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Review

Evaluation of New Genetic Toolkits and Their Role for Ethanol Production in Cyanobacteria

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Abstract: Since the public awareness for climate change has risen, increasing scientific effort has been made to find and develop alternative resources and production processes to reduce the dependency on petrol-based fuels and chemicals of our society. Among others, the biotechnological fuel production, as for example fermenting sugar-rich crops to ethanol, is one of the main strategies. For this purpose, various classical production systems like *Escherichia coli* or *Saccharomyces cerevisiae* are used and have been optimized via genetic modifications. Despite the progress made, this strategy competes for nutritional resources and agricultural land. To overcome this problem, various attempts were made for direct photosynthetic driven ethanol synthesis with different microalgal species including cyanobacteria. However, compared to existing platforms, the development of cyanobacteria as photoautotrophic cell factories has just started, and accordingly, the ethanol yield of established production systems is still unreached. This is mainly attributed to low ethanol tolerance levels of cyanobacteria and there is still potential for optimizing the cyanobacteria towards alternative gene expression systems. Meanwhile, several improvements were made by establishing new toolboxes for synthetic biology offering new possibilities for advanced genetic modifications of cyanobacteria. Here, current achievements and innovations of those new molecular tools are discussed.

Keywords: biofuel; synthetic biology; expression system; *Synechocystis* sp. PCC6803

1. Introduction

In the 18th century, the industrialization and with it the consumption of fossil fuels started. Since then, the global energy demand has risen due to the growing population and fossil fuels form the basis for the global energy supply [1]. However, these fuels are not renewable and their consumption sustainably affects ecological consequences, like the continuously increasing CO_2 emissions. Therefore, the need for alternative, renewable and eco-friendly energy sources increases while fossil resources are becoming more and more exhausted [2–4]. The development of renewable energies to maintain the ecological growth of the planet while protecting the environment is a big challenge. Using the harmful CO_2 for the production of highly needed biofuels is a promising approach, which has to be further investigated. So far, several feedstocks have been used to produce biofuels like bioethanol and biodiesel. In the beginning, food crops like corn, sugarcane, soybean, wheat, rapeseed and palm were used for their production. As all of these first generation feedstocks are based on food, they compete with the nutritional demands of the increasing population. Additionally, arable land is limited on Earth, which causes food prices to rise further if food will be used for biofuel production [5,6]. In comparison, the second generation feedstocks include non-edible resources, like lignocellulosic plants such as cereal straw, forest residues and vegetative grasses. They serve as substrates for ethanol producers such as *Saccharomyces cerevisiae* or *Zymomonas mobilis*. Here, pre-treatment of these materials to release the sugars is needed, sophisticated technologies are required and commercialization is the major limitation [6]. Further investigations consider the direct conversion of CO_2 into biofuels using

photosynthesis and the use of microalgae as a third generation feedstock. In general, microalgae are unique small bio-factories containing a large number of industrially valuable substances like fatty acids (e.g., PUFAs—poly unsaturated fatty acids), pigments (e.g., carotenoids and chlorophylls) and proteins, which enable the use of these microorganisms for a huge variety of applications like nutrition, fertilizers, cosmetics and energy feedstocks [6,7]. Furthermore, microalgae have higher growth rates compared to terrestrial crops while requiring ~49–132 times less area compared to soybean crops [6,8]. Moreover, their cultivation is not bound to valuable cultivable land. The great diversity of microalgae, which includes both eukaryotes and prokaryotes, offers great potential for their versatile use in biofuel production. Because of the lipids contained, up to 60–65% of dry weight eukaryotic microalgae can be used for the production of biodiesel or alternatively as feedstock for the production of ethanol [5,9]. The direct photosynthetic driven ethanol production would combine the general advantages of a more ecological biofuel production process without need of a fermentation feedstock, cultivated on valuable arable land. Therefore, costs and process time for pre-treatment of fermentation feedstock are eliminated. Due to extensive research over the last decades, the direct, photosynthetic driven production of ethanol could be realized by genetically modified cyanobacteria via the overexpression of genes. Here, the main improvements were made with special representatives, namely *Synecococcus elongatus* and *Synechocystis* sp. PCC6803 (*Synechocystis*) for cyanobacteria. A huge progress in evaluation of photosynthesis, algal metabolism and methods has been made with these model organisms. The genomes of both organisms are sequenced and various methods and genetic toolkits have been and are being established [10,11]. Even though the productivity is still behind compared to other production processes, recently developed genetic toolkits were not used for additional improvements. For a cost-efficient production, further improvement of cultivation techniques, like the design of highly efficient photobioreactors, has to be done.

Since the highest bioethanol titre out of cyanobacteria could be produced with genetically modified *Synechocystis*, it will be evaluated in more detail and compared to existing production systems. Mainly, the previously used toolkits, their application for bioethanol production as well as further potential improvements will be summarized and discussed.

2. Comparison of Ethanol Production

Choosing a suitable production system for a certain product turns out to be challenging, as each system provides different assets and drawbacks depending on the aim to be obtained. Important parameters for the production of ethanol are the capability of an organism to synthesize ethanol, their tolerance to the substance and a cheap way to gain high yield [12]. As common expression systems like mammalian cell lines and plants are either too expensive in cultivation or not capable of synthesizing ethanol, these expression systems are not further discussed in this work [13,14]. In contrast, bacteria such as the model organism *Escherichia coli* or yeasts like *S. cerevisiae* convert sugars into ethanol during fermentation. Therefore, they are used in the first two generations of biofuel production [15,16]. Both organisms are fast-growing with doubling times of 30 min and 90 min respectively, easy in handling, reaching high cell densities and, hence, are able to synthesize high yields of bioethanol due to the huge amount of available molecular tools for improving the production rate [14,17–19]. Therefore, those organisms already contribute to a more ecological biofuel production. However, the media contains organic carbon sources, which need to be produced prior the fermentation step leading to increasing culture media costs [20]. In contrast, cyanobacteria perform photosynthesis by fixing atmospheric CO_2 in the presence of light as carbon source (see Table 1) [21]. Since salts are the only supplements in the culture media, the nutritional costs of cyanobacteria like *Synechocystis* or *Synechococcus* sp. are less compared to those of bacteria and yeast. In addition, there is no competition with agricultural land for the production of fermentation feedstock, by direct photosynthesis driven ethanol production with cyanobacteria [7,21]. So far, cyanobacteria have to be genetically modified to synthesize ethanol. Therefore, the cultivation has to take place in an enclosed photobioreactor [22]. As production yield of cyanobacteria is linked to the photosynthetic efficiency, irradiation is an important cultivation

parameter. Hence, lacking irradiation during night, under ambient light conditions and for some species by excessive solar radiation during summer decrease the overall production efficiency [23]. This is because intracellular storage substances are metabolized leading to a decrease in product yield. In order to prevent this, artificial light sources are needed during night, which increase the overall cultivation cost [24]. Alternatively, for some heterotrophic cyanobacteria, like *Synechocystis*, the addition of an organic carbon source in the dark phase could prevent the product degradation, whereby again the cultivation costs would increase [23]. For the overall cost-efficient large-scale production, the design of a photobioreactor and the general production process has to be optimized for the ethanol production, which includes the possibilities for downstream processing [25,26]. Since ethanol is secreted from all described organisms, costly cell disruption methods are not needed and, due to its volatility, it can be harvested within a distillation process [20,22].

Table 1. Comparison between cyanobacterial and currently used ethanol production systems, based on process relevant parameters.

Process Parameters	Bacteria (e.g., *E. coli*)	Yeast (e.g., *S. cerevisiae*)	Cyanobacteria (e.g., *Synechocystis*)
Doubling Time	30 min[a]	90 min[a]	8–12 h[b]
Carbon Source	Sugar[c]	Sugar[c,d]	CO_2[c,e]
Feedstock Processing	Feedstock:[d] Cultivation Pre-treatment	Feedstock:[d] Cultivation Pre-treatment	non
Ethanol Production	Heterotrophic[e]	Heterotrophic[d]	Photoautotrophic[f]
Downstream Processing	Ethanol separation and purification	Ethanol separation and purification	Ethanol separation and purification
Productivity (g L^{-1})	20.7 per 96 h[c]	130.12 per 65 h[g]	5.5 per 26 days[i]
Space Time Yield (g L^{-1} d^{-1})	5.18[c]	104.52[h]	0.285[j]

[a] [14]; [b] [27]; [c] [21]; [d] [28]; [e] [16]; [f] [22]; [g] [29]; [h] [30]; [i] [31]; [j] [32].

However, since the produced ethanol yield in cyanobacteria is still very low (see Table 1), for industrial applications a distillation process would be too costly. Therefore, alternative methods like for example different stripping methods need to be investigated [12,33]. Currently, so far the highest productivity of 0.285 g L^{-1} d^{-1} in cyanobacteria was achieved by Lopes da Silva et al. [32] and the highest ethanol titre of 5.50 g L^{-1} in 26 days was reached by Gao et al. [31]. Compared to the other model organisms like *S. cerevisiae* or *E. coli* with 130 g L^{-1} in 65 h and 20.7 g L^{-1} in 96 h, respectively, there is still a big step to be made in cyanobacteria [21]. Since the first production of an industrial relevant substance extracted from cyanobacteria was in 1998, these organisms have been used as a production system for valuable products since the turn of the century [23,34]. Compared to the most commonly utilized organism *E. coli*, which has been used for decades, microalgae are a very young expression system [35]. Therefore, the research on new molecular toolkits to increase production yields and lowering the production costs for cyanobacteria are just at the beginning, but may in the end lead to a more sustainable biofuel production.

3. New Toolkits

Over the years, an extensive research effort was made to develop tools for genetic optimizations for cyanobacteria like *Synechocystis* (Figure 1).

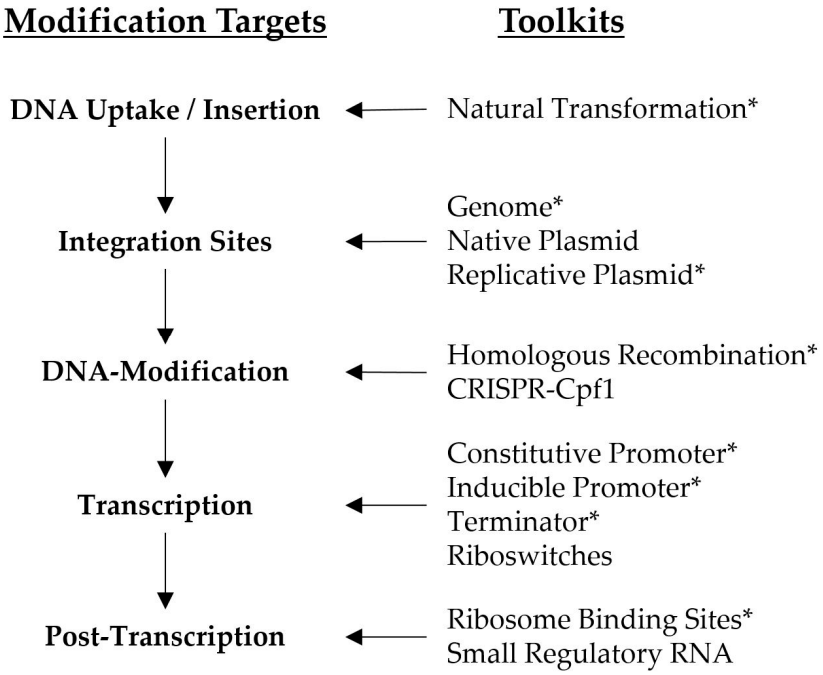

Figure 1. Overview of molecular toolkits and their modification targets in the cellular process of gene expression for *Synechocystis* sp. PCC6803. CRISPR (clustered regularly interspaced short palindromic repeats); *—Toolkits applied for ethanol production in *Synechocystis* sp. PCC6803 so far.

3.1. DNA Introduction and Modification

As a prokaryote, the intracellular organisation of cyanobacteria is less complex compared to eukaryotic microalgae. Therefore, exogenous DNA can only be integrated as an autonomously replicative plasmid DNA into one of the naturally occurring plasmids or the host genome. Thereby, the polyploidy of *Synechocystis* has to be considered. Depending on physiological and environmental parameters, the number of genome copies is highly variable and may range between dozens to over 200 genome copies per cell [36,37]. For this reason, a segregation step is necessary after each transformation, to ensure that all genome copies carry the modified DNA sequence, leading to a stable integration or knock-out of the desired DNA sequence [38,39]. The different parameters for the transformation of *Synechocystis* were systematically characterized by Kufryk et al. [40] and Zang et al. [41]. Among the tested methods, natural transformation is the most favourable one with the highest transformation efficiency [41]. Besides other classical parameters, like the amount and structure of the transformed DNA or the incubation parameters (time, w/ o light, etc.), the knock-out of the gene *sll1354*, annotated as a single-stranded-DNA-specific exonuclease *recJ*, resulted in a 100-fold increase of the transformation efficiency of replicative plasmids [40]. If genomic integration is desired, double homologous recombination is the method of choice. Zang et al. [41] also tested the impact of the length of the homologous sites and determined a positive correlation between the length of the homologous sites and the transformation efficiency. Different sites are used for genomic integration and show no phenotypic effect [31,39,42,43]. Besides genomic integration, different approaches for plasmid-based expression were attempted. On the one hand, the integration of expression cassettes into the native plasmids pCA2.4 and pCC5.2 were tested and resulted in an 8- to 20-fold higher reporter gene fluorescence compared to genomic integration [39,43]. On the other hand, self-replicating plasmids with different origins of replications as expression platforms were investigated. Here, plasmids harbouring either the heterologous broad-host range origin RSF1010 or the native origins of pCA2.4, pCB2.4 or pCC5.2 were compared with each other [44,45]. Comparing eYFP (enhanced yellow fluorescence protein) fluorescence intensities between RSF1010, pCA2.4 and pCB2.4 based shuttle vectors, 50% higher intensities were reached using the native origins [45]. When determining

the relative copy number of RSF1010 and pCC5.2 based shuttle vectors, results for the RSF1010 based plasmid indicate that it is maintained with a similar copy number to the genome of *Synechocystis*. In contrast, pCC5.2 is maintained in a 5 to 10 times higher copy number compared to the host genome, depending on the genomic reference gene [44]. These results suggest, that native origins are more favourable for shuttle vector applications with the aim of high expression rates.

Before CRISPR-Cas9 (clustered regularly interspaced short palindromic repeats and CRISPR associated protein 9) and related methods were established, genome-editing technologies like TALEN (transcription activator like effector nucleases) and ZFN (zinc finger nucleases) were not applied in *Synechocystis* [46]. Though CRISPR-Cas9 was successfully established in other organisms, the permanent expression of conventional Cas9 seems to be toxic to cyanobacteria in general [47–49]. However, the transient expression of the *cas9* gene lead to targeted gene modifications *S. elongatus* UTEX 2973 [49]. With *S. elongatus* UTEX 2973, *Anabaena sp.* PCC7120 and *Synechocystis*, the nuclease Cpf1 from *Francisella novicida* was tested as an alternative to the Cas9 protein. In addition to the strongly reduced toxicity, successful marker-less gene integrations, knock-outs and point mutations were demonstrated in all of the tested organisms using the CRISPR-Cpf1 system [48]. To resolve the issues for specific homozygous knock-out or knock-down, CRISPRi (CRISPR interference) was developed. This variant of the CRISPR-Cas9 system carries a point mutation in the Cas9 (dCas9) protein and, thereby, its nuclease activity is eliminated. In the research data presented so far for *Synechocystis*, the dCas9 protein does not show any cell toxicity and depending on the target gene, a repression rate between 50% to 95% was reached [50,51].

3.2. Transcription

As each promoter has its own characteristics, it is essential to choose the appropriate promoter for different applications. For *Synechocystis*, a wide selection of different promoter sequences is available. Besides their general control status, whether they are constitutive or inducible, their strength is the key parameter. For inducible promoters, further parameters have to be assessed, such as the fold-change, the relative difference between the induced and uninduced expression rate of the regulated genes, the inducer substance itself and the possible need for a repressor.

Since cultivation of cyanobacteria is most often performed with continuous illumination, promoters associated with the photosystem are classified as constitutive. Additionally, since photosystem-genes have to be highly expressed, their promoters normally belong to the strongest promoters in microalgae. In *Synechocystis*, the promoters for the large subunit of RuBisCo (Ribulose-1,5-bisphosphate carboxylase/oxygenase) P_{rbcL}, the D1 subunit of photosystem II P_{psbA2} or the β-subunit of phycocyanin P_{cpc560}, also called P_{cpcB}, are examples of such strong constitutive promoters. To further enhance their expression strength, different variants of these three promoters were generated and tested. The highest expression rates were reached with full length sequences of P_{cpc560} and P_{rbcL} [52,53], whereas the expression rate of P_{psbA2} increased when shortened sequences were used as promoter [54]. Liu and Pakrasi [45] examined the strength of twelve different native promoters with P_{cpc560} as the strongest known promoter in *Synechocystis* so far. Using this promoter, a protein expression level of approximately 15% of the total soluble protein content was reached [53].

In the effort of developing a complete temporally controlled gene expression, different inducible promoters for *Synechocystis* have been described. Here, different ion induced promoters like the P_{nrsB} induced by Ni^{2+} or P_{ziaA} induced by Zn^{2+} are in use. However, those promoters share the problem of crosstalk between different metal ions. For this reason and the high sensitivity of those promoters, it is difficult to control the induction level, because even residual metal ions in not appropriately cleaned glassware may induce gene expression [54–58]. Aside from native promoters, different promoters from *E. coli* were adapted for *Synechocystis*. Here, the most prominent examples are the P_{trc1O} and P_{tetR} promoters. However, both of them come with different limitations. P_{trc1O} is reported to be one of the strongest promoters for *Synechocystis*, but due to its leakiness, the transcription cannot be repressed efficiently [39,45,52]. A good example for the difficulties of adapting *E. coli* originated promoters to

Synechocystis is the P_{tetR} promoter. Whereas in *E. coli* P_{tetR} is a very strong promoter, this is not the case in *Synechosystis*. Therefore, Huang and Lindblad [59] generated a promoter library by altering the promoter sequence and developed promoters of different strength and inducibility. Besides that, another major disadvantage of the P_{tetR} promoter is the light instability of the inducer substance anhydrotetracycline. Another very promising promoter library was presented by Albers et al. [60]. Their developed $P_{sca6\text{-}2}$ promoter showed a high expression rate combined with a good repression rate of 98.9 ± 0.9%.

In addition, terminator sequences are important for the transcription of heterologous genes. The strength of a terminator sequence varies with the genetic composition of the expression cassette. Therefore, different combinations of regulatory sequences for each expression cassette have to be tested [45].

3.3. Post-Transcriptional Control Elements

After transcription, further processes may interfere with the expression of heterologous genes. RBS (ribosome binding sites) are important for efficient translation initiation in *Synechocystis*. However, the behaviour of different RBS in combination with different promoters and genes appear to be rather unpredictable [45,54]. For *Synechocystis,* non-coding sRNA (small regulatory RNA sequences) are described as well. sRNAs are very important for post-transcriptional regulation and are involved in different cellular processes like stress response, nutrient assimilation and even in the regulation of photosynthesis. In general, there are two different mechanisms described for their regulatory function. Either, longer sRNAs bind their counter mRNA over a long anti-sense sequence and, thereby, mediate the degradation via a cellular RNase, or short sRNAs compete with the 16S rRNA for the Shine-Dalgarno sequence and thus prevent translation initiation [61–66]. Further RNA based regulatory elements are riboswitches. Those non-coding RNA molecules contain an aptamer domain for ligand binding and a regulatory domain, which is responsible for the regulation of gene expression. Riboswitches undergo a conformational change after ligand binding, thus activating their regulatory domain. In *Synechocystis,* the formation or disruption of a rho-independent transcription terminator is responsible for their regulatory activity [67]. Additionally, riboswitches can affect translation via blocking the RBS [46,66,68,69]. The great advantage of such riboswitches is their modular composition, whereby different aptamer and regulatory sequences may be combined. This effect may be used as an additional regulatory element for leaky promoters.

4. Bioethanol Production in Cyanobacteria

Due to the lack of a natural pathway for bioethanol synthesis in cyanobacteria, the two genes *adh* (alcoholdehydrogenase) and *pdc* (pyruvate decarboxylase) of the ethanol synthesis pathway were introduced into different cyanobacteria like *Synechocystis* and *S. elongatus* PCC7942 to produce bioethanol out of pyruvate over the intermediate product acetaldehyde. The first reported photoautotrophic bioethanol producing cyanobacterial strain was an *S. elongatus* PCC7942 strain reaching an ethanol titre of 0.23 g L^{-1} after 4 weeks, which corresponds to an approximate 0.0082 g L^{-1} d^{-1} STY (space-time-yield), expressing *adhII* and *pdc* from *Z. mobilis* (adh_{ZM} and pdc_{ZM}) [70]. Later Dexter and Fu [71] constructed a *Synechocystis* strain capable of producing an ethanol titre of 0.46 g L^{-1} in 6 days with an STY of 0.0766 g L^{-1} d^{-1} with the same two genes. Since then, great scientific effort has been made to improve the ethanol yield in *Synechocystis* (see Table 2) [12,31,58,72,73].

First of all, different combinations of heterogeneous and endogenous *adh* genes together with the pdc_{ZM} gene were tested. The usage of pdc_{ZM} and the endogenous *adhA* gene *slr1192* (adh_{SYN}) resulted in the highest ethanol yield with *Synechocystis* so far. Direct comparison of the ethanol productivity of *Synechocystis* strains with genomic integrated gene combinations of pdc_{ZM}-adh_{ZM} and pdc_{ZM}-adh_{SYN}, both under the control of the P_{rbcL} promoter, revealed a 50% higher ethanol production capacity of pdc_{ZM}-adh_{SYN} compared to pdc_{ZM}-adh_{ZM}. Enzymatic characterizations of adh_{SYN} and adh_{ZM} showed a 74,000 higher enzyme activity for adh_{SYN} in the presence of acetaldehyde and NADPH. Since the

NADP(H) pool in *Synechocystis* is 10-fold bigger than the NAD(H) pool, this is one reason for the higher ethanol production capacity of the heterologous pdc_{ZM} together with the endogenous adh_{SYN} [31]. Furthermore, it has been shown that the catalytic activity of pdc_{ZM} is a limiting factor, as the optimal molar ratio between *pdc*:*adh* would be 1:2.5 but is 1:30 to 1:40 so far [72].

Table 2. Summary of recent ethanol producing *Synechocystis* sp. PCC6803 strains, reached with a photoautotrophic cultivation, without any media supplements like glucose, metal oxides or $NADP^+$. Sorted according to their space time yield.

Space Time Yield ($g\ L^{-1}\ d^{-1}$)	Expression Cassette	Integration Number	Integration Site	Reference
0.0154	P_{rbcL} pdc_{ZM} adh_{ZM}	single	*slr0168* neutral site	Gao et al. [31]
0.0431	P_{rbcL} pdc_{ZM} adh_{SYN}	single	*slr0168* neutral site	Gao et al. [31]
0.0573	P_{rbcL} pdc_{ZM} adh_{SYN}	single	*phaAB* genes	Gao et al. [31]
0.0766	P_{psbA2} pdc_{ZM} adh_{ZM}	single	*psbA2* gene	Dexter and Fu [71]
0.141	P_{psbA1} pdc_{SC} alr_{Syn}	single	*psbA2* gene	Velmurugan and Incharoensakdi [74]
0.181	P_{psbA2} pdc_{ZM} adh_{ZM}	single	*psbA2* gene	Armshaw et al. [75] Lopes da Silva et al. [32]
0.212	P_{rbcL} pdc_{ZM} adh_{SYN}	double	*slr0168* neutral site *phaAB* genes	Gao et al. [31]
0.236	$P_{zia\ *2ext}$ adh_{SYN} pdc_{ZM}	single	RSF1010 based plasmid	Duehring et al. [76]
0.261	P_{petJ} pdc_{ZM} adh_{SYN}	single	RSF1010 based plasmid	Dienst et al. [73]
0.285	P_{psbA2} pdc_{ZM} adh_{ZM}	double	*psbA2* gene *phaAB* genes	Armshaw et al. [75] Lopes da Silva et al. [32]

This result is supported by the fact that a double integration to increase the copy number of the genes further increased the ethanol yield. However, no significant difference in growth and ethanol production capacity was observed between a strain with double integration of the whole pdc_{ZM}-adh_{SYN} construct and a strain harbouring two copies of pdc_{ZM} and one copy of adh_{SYN} [31,72]. Additionally, the triple integration of both genes did not improve the yield any further. According to the authors, this might be due to the maximal gene expression level, which has already been reached, and the limited availability of the precursor pyruvate or co-factors like NADP(H) [12,31,58,72]. Velmurugan and Incharoensakdi supplemented metal oxides, either MgO or Fe_2O_3, into the BG-11 media to enhance the NADP(H) regeneration and thereby doubled the ethanol productivity. Moreover, a completely different gene combination was introduced. The endogenous NADP(H) dependent aldehyde reductase *slr0941* (alr_{SYN}) replaced adh_{SYN} and the pyruvate decarboxylase *PDC1* (pdc_{SC}) of *Saccharomyces cerevisiae* the pdc_{ZM}, both under the control of the P_{psbA1} promoter. Overall, 4.85 g L^{-1} and 5.10 g L^{-1} were reached after 25 days of cultivation, which correspond to 0.194 g L^{-1} d^{-1} and 0.204 g L^{-1} d^{-1} STY respectively, with either MgO or Fe_2O_3 [74]. In order to increase the available pyruvate pool for the ethanol synthesizing pathway, a knock-out of the PHB (polyhydroxy butyrate) pathway with the ethanol synthesis expression cassette was tested, but unexpectedly this did not increase the ethanol yield [31]. Nevertheless the *Synechocystis* strain producing the highest ethanol titre of 5.50 g L^{-1} in 26 days, which corresponds to 0.212 g L^{-1} d^{-1} STY, was the strain generated by Gao et al. [31], with double integration of the pdc_{ZM}-adh_{SYN} expression cassette under the control of the P_{rbcL} promoter. Although as confirmed by Luan et al. [72] this result has to be attributed to the double integration of the expression cassette and not to the knock-out of the PHB pathway with one of the copies, because a similar STY was reached with their present strain without a knock-out of the PHB pathway.

Further approaches focused on the use of non-light induced promoters like the Zn^{2+} induced promoter P_{ziaA} or the Cu^{2+} repressed promoter P_{petJ} for the control of the expression cassette. For the construction of both ethanol producing *Synechocystis* strains the same pdc_{ZM} and adh_{SYN} genes were combined with the respective promoters onto a self-replicative plasmid harbouring a RSF1010

origin of replication. Whereas, for the P_{petJ} the native promoter sequence was used, the sequence of P_{ziaA} was extended with a part of the 5'UTR of the native *Synechocystis hspA* gene *sll1514* carrying an RBS sequence. Compared to ethanol synthesizing strain presented earlier by Gao et al. [31], both strains reached even higher STY of 0.236 g L^{-1} d^{-1} and 0.261 g L^{-1} d^{-1} respectively under a continuous induced cultivation [12,73,76]. While testing different mutated variants of the P_{ziaA} promoter for the usability in an ethanol producing strain, Duehring et al. [76] tested an uncoupled pdc_{ZM} and adh_{SYN} gene expression as well. In these strains, the pdc_{ZM} gene was under the control of one of the mutated P_{ziaA} promoters and the adh_{SYN} gene under the control of the light induced P_{rbcL} promoter. Though, with such a hybrid construct, the cellular concentration of cell toxic intermediate acetaldehyde increased over time. With the extended P_{ziaA} promoter and a joint transcription of adh_{Syn} and pdc_{ZM}, the cellular concentrations remained constant and were even lower compared to the hybrid construct strain. This result may be attributed to the changed arrangement within the operon, from pdc_{ZM}-adh_{Syn} to adh_{Syn}-pdc_{ZM} [76]. Hence, this supports previous results of Gao et al. [31] and Luan et al. [72] suggesting that, a higher gene dosage of adh_{SYN} may improve the overall ethanol synthesis efficiency. Regardless of the higher ethanol productivity and ethanol titre reached with the extended P_{ziaA} promoter adh_{SYN}-pdc_{ZM} expression cassette, growth of the compared hybrid strain was better [76]. This indicates that the redirection of carbon flux away from growth to product formation may contribute to impaired growth behaviour of ethanol producing *Synechocystis*. However, the most productive *Synechocystis* strain was presented by Armshaw et al. [75] which was further characterized by Lopes da Silva et al. [32]. The strain reached an ethanol titre of 2.6 g L^{-1} during a cultivation over 9 days, which corresponds to a STY of 0.285 g L^{-1} d^{-1}. In contrast, this was reached with an expression construct consisting of a P_{psbA2} promoter, pdc_{ZM} and the initially used adh_{ZM} which was double integrated into the genome of *Synechocystis* in a similar manner as published by Gao et al. [31]. Due to a reached STY of only 0.181 g L^{-1} d^{-1} with single integration of the same construct, without a PHB knock-out, this result conflicts with previous presented data and theories about rate limitations of ethanol synthesis by lower cellular NAD(H) pools. Nevertheless, this work underlines the importance of the available pyruvate pool and of high gene expression rate.

Although the ethanol yield has already been increased by improving existing methods, compared to other model organisms like *S. cerevisiae* or *E. coli*, there is still a big step to be made [21]. In order to achieve this, the ethanol tolerance of *Synechocystis* has to be taken into account, because a yield of 15 g L^{-1} is needed for commercial use. At this time, the *Synechocystis* wild type is 50% growth inhibited if this concentration is externally added to the media [12,32]. In various studies, the stress response of *Synechocystis* was further investigated and different genes and non-coding RNA sequences were identified to enhance the ethanol tolerance. During a long-term cultivation of *Synechocystis* under permanent, externally applied, butanol stress, cell cultures evolved a 2.5-fold higher tolerance over time. From these mutated cultures, single colony clones were isolated, re-sequenced and different loss of function mutations identified. Two genotypic combinations of mutations in *mcpA* gene *slr1044* and *envD* gene *slr0369* or *hik43* gene *slr0322* and *envD* were identified to be the reasons for the increased tolerance against ethanol, butanol and isopentanol. A generated mutant strain combining the knock-outs of *mcpA* and *envD* with an ethanol synthesis pathway reached a 40% increased ethanol titre from approx. 30 mg L^{-1} to 40 mg L^{-1} [77]. Therefore, cellular synthesized ethanol starts to impair cellular physiology way earlier than externally supplemented ethanol. Underlining an increased ethanol tolerance is an important factor for commercial photoautotrophic ethanol production with cyanobacteria. Lopes da Silva et al. [32] demonstrated with the ethanol producing strains generated by Armshaw et al. [75] a positive correlation between an increasing ethanol production rate and enhanced ethanol tolerance. Proteomic analysis, comparing wild type and ethanol producing *Synechocystis* strains, provided a deeper insight into the stress response. While an increased abundance of proteins responsible for oxidative stress response was measured, proteins involved in the phycobilisome light harvesting complex were reduced in their relative abundance. The comparison of relative protein abundance and relative mRNA levels of selected proteins revealed a corresponding regulation at transcriptomic

level [78]. Another proteomic analysis of wild type *Synechocystis* with externally applied ethanol stress revealed increased abundance of proteins involved in cell membrane adaptation [32,79]. As different sRNA species were identified to be involved in the stress response of *Synechocystis*, the overexpression of the sRNA Nc117 enhanced the ethanol tolerance. Other sRNA identified in the same experiment led to a reduced growth phenotype during ethanol stress compared to the wild type when overexpressed in the same manner [65]. These data underline on the one hand the great opportunities for the development of cyanobacterial cell factories offered by now available so called "-omics" technologies, but on the other hand prove the big knowledge gap in the cellular regulation mechanisms.

To summarize the available data for the purpose of photoautotrophic ethanol production with *Synechocystis*, a highly productive cell factory system relies on high expression rates of the necessary genes (see Table 2). If no suitable promoters are available, a change of the copy number of the expression construct may compensate the lack of high transcription rates up to a certain point. In this case, the double integration of the ethanol expression cassette led to an about 50% higher STY [31,32,75]. Additionally, the supply of precursor metabolites and co-factors is essential for a high production rate, although the data seem to be conflicting at this point [31,32,72,73,75,76]. Finally, as shown by Matsusako et al. [77], product toxicity may impair the efficiency of such a cell factory even at a very low production rate.

5. Conclusions

Currently, the productivity of bioethanol from cyanobacteria is still too low for an industrial application. Therefore, further optimizations for the use of genetically modified cyanobacteria like *Synechocystis* are needed [9,80]. With the application of the genetic toolkits summarized in this work, the potential of cyanobacteria could be better exploited. For example, all hitherto presented ethanol producing strains were cultivated in a continuous one-step cultivation process [12,31,72,76]. Here, the ethanol has been produced continuously during the growth phase and, thus, inhibited cell growth. The use of inducible promoters enables a two-stage process with a separated growth and production phase. In a first stage, high cell biomass could be accumulated, without all the negative effects of the ethanol production, to potentially produce more ethanol in the second phase [76]. As the overexpression of the two genes *adh* and *pdc* is necessary to produce ethanol in cyanobacteria and the *pdc* seems to be the process limiting gene, the expression rate could be increased by very strong promoters like P_{trc1O} or P_{cpc560}, which have not been used for bioethanol production so far [12,31]. The direct comparison of promoters results in a more than 10-fold higher fluorescence of the P_{trc1O} and P_{cpc560} compared to the P_{rbcL}, used by Gao et al. [31], which underlines the huge potential of increased bioethanol production [45]. These promoters combined with their optimal terminator and RBS as well as integration sites could increase the overall production rate even further. Another approach could include replicating plasmids with native origins of replication for ethanol production, which demonstrated higher transgene expression compared to genomic integration. To address the imbalanced enzymatic activity of adh_{SYN} and pdc_{ZM}, either their expression may be uncoupled from each other or different gene combinations could be tested in order to avoid the accumulation of the metabolic intermediate acetaldehyde, which is toxic to the cells [31,32,71,72,74–76].

Based on recent proteomics data, further targets for gene knock-outs or overexpressions could be identified. Additional to proteins, recently identified sRNA species, which are involved in the biofuel stress response offer great new possibilities to enhance ethanol productivity.

Improving the availability of the precursor pyruvate could be another starting point to increase the ethanol yield, which, for example, could be achieved by the increase of CO_2 fixation or the knock-out of competitive metabolic pathways like the glycogen synthesis pathway [12,72]. The increase of the photochemical efficiency by overexpression of genes participating in photosynthesis like the *flv3* gene, which encodes a flavodiiron protein shown by Hasunuma et al. [81], could be used for improving the productivity, too. Here, the overexpression of *flv3* lead to an increased availability of metabolites of the Calvin cycle and thus enhanced the growth rate and CO_2 fixation [81]. In another study, the

co-overexpression of a heterologous pyruvate kinase gene to increase the pyruvate pool in a lactic acid producing *Synechocystis* strain lead to an almost doubled lactic acid titre [82]. Those measures combined with the supplementation of metal oxides, to enhance the NADP(H) regeneration, could improve the metabolic flux towards the ethanol synthesizing pathway [74].

A general challenge in prokaryotes is the degradation of heterologous proteins by endogenous proteases. Here, several improvements were achieved to establish *E. coli* as a production system with the knock-out of several endogenous genes [83]. Applying the knowledge gained with *E. coli* together with the new toolkits in cyanobacteria could accelerate the progress of using the potential of these small biofactories to establish microalgae as alternative production systems for bioethanol.

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