
Metabolic Engineering of the Model Photoautotrophic Cyanobacterium *Synechocystis* for Ethanol Production: Optimization Strategies and Challenges

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Abstract

Photoautotrophic ethanol production using model cyanobacteria is an attractive technology that offers potential for sustainable ethanol production as a biofuel. Model strains of *Synechocystis* PCC6803 have been metabolically engineered to convert central metabolic intermediates such as pyruvate to acetaldehyde via cloned heterologous pyruvate decarboxylase and from acetaldehyde to ethanol via cloned homologous or heterologous alcohol dehydrogenase. While the technology is now proven, strategies are required to increase the ethanol levels through metabolic and genetic engineering and in addition, production and process strategies are required to make the process sustainable. Here we discuss both genetic and molecular strategies in combination with downstream strategies that are being applied while also discussing challenges to future application.

Keywords: *synechocystis*, ethanol metabolic engineering, challenges industrial production

1. Introduction

As an alternative to ethanol fermentation using carbohydrate substrates, the use of photoautotrophic cyanobacteria metabolically engineered to produce ethanol offers an interesting alternative for sustainable biofuel production. Cyanobacteria or Cyanophyta, the name deriving from their color, are a distinct phylum of bacteria, which are photoautotrophic getting energy from sunlight and carbon from carbon dioxide. They are the only photosynthetic bacteria that

Genetic construct	Strain	Rate per day (g.L ⁻¹ .day ⁻¹)	References
ZmPDC and ADH1 P _{rbLS}	<i>Synechococcus</i> PCC7942	0.0082	[4]
ZmPDC and ADH1 P _{psbA2}	<i>Synechocystis</i> PCC6803	0.0766	[5]
ZmPDC and slr1192	<i>Synechocystis</i> PCC6803	0.097	[6]
JCC1581 B Isolate	<i>Synechococcus</i> PCC7002	0.41	[7]
ZmPDC and slr1192 P _{ziaA}	<i>Synechocystis</i> PCC6803	0.236	[8]
ZmPDC and slr1192 P _{corT}	<i>Synechococcus</i> PCC7002	0.235	[8]
ZmPDC and slr1192 P _{rbc}	<i>Synechocystis</i> PCC6803	0.202	[9]
ZmPDC and slr1192 P _{petJ}	<i>Synechocystis</i> PCC6803	0.261	[10]
TK504 Plasmid P _{co}	ABICyanol1	0.552	[11]

Table 1. Ethanol yields (g.L⁻¹.day⁻¹) as reported for various constructs using the *Zymomonas mobilis* (Zm) *pdc* gene, a variety of ADH genes and various promoter constructs to express these genes.

can evolve oxygen. Model species such as *Synechocystis* sp. PCC6803 have received considerable attention because they can be relatively easily manipulated genetically and metabolically engineered to produce a wide range of potentially valuable products of biotechnological interest [1]. Considerable attention has focused on the potential to utilize sunlight and CO₂ to produce ethanol as a biofuel at yields comparable to other biological production systems. Although there have been reports of natural ethanol production during dark metabolism, reported levels are far too low for exploitation [2].

The interest in utilizing cyanobacteria as cell factories for ethanol production has been stimulated via flux balance analysis on ethanol yields, which estimate that the stoichiometric energy yield for ethanol compares well with other potential fuel metabolites [3]. The earliest reports of photoautotrophic metabolically engineered ethanol production came in *Synechococcus elongatus* PCC 7942 [4] where heterologous genes encoding pyruvate decarboxylase and alcohol dehydrogenase were expressed from the ethanol producer *Zymomonas mobilis*. This was followed by expression of the same constructs in *Synechocystis* sp. PCC6803 [5] with reported higher yields (**Table 1**).

This was followed by reports in several patents from the US biotechnology companies Algenol and Joule Unlimited who further manipulated the system to improve yields (**Table 1**). The reported yields are represented as a daily yield and often the production cycle can last up to 20 days such that the yields would be multiplied by the production days. However, with potential evaporative loss and degradation of ethanol by contaminants in non-axenic culture these yields are lower than would be needed for commercial production. Thus, much effort has been focusing on improving this yield level by metabolic and strain engineering.

2. The model strain and production of key intermediates

The first *Synechocystis* strain was originally isolated in Oakland, California in 1968 [12] and placed in the Pasteur Culture Collection as *Synechocystis* sp. PCC6803 and the American Type

Culture Collection as *Synechocystis* sp. ATCC27184. Over the years, many sub-strains emerged from the original strain, such as the *Synechocystis* sp. GT (Glucose Tolerant) strain. This GT strain was sent to the Kazusa Research Institute in Japan and became known as the 'Kazusa' strain. Other sub-strain are known as the 'Vermaas' strain [13], the 'China' strain [5] and indeed others sub-strains have been reported [14] such as 'Moscow', 'Amsterdam' and 'New Zealand' depending on the location of the research laboratories using the so-called original *Synechocystis* sp. PCC6803 strain. Many of these strains have undergone microevolution, which may be a feature of cyanobacterial strains growing in high light conditions under laboratory conditions [15] with such genetic changes being detected by genome sequencing. Many sub-strains have interesting variations, which may be of biotechnological interest such as low transformation rates, buoyancy and variation in growth rate.

Yields of product such as ethanol are highly dependent on the biomass produced during growth of engineered strains. When growing photoautotrophically at 30°C doubling times of *Synechocystis* sp. PCC6803 can vary between 10 and 15 h, with optimal conditions observed at light intensities of 40–70 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ [16]. In controlled photobioreactors higher growth rates can be achieved when optimal conditions are provided throughout a growth cycle. Because the flux of carbon is diverted in metabolic engineered strains, from pyruvate via many of the engineered pathways, this has the effect of lowering biomass yields and indeed the more ethanol as a product that is produced the greater the effect on biomass yield will be. In general, the relatively slow growth rates of *Synechocystis* may be attributed to many reasons, its photoautotrophic metabolism or its polyploid (multi-copy) genome; however, one of the key issues is its encoding genome optimized for photoautotrophy. *Synechocystis* sp. PCC6803 was the first cyanobacterium to have its genome sequenced [17] and since then many further sub-strains have been sequenced [15, 18, 19]. Analysis of genome data reveals that *Synechocystis* in the main does not possess transporters for vitamins, co-factors, amino acids or nucleotides and must encode synthesis pathways and synthesize essential building blocks from the energy of photosynthesis. The needs therefore for complex synthetic machinery for its photoautotrophic lifestyle coupled to polyploidy are key aspects of its relatively slow growth rate. This then may be exacerbated when this organism is used as a cell factory for products such as ethanol.

During photoautotrophic metabolism in *Synechocystis* an intermediate of the Calvin cycle, Ribulose 1,5-bisphosphate, is used to fix carbon dioxide to 3-phosphoglycerate. This can be converted to phosphoenolpyruvate (PEP) from 2-phosphoglycerate via enolase or travel back through the Calvin cycle. Pyruvate kinase (pk) is then used to convert PEP to pyruvate [20]. This central intermediate, pyruvate, can then be diverted via metabolic engineering to a number of potential biotechnological products including ethanol [1].

3. Key aspects of the engineered ethanol cassette in *Synechocystis*

To metabolically engineer *Synechocystis* as a cell factory a 'cassette' of genes and sequences are needed. A key ingredient of a functional ethanol cassette, suitable for expression in *Synechocystis*, is the functional expression of a pyruvate decarboxylase gene encoding the

enzyme pyruvate decarboxylase (PDC). The PDC produced converts the metabolic intermediate pyruvate to acetaldehyde, which is in turn converted to ethanol by engineered alcohol dehydrogenase (ADH) or by the native *Synechocystis* ADH.

Pyruvate decarboxylase (PDC, EC 4.1.1.1) carries out the decarboxylation of pyruvate to acetaldehyde in alcohol fermentations and requires thiamine diphosphate/pyrophosphate (ThDP) and the divalent cation Mg^{2+} as cofactors. Several other enzymes in various metabolic pathways also require these cofactors to function and it is believed that each of them use a similar mechanism of action. PDC can be found in fungi, plants and yeast and is not present in humans [21]. PDC genes have been observed and characterized from only a small number of bacterial species as it appears to be rather rare amongst prokaryotes. These include *Zymomonas mobilis*, [22], *Zymobacter palmae* [23], *Acetobacter pasteurianus* [24], *Gluconacetobacter diazotrophicus* [25], *Thermococcus quaymacensis* [26], *Geobacillus thermoglucosidasius* [27] and *Sarcina ventriculi* [28]. Although the *Zymomonas mobilis* PDC is the most extensively utilized in ethanol production there is much potential to utilize some of the other bacterial PDC's on the basis of pH optimum or lower K_m (see **Table 2**). With model organisms, such as *Synechocystis*, using a PDC with a lower K_m may increase the flux from pyruvate and couple the product acetaldehyde better with ADH resulting in higher ethanol yields. There is thus some scope for improvement of the ethanol cassette given that some of the newly characterized PDCs have better kinetics than the original *Zymomonas* PDC. All known PDC's have specific co-factor requirements and co-factor availability is an issue when expressing engineered cassettes. While there may be little problem with Mg^{2+} supply, the availability of ThDP will be limited as the host organism must synthesis it (as *Synechocystis* does not possess a thiamine transporter) [18]. Equally ThDP will be required for other cellular metabolic reactions and its availability will be squeezed by added engineered PDC. Hence if metabolic engineering were to result in high level expression of heterologous PDC, the limited availability of ThDP would pose limitations on its function

Bacterial host and enzyme	kM (mM) pyruvate	Optimum pH	Optimum temperature (°C)
<i>Gluconacetobacter diazotrophicus</i>	0.06 pH 5.0	5.0–5.5	45–50
	0.6 pH 6.0		
	1.2 pH 7.0		
<i>Zymobacter palmae</i>	0.24 pH 6.0	7.0	55
	0.71 pH 7.0		
<i>Acetobacter pasteurianus</i>	0.39 pH 5.0	3.5–6.5	65
	5.1 pH 7.0		
<i>Zymomonas mobilis</i>	0.43 pH 6.0	6.0–6.5	60
	0.94 pH 7.0		
<i>Sarcina ventriculi</i>	5.7 pH 6.5	6.3–6.7	N/A
	4.0 pH 7.0		
<i>Gluconobacter oxydans</i>	0.12 pH 5.0	4.5–5.0	53
	2.8 pH 7.0		

Table 2. Properties of known bacterial PDCs [23, 25, 27].

and perhaps also on other host enzymes that use ThDP as a co-factor. Thus this may affect competitiveness of engineered strains and in the long term, engineering a thiamine transporter may be needed in *Synechocystis* production strains to overcome such issues.

While there is a potential choice of PDCs to use, in practice most work so far has been carried out on the *Zymomonas mobilis* PDC. This enzyme is a homo tetramer of 240 kDa [29] and has an optimum pH of 6.0 [30]. Given the pH optimum for growth of *Synechocystis* is ~pH 8, full enzymatic function or co-factor binding [31] may be somewhat compromised by the pH difference between the enzyme optimum and the host pH optimum which may suggest looking at other potential PDC candidates.

4. *Zymomonas mobilis* and *Synechocystis* alcohol dehydrogenase (ADH)

In most reports on engineered ethanol cassettes the source of ADH has been *Zymomonas mobilis*. Two ADH isozymes are known to be present within the genome of *Zymomonas mobilis* - ADH I and ADH II (EC 1.1.1.1) [32]. For metabolic engineering of ethanol production the Fe²⁺ containing ADHII encoded by the *adhB* gene of *Zymomonas* has been utilized [5]. This enzyme has a pH optimum of pH 8.5 (as opposed to pH 6.5 for ADHI) and a cofactor requirement for Nicotinamide adenine dinucleotide (NADH) for the reduction of acetaldehyde to ethanol [33].

Unusually *Synechocystis* also encodes its own native ADH gene via the *adhA* gene (slr1192). This is a medium chain alcohol dehydrogenase, which catalyzes the reversible oxidation of alcohols to aldehydes or ketones [34]. The *Synechocystis* ADH encodes a 140 kDa zinc dependent enzyme with broad alcohol dehydrogenase activity and which interestingly is Nicotinamide adenine dinucleotide phosphate (NADPH) dependent as opposed to the *Zymomonas* activity, which is NADH dependent [34]. Indeed *Synechocystis* has been reported to possess multiple *adh* genes but does not contain a native *pdC* gene [35] suggesting that the native ADH may play an as yet unknown function in the cyanobacterium. Indeed the *Synechocystis adh* gene has been substituted for the *Zymomonas* gene [9] and functions very well. Recently we have reported [36] metabolic engineered cassettes with a copy of the *Zymomonas mobilis adh* gene and the native *Synechocystis adh* gene with increased ethanol producing activity. This may occur because the two activities rely on different co-factors NADH (*Zymomonas*) and NADP (*Synechocystis*) which may spread the co-factor requirement and availability within the cell [36].

5. Construction of functional ethanol cassettes in *Synechocystis*

In general, terms the construction of an ethanol cassette follows the basic components as reported [4, 5]. The *Zymomonas mobilis pdC* gene is amplified and fused with the *Zymomonas mobilis adhB* gene under the control of an inducible promoter. The light inducible P_{psbA2} promoter is often utilized but other promoters have also been evaluated [37]. There is then the need for a strong selection of the cassette encoded generally by an antibiotic resistance determinant such as kanamycin or zeocin [36]. Homology sequences are needed at both ends of the

cassette to allow homologous integration into a neutral site within the organism (see **Figure 1**). The cassette utilized by Dexter and Fu [5] utilized the *psbA2* gene as a neutral site for integration but recently a number of other neutral sites have been discovered [38]. Indeed homologous integration has been used as a mechanism of integrating cassettes into functional competing genes, as a knock out mechanism also. This occurs where the cassette is integrated via use of homologous ends into genes such as the *pha* genes whereby integration knocks out the synthesis of polyhydroxyalkanoate (PHA) a competing pathway for pyruvate use [9, 36]. Using the *pha* genes as an integration site effectively increases the flux of pyruvate to ethanol by blocking alternative storage of photosynthetic products.

In attempts to increase ethanol production, gene dosage has been utilized such that two cassettes have been integrated at different sites giving potentially twice the gene copy number and protein expression level of PDC and ADH [9, 36]. While this strategy has been shown to increase the levels of ethanol produced it may be that given the polyploid nature of *Synechocystis* putting in and stabilizing two cassette copies which would be multiplied by some 50 copies (due to polyploidy) may be reaching the very limits of gene dosage with this metabolic engineering strategy.

Figure 1 illustrates the construction of an ethanol cassette pUL004 Kan. This cassette [36] consists of the *Zmpdc* coupled to the *Synechocystis adhA* gene with a kanamycin resistance determinant from the ICE R391. The genes are controlled via the P_{psbA2} light inducible promoter. The cassette contains 500 bp of DNA at each end with homology to a neutral integration site, in this case the *psbA2* gene. This construct is housed in pUC18 and replicated in *Escherichia coli* and termed pUL004. For integration, the plasmid pUL004 is transformed into *Synechocystis* whereupon

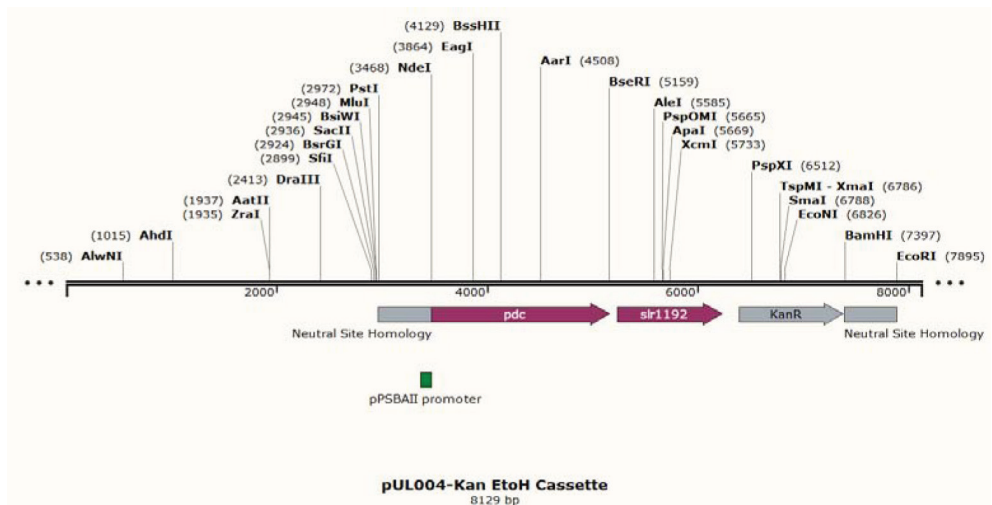


Figure 1. Structure of the ethanol cassette pUL004 [36]. The cassette contains the *Zymomonas pdc* gene (*Zmpdc*), the *Synechocystis* sp. PCC6803 *adh* gene (*slr1192*) and the kanamycin resistance determinant from the ICE R391 all under the control of the P_{psbA2} light inducible promoter. There is 500 bp at each end with homology to the neutral integration site and the construct is cloned into pUC18 for replication in *E. coli* prior to purification and transformation into *Synechocystis* PCC6803. Restriction sites within the cassette are also illustrated.

homology between the two 500 bp ends and the host chromosome leads to integration into the neutral site. In general, because of the polyploid nature of *Synechocystis*, selection for integration requires selection on increasing doses of kanamycin and PCR monitoring using primers across the neutral integration site. Initially many chromosomes will not contain an integrated cassette and this will show as a low molecular weight band (where no integration into the neutral site occurs). Those chromosomes that contain an integrated cassette will possess a higher molecular weight band where the cassette has integrated into the neutral site increasing the band size. At the initial stages, one would observe two bands one without and one with integration (one low and one high band). Following selection all chromosomes should contain a high molecular weight band (and no low molecular weight band) indicating that all chromosomes contain the cassette. This process illustrated in **Figure 2** (below) may take several weeks to segregate and stabilize. In the case of establishing an ethanol cassette, which provides no selective advantage on its host and in fact may be negative in selection terms as it causes diversion of pyruvate towards ethanol rather than biomass, selection and stabilization may take some time. Thus, strong selection

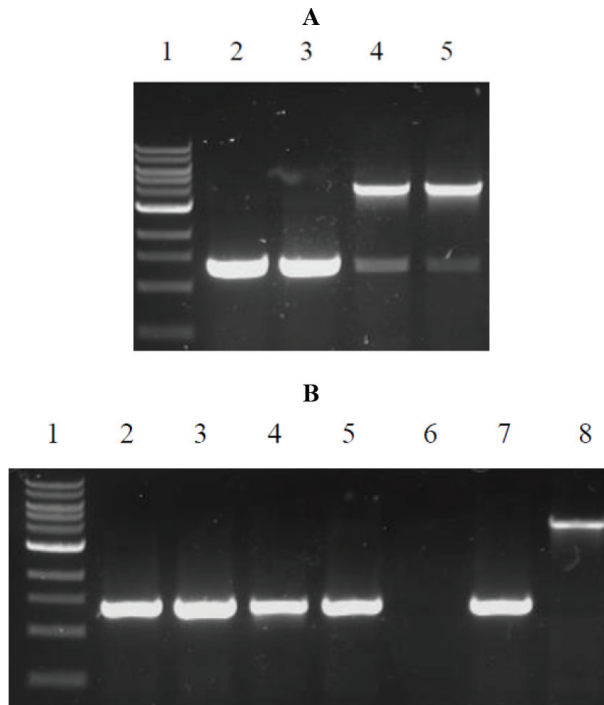


Figure 2. Agarose gel electrophoresis of PCR amplicons analyzing integration into the *psbA2* neutral site of pUL004. A) Lanes 2 and 3 (and B lanes 2,3,4,5,7) illustrate amplicons using primers to amplify across the neutral integration site which in these cases are all of low molecular weight indicating no integration into the neutral site. A) Lanes 4 and 5 illustrate that two amplicons are detected the lower band with no cassette and the higher band with the cassette integrated. This pattern is detected in strains with partial segregation of the cassette. B) upon selection strains harboring integrated cassettes in all chromosomes should resolve (the cassette is present in all chromosomes of the polyploid genome) as illustrated in lane 8 panel B. This band (lane 8 panel B) can then be removed and sequenced to verify integration. Lanes 1 a and B illustrate a molecular weight ladder to determine amplicons size.

and monitoring is required to realize integration and maintenance of such cassettes. To insert a second cassette a different neutral integration site (and hence different homologous sequences within the cassette are required) and a different antibiotic resistance determinant such as zeocin [36] is needed as part of the cassette construction.

6. Issues and methodologies to enhance ethanol production levels

6.1. Ploidy as an issue in cloning in *Synechocystis*

Strains of *Synechocystis* are polyploid with the chromosome number varying as a function of growth phase [39]. This causes issues with metabolic engineering and stabilization of engineered genes into chromosomal locations as one must select for integration into all chromosomal copies. In addition, the high polyploid level causes problems with generation and selection of mutants, which again must be fixed into all chromosomal copies.

Griese et al. using a real time PCR method demonstrated that the motile 'Moscow strain' of *Synechocystis* contained 58 genome copies per cell at both the log and stationary phases, while the GT 'Vermass strain' contained somewhat less with 42 chromosome copies during the same time period [39]. The 'Kazuza' strain had been reported to possess 12 copies but may have altered its ploidy because of laboratory growth over extended periods [40]. High copy number during growth has also been seen in several archaeal and other bacterial species [41, 42] but the levels reported in *Synechocystis* are amongst the highest chromosome copy numbers recorded for any cyanobacterial species or indeed prokaryote. Given the high chromosome number, the energy expended on its maintenance is high and contributes significantly to the slow growth rate of many polyploid cyanobacteria. Equally, this high chromosome number poses added difficulties in genetic and metabolic engineering in requiring many rounds of selection and screening to achieve stable integration of cloned genes. One possibility of limiting the polyploidy may be to culture production strains in limited phosphate containing media as this may have the effect of limiting phosphate availability for DNA synthesis and can limit the chromosome number, making it easier to establish recombinants and ease stabilization of chromosomally inserted cassettes.

6.2. Gene dosage

The initial cloning strategies [4, 5] used one copy of the ethanol cassette inserted into a chromosomal neutral site. To enhance productivity two copies of the cassette were then utilized [9, 36]. This had the effect of increasing productivity all be it at the expense of biomass and indeed stability during production. Attempts in our laboratory to generate strains with three cassette copies per cell have thus far failed. This suggests there could be a limit to the gene dosage that can be utilized for ethanol cassettes at least. This limit may be due to several factors and many of these factors may combine to limit production. There is the ploidy issue such that during growth if the ploidy level is some 50 copies [39] then with one cassette the copy number is already 50, two copies would mean it would be approximately 100 and the cell may not be able to tolerate more. There may also be instability issues with recombination events between similar cassette sequences. In addition, there may be the issue of ability to supply

the co-factors ThDP, NADH and NADPH for this level of enzyme expression. There may be additional factors such as limitation of pyruvate for other essential cellular functions if high levels of enzyme activity are utilizing it to react to ethanol. This in turn may affect biomass production and synthesis of essential cell components and thus triggering a stress response. In addition, given the negative effect ethanol has on growth there may be the selective pressure to mutate the cassettes selecting for faster growing strains which do not have the burden of ethanol production. The nature of all these possibilities may need to be examined in more detail to generate optimal strains going forward.

That gene dosage can have an effect on production has been demonstrated by utilizing the small native *Synechocystis* plasmid pCA2.4 [43]. This plasmid has a copy number of seven per chromosome copy, thus potentially greater than 300 copies per cell. Cloning of the yellow fluorescent protein (YFP) into a neutral site on this highly stable plasmid resulted in >100 fold increase production levels of YFP relative to a chromosomal insert indicating the potential of gene dosage within *Synechocystis* [43] all be it in this case with a non-burdening or non-toxic product.

6.3. Promoter constructs

Most productivity studies for ethanol in *Synechocystis* have been carried out with the light inducible P_{psbA2} promoter [4, 5, 9, 36]. However, a number of other promoters have been examined specifically to improve yields (see **Table 1**). Recently heterologous strong promoters P_{trc} [44], P_{rmpB} [45] and P_{lac} [46] have been used for butanol, lactate and ethylene production respectively. Use of the super promoter $P_{cpc560'}$ [47] was shown to produce functional proteins at a level of up to 15% of total soluble protein in *Synechocystis* sp. PCC6803, a level comparable to that produced in *E. coli*. This promoter appears to have 14 predicted transcription binding sites, which appear to be key to its high expression level [47]. Many of these promoters are always on and may not be optimal for controlled expression however.

A number of controllable promoters have also been analyzed [37] with the most useful being the Ni^{++} Co^{++} inducible, $P_{hrsB'}$ which gives relatively silent expression in the un-induced state and can be induced some 40 fold to approximately the level of the P_{psbA2} promoter with inducer. Such promoters may allow tuneable promoter activity for ethanol production. Always on promoters, do not allow biomass to be generated as might happen in the yeast system where removal of aeration during production leads to the switch to anaerobic metabolism and ethanol productivity following adequate biomass production. This decoupling of growth from ethanol production could be achieved by tuneable promoters and has been reported [48] where by a riboswitch was incorporated in an ethanol cassette following the P_{psbA2} promoter. Such riboswitches can be induced by theophylline and has been used as a *proof of concept* to decouple biomass from ethanol production [48].

6.4. Knockout of competing pathways as an aid to greater production

Manipulation of carbon flux within the cell factory *Synechocystis* has been used to increase production of metabolically engineered products. Photoautotrophic growth in the light results in accumulation of a number of storage compounds in *Synechocystis* including the major storage polymers glycogen and polyhydroxyalkanoates (PHA), the best characterized

being polyhydroxybutyrate (PHB) [49]. Mutants deficient in accumulation of such storage compounds have been used to express metabolic engineered pathways such as in the production of lactate [50]. Here diverting flux away from storage has been demonstrated to have positive effects on production. In a similar way, inserting the ethanol cassette directly into the *pha* genes has also been shown to increase production of ethanol [9, 36].

Increasing levels of substrate, in this case pyruvate, have also been used to increase yield in metabolic engineered strains. Expressing the enzyme pyruvate kinase (PK), which transfers a phosphate group from PEP to ADP forming Pyruvate [51], has been shown to increase flux to product [52]. Thus, there appears to be some potential for manipulating the flux pathways to and from pyruvate as a means of increasing product yield, which may prove useful when coupled to ethanol production.

6.5. Mutagenesis strategies

Mutagenesis and mutant selection has been developed in *Synechocystis* however; the use of random mutagenesis is difficult. This stems from the polyploid nature of the organism and the need to establish the mutant genotype in all chromosomes before the phenotype is apparent. A novel microfluidics strategy has been developed as an aid to select mutants with higher ethanol production levels [53]. This micro-droplet technique can detect increased ethanol from single cells of engineered *Synechocystis* in micro-droplets. The technique is based on an enzymatic assay, which couples ethanol levels produced within the micro-droplet directly to resorufin, a fluorescent compound. The extra fluorescence apparent with a high ethanol producer can be detected and the droplet containing the higher producer collected [53]. Passage of large quantities of metabolic engineered ethanol producing *Synechocystis*, through the system coupled to laser detection and separation of high fluorescent strains facilitates separation of higher ethanol producers [53]. Such a technique could be used to rapidly screen a large library of transposon insertion mutants, a cloned library of genes potentially enhancing ethanol production (such as PK) or directed insertion libraries (such as PHA) to select higher producers.

6.6. Improving carbon capture

Several mechanisms of carbon accumulation have been described to operate in *Synechocystis*, which include both bicarbonate and CO₂ transporters [54, 55]. These systems include the high affinity bicarbonate transporter BCT1 (locus slr0040–44), the sodium dependent bicarbonate transporter *SbtA* (slr1512), the medium affinity bicarbonate transporter *BicA* (locus slr0834) and the multi component CO₂ transporters NDH [56]. Theoretically, manipulation of transporters could provide more carbon for fixation and conversion to products such as ethanol. While some of the transporters are multi subunit complexes and could be difficult to express to functional activities, some are single gene encoded activities and more easily amenable to metabolic engineering. The *BicA* protein was expressed in *Synechocystis* [57] by engineering the strain to contain additional inducible copies. Studies revealed that this strain resulted in enhanced biomass yields. We confirmed that expressing *BicA* did in fact increase biomass whereas expressing *sbtA* in our hands did not (O’Riordan, Armsshaw and Pembroke, unpublished 2018). This offers a proof of concept that increasing carbon flux can affect productivity and may have applications in enhancement of product yield. Other

strategies also show potential in *Synechocystis*. Manipulating the Calvin-Benson-Bassham cycle, has been proposed as a strategy for improving cyanobacterial growth and product metabolites [58]. Four enzymes of the cycle, ribulose-1, 5-bisphosphate carboxylase/oxygenase (RuBisCO), fructose-1, 6/sedoheptulose-1, 7-bisphosphatase, transketolase and aldolase were co-expressed with an ethanol cassette (containing *pdh* and *adh*) expressed with the P_{nrsB} promoter in *Synechocystis*. In all cases there was a 55, 67, 37 and 69% increase respectively in ethanol production and also a relative increase in biomass [58], indicating the potential of increasing carbon flux within the cell factory as a means of improving product yield.

6.7. Neutral sites for integration

As integrative vectors, which utilize homologous recombination into the chromosome, are widespread when metabolic engineering *Synechocystis* [59] the characterization of integration sites, termed neutral sites, is important, particularly in terms of functionality and stability of insert. Pinto et al. carried out a systematic study of neutral sites using insertion and deletion at the site and expression of the green fluorescent protein module [38]. Although a large number and variety of sites were chosen based on genomic and sequence analysis further analysis revealed that many of these were unsuitable. Location of genuine neutral sites appears to be complicated by the possibility that non-coding regions may possess cis-acting sites thus a systematic assay using trials was needed [38]. Investigation indicated that not all neutral sites were the same and that insertion in some caused some growth defects relative to wild type. This systematic review points to a new and validated set of potential sites that can be utilized going forward which is important given the popularity of integration as the preferred tool for metabolic engineering in *Synechocystis*.

6.8. Replicative plasmids

Replicative plasmids have been utilized for genetic engineering in *Synechocystis* [60] and many are based on the broad host range chassis of the IncQ plasmid, RSF1010, which functions in *Synechocystis*. While this chassis allows ease of construction of inserts and can replicate in *E. coli* for generating transformation material [61] they are not widely utilized. Replicative plasmids in *Synechocystis* suffer from a number of drawbacks including recombination back into the chromosome, loss of the vector without selective pressure and unusual effects on transcription within the vector [38]. Also given the polyploid nature of the organism, segregation and maintenance of such vectors can be problematic.

6.9. Tolerance to ethanol

For high level, production of ethanol within *Synechocystis* it has been estimated that yields would need to be above 15 g.L⁻¹ but indeed as high as possible from a commercial perspective [62]. Currently levels of production are nowhere near these levels. However were such levels, approaching 15 g.L⁻¹, to be reached the tolerance of the organism to ethanol could become an issue, as this would stress the cell factory. In competitive yeast fermentation systems up to 20% ethanol (v/v) has been reported [63], which is far from the current production capacity of metabolically engineered *Synechocystis* (**Table 1**). However, in preparation for strains that

would have this capacity it is important to determine the level of tolerance to ethanol in engineered strains and examine toxic or stress related effects. Proteomic analysis has been used to determine the response of engineered strains [64] with current ethanol production levels and the response of strains with ethanol added up to projected or expected ethanol production levels [65]. In the case of added ethanol incubation with 1.5% (v/v) of ethanol for 24 h reduced growth of *Synechocystis* by 50% with cell aggregation visible [65]. Proteome analysis revealed some 32 unique proteins up-regulated and some 42 down-regulated after 24 h. This number of altered proteins increased after 48 h. incubation. Many of these proteins were demonstrated to be involved in the common stress response such as those associated with oxidative stress [65], transporters, cell-membrane modifying proteins and proteins associated with the photosystems. Many of the altered protein observed in the proteome response in *Synechocystis* were similar to those observed in the tolerance response of *Zymomonas mobilis* [66]. Proteomic analysis has also been observed on metabolic engineered *Synechocystis* producing levels as outlined in **Table 1** [64]. Here some 60–70% of the carbon fixed was converted to ethanol via a single ethanol cassette. At this rate of ethanol production there was no significant stress response observed rather there was a realignment of systems. Some upregulation of carbon concentrating mechanisms were observed, as were enzymes of the Calvin cycle and photosynthesis antennae proteins [64]. Interestingly the *thiC* gene, encoding phosphomethylpyrimidine synthase involved in thDP synthesis (the PDC-cofactor), was also up-regulated suggesting that even at this low level of ethanol production that co-factor availability was limited [64].

Tolerance has also been examined via transcriptomic analysis following exogenous ethanol addition [67] with 1.2–3% ethanol addition to wild type *Synechocystis*. Addition of 1.5% caused a 50% reduction in growth rate with visible aggregation suggesting stress. Many of the genes up-regulated in the transcriptomic study were associated with energy metabolism particularly photosynthesis. The results observed were broadly in line those observed in the proteomic studies [64].

7. Linking metabolic engineering of *Synechocystis* to production

While progress is being made with metabolic engineering for ethanol production and establishing *Synechocystis* as a cell factory there needs also be to an understanding of the production landscape when developing the system at industrial scale. Generation of a viable ethanol producing photoautotroph will necessitate rolling out of a production system to commercial level. Thus, at one level, there are the limitations and possibilities of metabolic engineering which have been discussed above but there is a second level that also needs to be addressed, that of the production environment to realize the potential of metabolically engineered strains. Indeed a fuller understanding of the requirements at this stage can help inform the strategies used for optimal metabolic engineering of potential production candidates.

7.1. Overall process life cycle analysis

Implementation of an industrial process for ethanol production from cyanobacteria will be the next stage of development once the challenges of metabolic engineering have been addressed.

Development of the downstream aspects of production will require optimization of several parameters and a more favorable economic outlook. Capital expenditure (CapEx) will be a key driving force with many components needing to be considered. Chief amongst these is the nature of the producing organisms being a recombinant strain. This poses potential safety and containment considerations, which would add to the economics of plant construction and operation. The need for sunlight (which may limit location of production facilities) or continuous LCD exposure again adds costs with either cyclic day exposure in high light climates or continuous growth with added light, which would come with an added energy cost. Equally, calculations of volumes that would be needed suggest large CapEx expenditure on plant, large water requirements and effluent processing costs. Many geographical areas that have high sunlight with marginal land, such as desert areas, at first sight might seem suitable but will suffer from water limitations. Other issues that are related to CapEx relate to the growth of the production strains themselves and the provision of optimal conditions for growth and production. Currently as one diverts photosynthetic intermediates to ethanol, one is affecting the flux to biomass. The more ethanol that is produced the slower the growth and the less biomass that can be produced. This impinges significantly on the growth rate and hence competitiveness of production strains. Given that, growth under sterile conditions in photobioreactors would be economically unsustainable (due to cost and the low value of the product ethanol); competitor contamination would need to be built into the growth cycle. Thus, slow growth of producers would have two major potential consequences that could affect the process. Firstly, there may be mutational selection for faster growers, which have lost the engineered ethanol cassette reducing the yield during production, and secondly given that axenic conditions could not be maintained during aseptic but non-sterile culturing, contaminants could easily outgrow the engineered strains. Strategies that might mitigate this could be the addition of mutualistic consortia, which might stimulate the production strains by providing vitamins or co-factors while limiting the growth of contaminants [68]. Thus, strategies that would aid production at large scale would need to be factored in at the initial stages of metabolic engineering.

7.2. Reactor design for large-scale economic production

The need for significant scale up of photoautotrophic ethanol production in a high light environment can add significantly to initial CapEx. Within the reactor system itself, several components may need significant attention. It is impractical for low value ethanol products, at least in comparison to current fuel costs, for growth and production to be carried out in sterile photobioreactors (PBRs) with full control over light, and key physiological conditions. Although the technologies for such photobioreactors are well developed their practicality can reasonably only be considered suitable for high value products [69]. In addition to containment issues, there are issues with inoculum development for non-axenic culturing to insure that initial inoculum is stable, productive and clonal. Depending on the plant size, this may require significant CapEx.

The most frequent types of PBRs proposed are non-sterile horizontal tubular or vertical flat panel PBRs, which have several limitations including: (a) cost, which have been estimated at €2400 m² for small scale, reducing in cost slightly with scale [70]. This would result in a cost

of some €12.6 kg⁻¹ [71], (b) High energy consumption [72] from mixing, CO₂ supply, pumping, separations, cleaning, and (c) Maintenance, cleaning and labor costs [70], (d) The reactor design must be able to withstand photo-oxidation, prevent evaporative loss of product, while maintaining axenic conditions as long as possible. Given the generally slow growth, rates of cyanobacterial species, largely because of the photoautotrophic lifestyle, need to manufacture most of their metabolites, maintain a polyploid genome because of the high sunlight and UV exposure the design of PBRs suitable for low value ethanol production from cyanobacteria is a challenge.

In production terms once one moves away from a controlled PBR design one halves the production cycle and level of photosynthetic production due to the night-day diurnal cycle and in addition there is less process control over the operation. Many approaches have been taken in an attempt to reduce cost; this has included use of bicarbonate-based systems for supply of carbon following carbon capture [73]. This may have significant cost savings in terms of CO₂ sparging, transport costs and CO₂ loss due to outgassing. Bag type culturing [70] which can be once off or be reusable can offer another potential solution. This may mitigate against some of the limitations of more traditional PBRs. Controlling contaminants in non-axenic culture might be carried out by use of pH as a control mechanism for limiting contamination, however this may necessitate use of more alkaliphilic cyanobacterial species [73]. Indeed adapting the production strain to the process or vice versa may offer a way forward in developing optimal reactor configurations with reduced CapEx. Thus incorporating knowledge of the production cycle, the types of conditions required for growth into a metabolic engineering strategy can be important during initial development of strains and strategies.

7.3. Temperature control, energy and evaporative loss

By virtue of the fact that ethanol-producing cyanobacteria will be recombinant strains, the current experimental systems tend to be enclosed due to regulatory constraints with GMO's. In geographical locations which are suitable for maximal sunlight and hence photosynthesis, enclosing a facility may raise issues with temperature control unless this is designed into the build. Direct exposure to air circulation or venting may also not be feasible due to safety issues while heat buildup beyond optimal growth temperatures, such as 30°C for *Synechocystis*, may easily occur. In such cases, utilizing a thermophilic or thermotolerant strain as a cell factory may be more feasible although this is currently not being done. In addition evaporative loss of the product ethanol may occur given the rather long growth and production rates, thus strategies to constantly remove and collect product during production may be essential, which might mitigate against needing ethanol tolerant strains. These issues illustrate the potential interplay between knowledge of the production system and the metabolic engineering needs and strategies.

Thus to ensure maximal production and recovery of ethanol, systems may need to be engineered to trap and recover ethanol during production which again may add considerably to CapEx. Jorquera et al. estimated, in a comparative analysis of power consumption of different photosynthetic reactors that horizontal tubular PBRs consumed 2500 W.m³, which reduced to

54 W.m³ for flat panel PBRs and to 3.7 W.m³ for raceway systems [74]. However mixing rates are quite different in the different systems such that in raceway systems there is little mixing, which effects movement of producing cells into light and poor mass transfer limiting overall productivity. Thus, power consumption unless linked to wind or solar in an integrated way may be a key hurdle to overall process efficiency and economy.

7.4. Ethanol recovery from production media

Lignocellulose based fermentations tend to be more dilute than starch based systems due to the presence of hemicellulose which increases viscosity and the presence of fermentation based inhibitors [75, 76]. This is currently similar in terms of cyanobacterial production of ethanol, which is also dilute and low in terms of yield. Recovery of ethanol from dilute production streams in an energy efficient and economical manner poses significant technical difficulties. Traditionally ethanol is recovered via distillation, however in the case of biofuel ethanol from cyanobacterial production the energy costs of distillation would be far too high particularly from dilute streams. It has been estimated that in a well-integrated lignocellulose to ethanol plant the process would require 4350 MJ.m⁻³ equivalent to approximately 20% of the energy content of the ethanol produced [76]. Thus, alternatives to distillation are needed to drive economy from cyanobacterial production systems. A number of techniques are available which may be suitable for the recovery of ethanol from cyanobacterial production such as membrane permeation or pervaporation, vacuum stripping, gas stripping, solvent extraction, adsorption and various hybrid processes [76]. However, the efficiency is dependent on the initial ethanol concentration (which is currently low for metabolically engineered *Synechocystis*) and often multiple cycles of processing would be required to achieve optimal yield. Thus, there are currently significant challenges to optimal recovery from dilute streams such as cyanobacterial systems although the potential for energy savings over distillation are possible. This thus implies that the higher the yield initially the better in terms of downstream processing, hence the current focus on optimizing metabolic engineering for yield.

8. Conclusions and perspectives

The basic proof of concept for photoautotrophic ethanol production from model cyanobacteria such as *Synechocystis* has been carried out. Strategies for increasing ethanol yields are currently being investigated but there are challenges going forward. These include the effect of ethanol synthesis on the metabolism of the producing strain, which include understanding and optimizing carbon flux, the tolerance of the organism to ethanol, growth and production rates and the challenges of integrating a production strategy that can inform the metabolic engineering strategy. Indeed the lessons learned from model organisms such as *Synechocystis* may need to be applied to different candidate strains which grow faster, can be genetically modified, are more robust in non-axenic culture, are more competitive or are more tolerant to the product once yields are increased. It is very much a case of much work done but significant challenges to future implementation of a viable production system.

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