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...
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 Ppcp and PpsbA, have been widely used. Improved variants of the Ppcp 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 Ppcp 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 to introduce isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible transcription initiation systems. Two lac operon 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 *Ppcp* 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*, *Ptacl* and *P17* (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
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-B00344 (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 in all constructs from the same transcription and translation initiation unit in the presence of different GOIs. We cloned PtrcO1-RBS* upstream of the codon-optimized hydA from Chlamydomonas reinhardtii and PtrcO1-B00344 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-adaptor 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-adaptor 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: gcaaa-caagtgaagctgatgcr, RT HydA-R: ctcctatggcatatccctaa; RTMatE-F: cgtcagag-gaaatgaaatatatcg, RTMatE-R: gcatcaatttctttaaagaacaaaat; RTMatF-F: ctattccctcttattagaaaaag, RTMatF-R: cgtaattcaaattccttcgtaac; RTMatG-F: caaatcttcccctattagaaaag, RTMatG-R: ccagcaaccttctttctgctct; RT16S-F: ccaactcctctctcagaaaag, RT16S-R: ctgctggcaagggagtca.
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 - \textit{ggpS} and \textit{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 \textit{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 \textit{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 \textit{Synechocystis} 6803.

For modulation of the chassis intracellular oxygen concentration, important when introducing synthetic modules encoding O$_2$-sensitive
enzymes (such as hydrogenases), several Oxygen Consuming Devices (OCD) were designed and synthesized. The OCDs are comprised of the ccoE 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 5L photobioreactor (3L 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-Δε mutant displayed a fourfold increased electron transport rate in comparison with the wild type. However, only 25% of the electrons produced in the Olive-Δε 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 Synecocysts 6803 that would present a biological barrier against the spread of genetically modified Synecocysts 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
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

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**Table 1**

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

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<th>FNR variants</th>
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<td>FNRL/s</td>
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<tr>
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<td>Fd-D22A</td>
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<tr>
<td>FNRL_K75A</td>
<td>Fd-D61A</td>
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<tr>
<td>FNRL_K75D</td>
<td>Fd-D22A-D61A</td>
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<td>FNRL_K78A</td>
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<td>FNRL_K75D-K78D</td>
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**Fig. 8.** The native TAS (A) and engineered effector/inhibitor device 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<sub>ind</sub>, inducible promoter; P<sub>const</sub>, 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 Zn2+ 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 13C 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>
<thead>
<tr>
<th>Promoter-nuclease</th>
<th>Promoter-nuclease inhibitor</th>
<th>Switch activity</th>
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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 during peptide separation are substantially increased (Fig. 9). As a further application, genetic changes giving 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 H2 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 H2 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
models 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 CO2 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 at- tenuation 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 CO2 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 CO₂ 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 revitalisation 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

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Fig. 10. The flat panel PhotoBioReactor (PBR) system (maximum working volume 1300 L) is composed of 20 vertical parallel plates 50 L, 1150 x 1150 x 50 mm (W x H x 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 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).

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### 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.

<table>
<thead>
<tr>
<th>Daytime</th>
<th>DW (g L$^{-1}$)</th>
<th>Protein (%)</th>
<th>Carbohydrate (%)</th>
<th>Chlorophyll (%)</th>
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<tr>
<td>08:00</td>
<td>0.32 ± 0.01</td>
<td>41.0 ± 1.0</td>
<td>12.6 ± 0.3</td>
<td>1.9 ± 0.1</td>
</tr>
<tr>
<td>10:00</td>
<td>0.33 ± 0.06</td>
<td>43.9 ± 7.5</td>
<td>13.0 ± 2.2</td>
<td>1.8 ± 0.1</td>
</tr>
<tr>
<td>12:00</td>
<td>0.34 ± 0.00</td>
<td>46.3 ± 0.6</td>
<td>15.0 ± 3.3</td>
<td>1.9 ± 0.1</td>
</tr>
<tr>
<td>14:00</td>
<td>0.42 ± 0.01</td>
<td>42.1 ± 0.7</td>
<td>16.3 ± 0.3</td>
<td>1.8 ± 0.1</td>
</tr>
<tr>
<td>16:00</td>
<td>0.44 ± 0.03</td>
<td>42.0 ± 2.7</td>
<td>20.1 ± 1.3</td>
<td>1.7 ± 0.1</td>
</tr>
<tr>
<td>18:00</td>
<td>0.51 ± 0.06</td>
<td>40.9 ± 5.2</td>
<td>20.5 ± 2.6</td>
<td>1.6 ± 0.1</td>
</tr>
</tbody>
</table>
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].

Acknowledgement

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Declaration of author contributions

Statement of informed consent, human/animal rights

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

References


