximum working volume 1300 L) is composed of 20 vertical parallel plates 50 L, 1150 × 1150 × 50 mm ($W \times H \times D$), in direct communication with each other, left. Daily variation of maximum quantum efficiency of PSII photochemistry (F_v/F_m) and effective photochemical quantum yield of PSII ($\Delta F/F_m'$) right. Data are shown as mean ± SD, n = 3.

installed near the CNR experimental area of Sesto Fiorentino (Firenze), latitude 43.8° N, longitude 11.2° E. The PBR system (working volume of 1350 L) is composed of 20 vertical parallel plates, each of 50 L in volume measuring 1150 × 1150 × 50 mm (W × H × D), in direct communication with each other (Fig. 10). The inlet of the culture was achieved from the bottom part of each plate through two manifolds (i.e. 9 cm), while the outlet by two manifolds located in the upper part of the plates through which the culture flows back to the degasser. Both the inlet and outlet culture manifolds were constructed with transparent Plexiglas®, therefore, the culture circulates in a parallel mode (i.e. parallel independent compartments). With this type of circulation, the mixing time (T_{mix}) is reduced to about 2 min, which strongly facilitated pH control, CO_2 and nutrient supply, and O_2 degassing of the culture. The plate frames were initially made from PVC, which proved to be inadequate to cope with the temperature stress (up to 70–80 °C in summer, and below zero in winter). Therefore, new replacement ones were made from stainless steel. On top of each plate, there are four ports for accessing the culture (i.e., culture sampling, pH, temperature, oxygen probes). The glass windows of the PBR (1150 × 1150 mm) were made from tempered glass and fixed onto the frame with 32 metal screws (10 mm diameter).

Moreover, *Synechocystis* 6803 cultures at a concentration of 0.31 g L^{-1} (DW) were inoculated, in this large-scale system, maintained at a constant temperature of 28 °C during the light period and 20–22 °C at night (Fig. 10). The experiments were carried out with the plates placed at a distance of 0.5 m from each other. The culture behavior was regularly monitored through measurements of chlorophyll fluorescence and dry weight.

One major problem that occurred during the mass cultivation of *Synechocystis* was the contamination by a flagellate belonging to the species of *Poterioochromonas* (Synurophyceae). It is a very efficient phagotroph, with high growth rates, and *Synechocystis* represented its feeding prey. However, culturing *Synechocystis* at a pH above 11 proved to be a useful strategy to control the contamination with little effect on both culture productivity (less than 10% reduction) and biochemical composition of the biomass [49].

The time course of the maximum quantum yield of PSII, F_v/F_m, of the cultures grown in the PBR is shown in Fig. 10. The maximum F_v/F_m ratio decreased at noon to 72.2% of the initial morning value, indicating that the cultures were subject to photoinhibition, despite the high ratio between illuminated area and ground area occupied the reactor, which should allow a reduction of the light intensity on the plates (AR/AG) (Fig. 10). Indeed, portions of the plates were still exposed to high light (above 1000 μmol photons $m^{-2} s^{-1}$). The productivity of the culture was 185 mg L^{-1} day. The protein content of the biomass grown in the PBR decreased as carbohydrates content increased (Table 3).

Additionally, outdoor experiments of hydrogen production were carried out in a 50 L tubular PBR according to an indirect biophotolysis process. The PBR was inoculated with laboratory grown cultures and filled up with $BG11_0$ medium, obtaining a biomass concentration of 0.36 g L^{-1} and chlorophyll concentration of 7.48 ± 0.06 mg L^{-1}. Cell growth proceeded until all available nitrate was consumed. During the outdoor experiments, temperature was maintained at 28.0 ± 0.5 °C, with an initial pH of 7.5. *Synechocystis* cells started to accumulate carbohydrates during the day, and partially consumed them at night through respiration. At the end of the third day, before the start of the dark anoxic phase, carbohydrate content was 62.5 ± 0.4% of dry weight, while cell biomass concentration had increased from 0.36 ± 0.03 to 0.78 ± 0.01 g L^{-1} (Fig. 11). At the end of the carbohydrate accumulation phase, the cultures were bubbled with N_2 gas and covered with a black sheet to prevent light penetration. Hydrogen release started immediately after degassing for a final total output of 312 mL PBR^{-1} (i.e., 285 mL in the headspace plus 27 mL dissolved in the culture medium) showing a maximum hydrogen production rate of 0.778 mL H_2 $L^{-1} h^{-1}$ (Fig. 11). During this phase 0.06 g L^{-1} of carbohydrates were fermented (i.e., 2.8 g per reactor). Indeed, this study [48] represents the first attempt to scale up hydrogen production with *Synechocystis* 6803 cells cultivated outdoors under solar illumination, in a 50 L tubular PBR. One benefit of the indirect light driven process may come from taking advantage of the natural light/dark cycle, which strongly facilitates the separation of aerobic and anaerobic phases. However, further investigation is necessary to consistently improve the growth and carbohydrate accumulation in *Synechocystis* during the light driven phase, and reduce hydrogen fermentation in the dark anoxic conditions.

10. Data management and visualization

The CyanoFactory research consortium set the stage for a unifying goal to engineer a cyanobacterium into producing desired bio-molecules. In particular, the partners successfully worked at the interface between experimental and computational biology, which implies different understanding of data. The CyanoFactory KnowledgeBase (CFKB), which is a massive expansion of the WholeCell KB, provides a central data hub for members of the consortium and for disseminating project data to the research community. Thus, it provides ways for an improved collaboration between all partners within the CyanoFactory consortium. All partners were experts in different fields from microbial biotechnology or metabolic modelling, up to synthetic biology. The goal of CFKB was to bridge the gap between bio-engineers and bioinformaticians by providing user-friendly functionalities for working with experimental data and for visualizing and contextualizing it in different ways. Besides experimental data, further data is obtained from other biological databases.

Life science research is dominated by two conditions: interdisciplinarity and high-throughput. The former leads to highly diverse datasets from a content point of view while high-throughput yields massive amounts of data. Both aspects are reflected by the byte-growth of public bio-databases and the diversity of specialized databases. However, quite often more data leads to less understanding. Driven by the methodology of systems biology, a holistic view of genetic and metabolic regulatory processes is demanded. One important goal is the application of these systemic data for in silico modelling of biological processes or, ultimately, biological systems, i.e., cells, tissues, organisms. One step towards this goal was the successful prediction of the phenotype from the genotype in *Mycoplasma genitalium* [50]. The basis to solve this challenge was a database named WholeCell Knowledge Base (WholeCell KB) [51]. It contains experimental results from over 900 publications and includes more than 1900 experimentally observed parameters. Importantly, all data have been validated and curated by scientists.

Another important manually curated database is Brenda (brenda-enzymes.org), which contains over 1.5 million manually curated enzyme parameters. In contrast, GenBank (ncbi.nlm.nih.gov) contains around 180 million individual and 190 million complete genome shotgun sequences that are partially manually uploaded but not curated.

With the rising amount of biological data and the increasing capabilities of computer hardware, many attempts have been undertaken

Table 3
Daily variation of biomass (dry weight, DW), protein, carbohydrate and chlorophyll (Chl) contents of a *Synechocystis* 6803 culture in the flat panel PBR. Data are shown as mean ± SD, n = 3.

Daytime	DW (g L^{-1})	Protein (%)	Carbohydrate (%)	Chlorophyll (%)
08:00	0.32 ± 0.01	41.0 ± 1.0	12.6 ± 0.3	1.9 ± 0.1
10:00	0.33 ± 0.06	43.9 ± 7.5	13.0 ± 2.2	1.8 ± 0.1
12:00	0.34 ± 0.00	46.3 ± 0.6	15.2 ± 0.3	1.9 ± 0.1
14:00	0.42 ± 0.01	42.1 ± 0.7	16.3 ± 0.3	1.8 ± 0.1
16:00	0.44 ± 0.03	42.0 ± 2.7	20.1 ± 1.3	1.7 ± 0.1
18:00	0.51 ± 0.06	40.9 ± 5.2	20.5 ± 2.6	1.6 ± 0.1

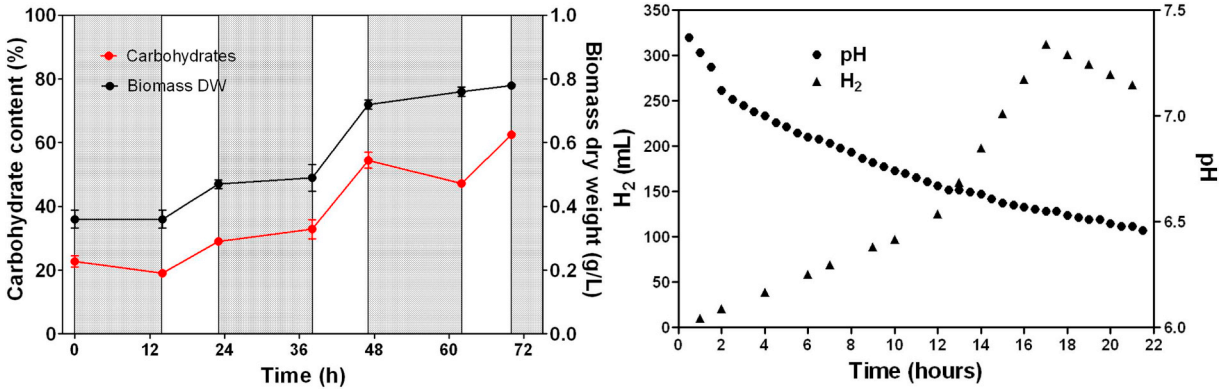

Fig. 11. Carbohydrate content and biomass concentration in a *Synechocystis* 6803 cell culture during an outdoor experiment. During the first 72 h, carbohydrate accumulation take place; at 72 h, the dark anaerobic/hydrogen production phase starts (day-white bars, night-gray bars), left. Data are shown as mean ± SD, n = 3. pH value and H_2 (mL) accumulation of the *Synechocystis* cell culture under a dark and anaerobic condition, right. Data from a single, representative experiment is shown.

to automatically harvest, store, cross-link and provide biological data in databases and databases of databases (i.e., data warehouses) [52]. However, automatically generated data collections are of limited value, especially in the context of large scale biological engineering as envisioned by the field of synthetic biology. Here, computer models of biological processes build the fundament to targeted (instead of trial-and-error) genetic engineering.

CFKB is a productive knowledge base, which handles all the information from our partners. Its advantage is that, besides holding information, it provides different visualization techniques and cross-links to other data sources. Uploading of experimental data is supported in different formats. The warehouse provides import functionality for FASTA, GenBank and System Biology Markup Language (SBML). The import runs as a background job and is automatically merged into the current dataset upon completion. All modifications to the knowledge base are logged, therefore, changes to all items can be retrieved and rolled back if necessary. Exports are possible in the import formats and furthermore in machine readable XML or JSON formats. The access to individual resources can be restricted by using permissions.

Besides the hierarchical view of the warehouse the user can group selected data in so called “baskets”. A user can create different baskets and group relevant items in them. The general structure of the organism is visualized by using a chromosome viewer. The viewer is fully interactive and provides filtering functionalities. Additional metadata is displayed beneath the genes.

Metabolic processes and interactions of biochemical components are visualized using the *Process Description Language* of the Systems Biology Graphical Notation (SBGN) [53]. SBGN represents the metabolic model of the organism in a way detailed enough for biochemists and is machine readable, therefore, supporting mathematical simulations inside the model. It should, however, be noted that SBGN proved to be confusing to the human eye. Thus, traditional visualizations, such as Boehringer Pathway Maps [54] or KEGG maps [55], may be preferred.

Metabolic modelling is provided as part of a CyanoDesign plug-in. Flux balance analysis (FBA) is used for the reconstruction of metabolic networks of organisms. A metabolic network consists of multiple enzymatic reactions with metabolites contained in a stoichiometric matrix (positive for production, negative for degradation). This network is solved using a linear solving method. The motivation behind CyanoDesign is allowing the bio-engineer to change the metabolic network in silico and to get a prediction, how the organism is likely to behave. A modelling approach saves valuable time because it gives hints how mutants of the organism may behave, resulting in a high amount of saved work in the lab. FBA is done using the library PyNetMet [56] and for improved quality of the simulation results the addition of more advanced algorithms like “Minimization of Metabolic Adjustment” (MOMA) can be used.

11. Conclusion

The European project CyanoFactory brought together ten partners (universities, research organizations, and enterprises) with a common goal to develop synthetic biology and photobioreactors, needed technologies to engineer cyanobacteria to produce chemicals, including fuels, in direct processes [57]. Molecular tools for the design and construction to engineer cyanobacteria were developed and have been used by the partners, and others, to demonstrate, e.g., that cells of *Synechocystis* PCC 6803 engineered for enhanced growth show increased ethanol production and higher biofuel to biomass ratio [58], introduction and characterization of a functional oxygen consuming device in *E. coli* cells [14], and the generation of a functional, semisynthetic [FeFe]-hydrogenase in *Synechocystis* cells [11]. Knowledge and competence to build and operate efficient photobioreactors outdoors have been established. Additionally, recent reviews include hydrogen production using novel photosynthetic cell factories with a focus on the design of an efficient cyanobacterial cell [59] and engineering cyanobacteria for biofuel production [1].

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Declaration of author contributions

The conception, design, and supervision of the project, and coordination of writing the manuscript, were done by P. Lindblad, M. Diano, M. Dolinar, M. Rögner, H.-J. Schmitz, P. Tamagnini, G. Torzillo, J.F. Urchueguía, P.C. Wright and R. Wünschiers. Experiments and generation of results, analysis and interpretation of data were done by D. Fuente, F. Borbe, B. Cicchi, J.A. Conejero, N. Couto, H. Čelešnik, S. Esposito, C. Evans, E.A. Ferreira, J. Keller, N. Khanna, G. Kind, A. Landels, L. Lemus, J. Noirel, S. Ocklenburg, P. Oliveira, C·C. Pacheco, J.L. Parker, J. Pereira, T.K. Pham, F. Pinto, S. Rexroth, A.M. Silva Benavides, M. Siurana, E. Touloupakis, A. Wegelius, K. Wiegand and M. Wutschel, the latter together with the supervisors. Text edition was performed by P. Lindblad and D. Fuente.

Statement of informed consent, human/animal rights

No conflicts, informed consent, human or animal rights applicable.

References

[1] R. Miao, A. Wegelius, C. Durall, F. Liang, N. Khanna, P. Lindblad, Engineering cyanobacteria for biofuel production, in: P. Hallenbeck (Ed.), Modern Topics in the Phototrophic Prokaryotes: Environmental and Applied Aspects, Springer International Publishing, Switzerland, 2017, pp. 351–393, , https://doi.org/10.1007/978-3-319-46261-5_11 chapter 11.

[2] J. Zhou, H. Zhang, H. Meng, Y. Zhu, G. Bao, Y. Zhang, Y. Li, Y. Ma, Discovery of a super-strong promoter enables efficient production of heterologous proteins in cyanobacteria, Sci. Rep. 4 (2014) 4500, , https://doi.org/10.1038/srep04500.

[3] C.J. Ramey, A. Barón-Sola, H.R. Aucoin, N.R. Boyle, Genome engineering in cyanobacteria: where we are and where we need to go, ACS Synth. Biol. 4 (11) (2015) 1186–1196, https://doi.org/10.1021/acssynbio.5b00043.

[4] J. Zhou, T. Zhu, Z. Cai, Y. Li, From cyanochemicals to cyanofactories: a review and perspective, Microb. Cell Factories 15 (1) (2016) 2, https://doi.org/10.1186/s12934-015-0405-3.

[5] A.L. Markley, M.B. Begemann, R.E. Clarke, G.C. Gordon, B.F. Pfleger, Synthetic biology toolbox for controlling gene expression in the cyanobacterium *Synechococcus* sp. strain PCC 7002, ACS Synth. Biol. 4 (5) (2015) 595–603, https://doi.org/10.1021/sb500260k.

[6] H.-H. Huang, D. Camsund, P. Lindblad, T. Heidorn, Design and characterization of molecular tools for a synthetic biology approach towards developing cyanobacterial biotechnology, Nucleic Acids Res. 38 (8) (2010) 2577–2593, https://doi.org/10.1093/nar/gkq164.

[7] E.A. Ferreira, C.C. Pacheco, F. Pinto, J. Pereira, P. Lamosa, P. Oliveira, B. Kirov, A. Jaramillo, P. Tamagnini, Expanding the toolbox for *Synechocystis* sp. PCC 6803: characterization of a set of novel promoters and validation of replicative vectors, Synth. Biol. 3 (1) (2018) ysy014, https://doi.org/10.1093/synbio/ysy014.

[8] T. Heidorn, D. Camsund, H.-H. Huang, P. Lindberg, P. Oliveira, K. Stensjö, P. Lindblad, Synthetic biology in cyanobacteria engineering and analyzing novel functions, Methods Enzymol. 497 (2011) 539–579 https://doi.org/10.1016/B978-0-12-385075-1.00024-X.

[9] S. Cardinale, A.P. Arkin, Contextualizing context for synthetic biology - identifying causes of failure of synthetic biological systems, Biotechnol. J. 7 (7) (2012) 856–866, https://doi.org/10.1002/biot.201200085.

[10] V.K. Mutalik, J.C. Guimaraes, G. Cambray, C. Lam, M.J. Christoffersen, Q.-A. Mai, A.B. Tran, M. Paull, J.D. Keasling, A.P. Arkin, D. Endy, Precise and reliable gene expression via standard transcription and translation initiation elements, Nat. Methods 10 (4) (2013) 354–360, https://doi.org/10.1038/nmeth.2404.

[11] A. Wegelius, N. Khanna, C. Esmieu, G.D. Barone, F. Pinto, P. Tamagnini, G. Berggren, P. Lindblad, Generation of a functional, semisynthetic [FeFe]-hydrogenase in a photosynthetic microorganism, Energy Environ. Sci. 11 (11) (2018) 3163–3167, https://doi.org/10.1039/C8EE01975D.

[12] F. Pinto, C.C. Pacheco, P. Oliveira, A. Montagud, A. Landels, N. Couto, P.C. Wright, J.F. Urchueguía, P. Tamagnini, Improving a *Synechocystis*-based photoautotrophic chassis through systematic genome mapping and validation of neutral sites, DNA Res. 22 (6) (2015) 425–437, https://doi.org/10.1093/dnares/dsv024.

[13] P. Oliveira, N.M. Martins, M. Santos, F. Pinto, Z. Büttel, N.A.S. Couto, P.C. Wright, P. Tamagnini, The versatile TolC-like Slr1270 in the cyanobacterium *Synechocystis* sp. PCC 6803, Environ. Microbiol. 18 (2) (2016) 486–502, https://doi.org/10.1111/1462-2920.13172.

[14] C.C. Pacheco, Z. Büttel, F. Pinto, G. Rodrigo, J. Carrera, A. Jaramillo, P. Tamagnini, Modulation of intracellular O_2 concentration in *Escherichia coli* strains using Oxygen Consuming Devices (OCDs), ACS Synth. Biol. 7 (7) (2018) 1742–1752, https://doi.org/10.1021/acssynbio.7b00428.

[15] M. Nyberg, T. Heidorn, P. Lindblad, Hydrogen production by the engineered cyanobacterial strain *Nostoc* PCC 7120 Δ*hup*W examined in a flat panel photobioreactor system, J. Biotechnol. 215 (2015) 35–43, https://doi.org/10.1016/j.jbiotec.2015.08.028.

[16] J.-H. Kwon, G. Bernát, H. Wagner, M. Rögner, S. Rexroth, Reduced light-harvesting antenna: consequences on cyanobacterial metabolism and photosynthetic productivity, Algal Res. 2 (2013) 188–195, https://doi.org/10.1016/j.algal.2013.04.008.

[17] L.E. Page, M. Liberton, H.B. Pakrasi, Reduction of photoautotrophic productivity in the cyanobacterium *Synechocystis* sp. strain PCC 6803 by phycobilisome antenna truncation, Appl. Environ. Microbiol. 78 (17) (2012) 6349–6351, https://doi.org/10.1128/AEM.00499-12.

[18] M. Imashimizu, G. Bernát, E.-I. Sunamura, M. Broekmans, H. Konno, K. Isato, ... T. Hisabori, Regulation of F0F1-ATPase from *Synechocystis* sp. PCC 6803 by γ and ϵ subunits is significant for light/dark adaptation, J. Biol. Chem. 286 (30) (2011) 26595–26602, https://doi.org/10.1074/jbc.M111.234138.

[19] P. Liauw, T. Mashiba, M. Kopczak, K. Wiegand, N. Muraki, H. Kubota, Y. Kawano, M. Ikeuchi, T. Hase, M. Rögner, G. Kurisu, Cloning, expression, crystallization and preliminary X-ray studies of the ferredoxin-NAD(P)+ reductase from the thermophilic cyanobacterium *Thermosynechococcus elongatus* BP-1, Acta Crystallogr. Sect. F: Struct. Biol. Cryst. Commun. 68 (9) (2012) 1048–1051, https://doi.org/10.1107/S1744309112031910.

[20] K. Wiegand, M. Winkler, S. Rumpel, D. Kannchen, S. Rexroth, T. Hase, C. Farès, T. Happe, W. Lubitz, M. Rögner, Rational redesign of the ferredoxin-NADP+-oxidoreductase/ferredoxin-interaction for photosynthesis-dependent H2-production, Biochimica Biophysica Acta 1859 (4) (2018) 253–262, https://doi.org/10.1016/j.bbabio.2018.01.006.

[21] O. Wright, G.-B. Stan, T. Ellis, Building-in biosafety for synthetic biology, Microbiology 159 (7) (2013) 1221–1235, https://doi.org/10.1099/mic.0.066308-0.

[22] H. Čelešnik, A. Tanšek, A. Tahirović, A. Vižintin, J. Mustar, V. Vidmar, M. Dolinar, Biosafety of biotechnologically important microalgae: intrinsic suicide switch implementation in cyanobacterium *Synechocystis* sp. PCC 6803, Biology Open 5 (4) (2016) 519–528, https://doi.org/10.1242/bio.017129.

[23] S. Kopfmann, S.K. Roesch, W.R. Hess, Type II toxin—antitoxin systems in the Unicellular Cyanobacterium *Synechocystis* sp. PCC 6803, Toxins 8 (7) (2016) 228, https://doi.org/10.3390/toxins8070228.

[24] A.M. Muro-Pastor, E. Flores, A. Herrero, C.P. Wolk, Identification, genetic analysis and characterization of a sugar-non-specific nuclease from the cyanobacteriu *Anabaena* sp. PCC 7120, Mol. Microbiol. 6 (20) (1992) 3021–3030, https://doi.org/10.1111/j.1365-2958.1992.tb01760.x.

[25] A.F.M. Altelaar, C.K. Frese, C. Preisinger, M.L. Hennrich, A.W. Schram, H.T.M. Timmers, M. Heck, S. Mohammed, Benchmarking stable isotope labeling based quantitative proteomics, J. Proteome 88 (2013) 14–26, https://doi.org/10.1016/j.jprot.2012.10.009.

[26] E. Brunk, K.W. George, J. Alonso-Gutierrez, M. Thompson, E. Baidoo, G. Wang, C.J. Petzold, D. McCloskey, J. Monk, L. Yang, E.J. O'Brien, T.S. Batth, H.G. Martin, A. Feist, P.D. Adams, J.D. Keasling, B.O. Palsson, T.S. Lee, Characterizing strain variation in engineered *E. coli* using a multi-omics-based workflow, Cell Systems 2 (5) (2016) 335–346, https://doi.org/10.1016/j.cels.2016.04.004.

[27] A. Landels, C. Evans, J. Noirel, P.C. Wright, Advances in proteomics for production strain analysis, Curr. Opin. Biotechnol. 35 (2015) 111–117, https://doi.org/10.1016/j.copbio.2015.05.001.

[28] T. Kaneko, A. Tanaka, S. Sato, H. Kotani, T. Sazuka, ... S. Tabata, Sequence analysis of the genome of the unicellular cyanobacterium *Synechocystis* sp. strain PCC6803. I. Sequence features in the 1 Mb region from map positions 64% to 92% of the genome, DNA Res. 2 (4) (1995) 153–166, https://doi.org/10.1093/dnares/2.4.153.

[29] Takakazu Kaneko, S. Sato, H. Kotani, A. Tanaka, E. Asamizu, Y. Nakamura, et al., Sequence analysis of the genome of the unicellular cyanobacterium *Synechocystis* sp. strain PCC6803. II. Sequence determination of the entire genome and assignment of potential protein-coding regions, DNA Res. 3 (3) (1996) 109–136.

[30] V.F. Kalb, R.W. Bernlohr, A new spectrophotometric assay for protein in cell extracts, Anal. Biochem. 82 (2) (1977) 362–371, https://doi.org/10.1016/0003-2697(77)90173-7.

[31] A. Montagud, E. Navarro, P. Fernández de Córdoba, J.F. Urchueguía, K.R. Patil, Reconstruction and analysis of genome-scale metabolic model of a photosynthetic bacterium, BMC Syst. Biol. 4 (1) (2010) 156, https://doi.org/10.1186/1752-0509-4-156.

[32] A. Montagud, A. Zelezniak, E. Navarro, P. Fernández de Córdoba, J.F. Urchueguía, K.R. Patil, Flux coupling and transcriptional regulation within the metabolic network of the photosynthetic bacterium *Synechocystis* sp. PCC6803, Biotechnol. J. 6 (3) (2011) 330–342, https://doi.org/10.1002/biot.201000109.

[33] W. Baebprasert, S. Jantaro, W. Khetkorn, P. Lindblad, A. Incharoensakdi, Increased H_2 production in the cyanobacterium *Synechocystis* sp. strain PCC 6803 by redirecting the electron supply via genetic engineering of the nitrate assimilation pathway, Metab. Eng. 13 (5) (2011) 610–616, https://doi.org/10.1016/j.ymben.2011.07.004.

[34] F. Gutthann, M. Egert, A. Marques, J. Appel, Inhibition of respiration and nitrate assimilation enhances photohydrogen evolution under low oxygen concentrations in *Synechocystis* sp. PCC 6803, Biochimica et Biophysica Acta (BBA)-Bioenergetics 1767 (2) (2007) 161–169, https://doi.org/10.1016/j.bbabio.2006.12.003.

[35] K. Yizhak, T. Benyamini, W. Liebermeister, E. Ruppin, T. Shlomi, Integrating quantitative proteomics and metabolomics with a genome-scale metabolic network model, Bioinformatics (Oxford, England) 26 (12) (2010) i255–i260, https://doi.org/10.1093/bioinformatics/btq183.

[36] D.J. Lea-Smith, P. Bombelli, J.S. Dennis, S.A. Scott, A.G. Smith, C.J. Howe, Phycobilisome-deficient strains of *Synechocystis* sp. PCC 6803 have reduced size and require carbon-limiting conditions to exhibit enhanced productivity, Plant Physiol. 165 (2014) 705–714, https://doi.org/10.1104/pp.114.237206.

[37] D. Fuente, J. Keller, J.A. Conejero, M. Rögner, S. Rexroth, J.F. Urchueguía, Light distribution and spectral composition within cultures of micro-algae: quantitative modelling of the light field in photobioreactors, Algal Res. 23 (2017) 166–177, https://doi.org/10.1016/j.algal.2017.01.004.

[38] D. Fuente, C. Lizama, J.F. Urchueguía, J.A. Conejero, Estimation of the light field inside photosynthetic microorganism cultures through Mittag-Leffler functions at depleted light conditions, J. Quant. Spectrosc. Radiat. Transf. 204 (2018) 23–26, https://doi.org/10.1016/j.jqsrt.2017.08.012 Supplement C.

[39] P.M. Schenk, S.R. Thomas-Hall, E. Stephens, U.C. Marx, J.H. Mussgnug, C. Posten, O. Kruse, B. Hankamer, Second generation biofuels: high-efficiency microalgae for biodiesel production, Bioenergy Research 1 (1) (2008) 20–43, https://doi.org/10.1007/s12155-008-9008-8.

[40] F.G. Acién Fernández, J.M. Fernández Sevilla, J.A. Sánchez Pérez, E. Molina Grima, Y. Chisti, Airlift-driven external-loop tubular photobioreactors for outdoor production of microalgae: assessment of design and performance, Chem. Eng. Sci. 56 (8) (2001) 2721–2732, https://doi.org/10.1016/S0009-2509(00)00521-2.

[41] F.G. Acién Fernández, J.M. Fernández Sevilla, E. Molina Grima, Costs analysis of microalgae production, Biofuels from Algae (2019) 551–566, https://doi.org/10.1016/B978-0-444-64192-2.00021-4.

[42] M. Morweiser, O. Kruse, B. Hankamer, C. Posten, Developments and perspectives of photobioreactors for biofuel production, Appl. Microbiol. Biotechnol. 87 (4) (2010) 1291–1301, https://doi.org/10.1007/s00253-010-2697-x.

[43] J.C. Weissman, R.P. Goebel, J.R. Benemann, Photobioreactor design: mixing, carbon utilization, and oxygen accumulation, Biotechnol. Bioeng. 31 (4) (1988) 336–344, https://doi.org/10.1002/bit.260310409.
[44] G. Torzillo, B. Pushparaj, J. Masojidek, A. Vonshak, Biological constraints in algal biotechnology, Biotechnol. Bioprocess Eng. 8 (6) (2003) 338–348, https://doi.org/10.1007/BF02949277.
[45] M.A. Meyer, A. Weiss, Life cycle costs for the optimized production of hydrogen and biogas from microalgae, Energy 78 (2014) 84–93, https://doi.org/10.1016/j.energy.2014.08.069.
[46] P. Bermann, P. Ripplinger, L. Beyer, W. Trösch, Disposable flat panel airlift photobioreactors, Chemie Ingenieur Technik 85 (1–2) (2013) 202–205, https://doi.org/10.1002/cite.201200132.
[47] C.-G. Lee, B.O. Palsson, High density algal photobioreactors using ligth-emitting diodes, Biotechnol. Bioeng. 44 (10) (1994) 1161–1167, https://doi.org/10.1002/bit.260441002.
[48] E. Touloupakis, A.M.S. Benavides, B. Cicchi, G. Torzillo, Growth and hydrogen production of outdoor cultures of *Synechocystis* PCC 6803, Algal Res. 18 (2016) 78–85, https://doi.org/10.1016/j.algal.2016.06.010.
[49] E. Touloupakis, B. Cicchi, A.M.S. Benavides, G. Torzillo, Effect of high pH on growth of *Synechocystis* sp. PCC 6803 cultures and their contamination by golden algae (*Poterioochromonas* sp.), Appl. Microbiol. Biotechnol. 100 (3) (2016) 1333–1341, https://doi.org/10.1007/s00253-015-7024-0.
[50] J.R. Karr, J.C. Sanghvi, D.N. Macklin, M.V. Gutschow, J.M. Jacobs, B. Bolival, M.W. Covert, A whole-cell computational model predicts phenotype from genotype, Cell 150 (2) (2012) 389–401, https://doi.org/10.1016/j.cell.2012.05.044.
[51] J.R. Karr, J.C. Sanghvi, D.N. Macklin, A. Arora, M.W. Covert, Whole CellKB: model organism databases for comprehensive whole-cell models, Nucleic Acids Res. 41 (D1) (2013) D787–D792, https://doi.org/10.1093/nar/gks1108.
[52] G. Kind, E. Zuchantke, R. Wünschiers, CyanoFactory knowledge base & synthetic biology - a plea for human curated bio-databases, Proceedings of the International Conference on Bioinformatics Models, Methods and Algorithms - Volume 1: BIOINFORMATICS, (BIOSTEC 2015), SciTePress, 2015, pp. 237–242, , https://doi.org/10.5220/0005285802370242.
[53] N. Le Novère, M. Hucka, H. Mi, S. Moodie, F. Schreiber, A. Sorokin, E. Demir, K. Wegner, M.I. Aladjem, S.M. Wimalaratne, F.T. Bergman, R. Gauges, P. Ghazal, H. Kawaji, L. Li, Y. Matsuoka, A. Villéger, S.E. Boyd, L. Calzone, M. Courtot, U. Dogrusoz, T.C. Freeman, A. Funahashi, S. Ghosh, A. Jouraku, S. Kim, F. Kolpakov, A. Luna, S. Sahle, E. Schmidt, S. Watterson, G. Wu, I. Goryanin, D.B. Kell, C. Sander, H. Sauro, J.L. Snoep, K. Kohn, H. Kitano, The systems biology graphical notation, Nat. Biotechnol. 27 (8) (2009) 735–741, https://doi.org/10.1038/nbt.1558.
[54] Y. Marcus, H. Altman-Gueta, Y. Wolff, M. Gurevitz, Rubisco mutagenesis provides new insight into limitations on photosynthesis and growth in *Synechocystis* PCC6803, J. Exp. Bot. 62 (12) (2011) 4173–4182, https://doi.org/10.1093/jxb/err116.
[55] M. Kanehisa, S. Goto, Y. Sato, M. Kawashima, M. Furumichi, M. Tanabe, Data, information, knowledge and principle: back to metabolism in KEGG, Nucleic Acids Res. 42 (D1) (2014) D199–D205, https://doi.org/10.1093/nar/gkt1076.
[56] D. Gamermann, A. Montagud Aquino, R.J. Infante, J. Triana, J.F. Urchueguía, P.J. de Córdoba Castellá, PyNetMet: python tools for efficient work with networks and metabolic models, Computational and Mathematical Biology 3 (5) (2014) 1–11 https://www.iconceptpress.com/journal/computational-and-mathematical-biology/article/?id=1402001135.
[57] R. Wünschiers, Making-of synthetic biology: The European CyanoFactory research consortium, in: K. Hagen, Engelhard Margret, G. Toepfer (Eds.), Ambivalences of Creating Life: Societal and Philosophical Dimensions of Synthetic Biology, Springer International Publishing, Cham, 2016, pp. 55–72, , https://doi.org/10.1007/978-3-319-21088-9_3.
[58] F. Liang, E. Englund, P. Lindberg, P. Lindblad, Engineered cyanobacteria with enhanced growth show increased ethanol production and higher biofuel to biomass ratio, Metab. Eng. 46 (2018) 51–59, https://doi.org/10.1016/j.ymben.2018.02.006.
[59] P. Lindblad, Hydrogen production using novel photosynthetic cell factories. Cyanobacterial hydrogen production: design of efficient organisms, in: M. Seibert, G. Torzillo (Eds.), Microalgal Hydrogen Production: Achievements and Perspectives, G. Royal Society of Chemistry, UK, 2018, pp. 323–334, , https://doi.org/10.1039/9781849737128-00323 chapter 13.