Chapter 3
Cyanobacterial Biofuel Production: Current Development, Challenges and Future Needs

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Abstract As the need to replace fossil fuels increases and global energy needs expand the drive to find alternative, sustainable sources of fuels have accelerated. Microbial sources are attractive because of the rapid growth rates of microorganisms and their potential techno-economic advantages. Cyanobacteria are prokaryotic photoautotrophs (utilise photosynthesis and CO_2 for energy and carbon needs), which have emerged as potentially ideal candidates as sources of sustainable biofuel producers once metabolically engineered to do so. Over the past decade, there has been much interest in utilising cyanobacterial model species as proof of concept to produce and overexpress a range of biofuel candidates ranging from ethanol, butanol and other compounds ranging from hydrogen to fatty acids. Research on model biofuel candidates has revealed the potential for biofuel production but also revealed a number of challenges to future development. These challenges range from (1) biological, concerning the genetic constructs, their expression, stability and tolerance to the recombinant biofuel product, (2) production efficiency and biofuel recovery strategies and (3) economic, concerning the viability of production at a scale relative to the market price of the biofuel. Here, various technical challenges will be addressed based on experience and insights gained from the production of ethanol in model cyanobacteria, where many of these challenges are identified and strategies for future development discussed based on the current state of the art.

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3.1 Introduction

The need to replace fossil fuels in an expanding energy market with sustainable fuel sources has never been greater. Much attention has focused on biofuels from microbial sources that are potentially sustainable; do not compete with land usage for food crops and which can potentially be genetically manipulated for high yield in an industrial production-type system. Attention has focused particularly on cyanobacterial species, which are prokaryotic photoautotrophs and derive their energy and carbon needs from sunlight and CO_2, respectively. This not only holds out the possibility of phototrophic biofuel production but also environmentally could impact carbon capture strategies by recycling CO_2.

Initial attention with cyanobacteria has focused on a broad range of compounds for metabolic engineering. These include 3-hydroxybutyrate (Wang et al. 2013), 1,2-propanediol (Li and Liao 2013), isobutanol, isobutyraldehyde (Atsumi et al. 2009; Varman et al. 2013a), 2,3-butanediol (Oliver et al. 2013; Savakis et al. 2013), isopropanol (Kusakabe et al. 2013), free fatty acids (Gao et al. 2012a; Kaiser et al. 2013), fatty alcohols (Tan et al. 2011; Yao et al. 2014), endogenously produced alka(e)nes (Schirmer et al. 2010; Wang et al. 2013), carotinoids (Lagarde et al. 2000), squalene (Englund et al. 2014), sesquiterpene β-caryophyllene (Reinsvold et al. 2011), isoprene (Bentley et al. 2014), terpenoids (Lin and Pakrasi 2019), limonene (Kiyota et al. 2014), heptadecane (Yoshino et al. 2015), ethylene (Takahama et al. 2003; Guerrero et al. 2012), β-Phellandrene (Formighieri and Melis 2014), poly-β-hydroxybutyrate (PHB) (Wu et al. 2002), heparasan (Sarnaik et al. 2019) polyhydroxyalkanoate (Lau et al. 2014), plant essential oils (Formighieri and Melis 2018), hydroxyl propionic acid (Lan et al. 2015) cellulose (Nobles and Brown 2008), sucrose and glucose/fructose carbon substrates (Niederholtmeyer et al. 2010; Ducat et al. 2012), farnesene (Halfmann et al. 2014), mannitol (Jacobsen and Frigaard 2014), lactic acid (Niederholtmeyer et al. 2010; Angermayr et al. 2012; Joseph et al. 2013; Varman et al. 2013b), acetone (Zhou et al. 2012), H_2 production—both directly (Khetkorn et al. 2017) and in microbial electrolytic cells (McCormick et al. 2013) and ethanol (Deng and Coleman 1999; Dexter and Fu 2009; Dexter et al. 2015). The strategies utilised in metabolically engineering and assessing production levels with these various systems have proven useful in providing optimisation strategies towards particular biofuel production systems in cyanobacteria.

Model cyanobacterial systems have been extensively utilised to provide proof of concept for biofuel production systems. Within the cyanobacteria, the model organisms *Synechocystis sp* PCC6803 and *Synechococcus sp* PCC7942, have been utilised most extensively. In both cases, key attributes for choosing these species were based on ease of genetic manipulation, availability of genetic systems for metabolic engineering, nucleotide sequence data for the organisms, mutant strain availability and knowledge of the organisms through extensive usage. There is currently little consensus as to what type of cyanobacteria would make an ideal 'production' candidate, and indeed this may differ depending on the end product. An industrial 'producer' would have to possess many additional traits that are not

necessarily optimal in a model strain. These might include competitiveness in an open reactor system necessary for low-value biofuel products, fast growth rates, better partitioning of biofuel to product, high production rates and tolerance to the end product to mention just a few. Then given that these traits may be found, the necessary knowledge on genetics and metabolic engineering within such a candidate may take time to develop. Thus, although there are many challenges, lessons are being learned from model organism manipulation and with single biofuels such as ethanol. These can inform strategies for other organisms and indeed other biofuel products.

3.2 Manipulation Strategies with Model Organisms for Ethanol as a Biofuel

Many potential cyanobacterial niches allow photosynthetic metabolism for only part of the life cycle and many cyanobacteria utilise energy and storage compounds generated during the light phase to promote dark metabolism (Rastegari et al. 2019). Equally because of the niche, for example in microbial mats or lake sediments, the environment may become anoxic requiring inhabiting cyanobacteria to adapt quickly to different metabolic situations. Many cyanobacteria possess fermentative pathways that allow rapid adaptive change to environmental conditions somewhat like a survival tool (Stal and Moezelaar 1997). Some cyanobacteria are therefore capable of producing ethanol principally via heterotrophic anaerobic metabolism and surveys have shown that many strains inhabiting mats are capable of ethanol production (Heyer and Krumbein 1991; Stal and Moezelaar 1997) but at levels far too low and under non-photoautotrophic conditions to be of any practical use. Hence, realistically the only way forward is via metabolic engineering for the production of most cyanobacterial biofuels, including ethanol. A key start point to metabolically engineer biofuel production such as ethanol is to understand the pathways of central metabolism and how key intermediates can be diverted or manipulated (Fig. 3.1).

Synechocystis sp. PCC6803 was one of the first cyanobacteria to be fully sequenced (Kaneko et al. 1996). There are a variety of sub-strains of this model organism but these have in general been derived from the original strain although have acquired a number of mutations as a result of laboratory passage. Currently, there are a number of sub-strain genome sequences available with many single nucleotide polymorphisms that can affect growth and productivity on occasion. The availability of the genome sequence of model organisms such as *Synechocystis* sp. PCC6803 allows the sequence to be analysed via the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database (Ogata et al. 1999). This allows prediction of the metabolic pathways encoded by the organism and allows rational strategies to be developed to examine where metabolic engineering can start (from what intermediate) and what the consequences may be for perturbation of that intermediate. In the case of bioethanol production as a biofuel candidate, the start point is pyruvate

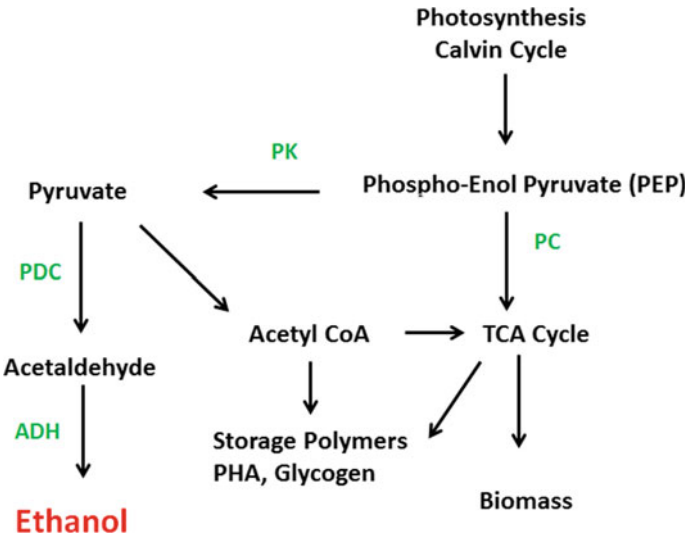

Fig. 3.1 Strategies for manipulating ethanol production in the model cyanobacterium, *Synechocystis* sp. PCC6803. The metabolic intermediate pyruvate can be converted to ethanol via the introduction of a genetic cassette expressing the *Zymomonas mobilis* pyruvate decarboxylase activity (*Pdc*), which channels pyruvate to acetaldehyde. This is in turn converted to ethanol by either the *Zymomonas mobilis* alcohol dehydrogenase II activity or by the native *Synechocystis Adh*A activity. Metabolic intermediates are channelled in the light to storage polymers and to the TCA cycle for biomass needs. Further manipulation of the pathways could be achieved by expressing pyruvate kinase (PK) to increase pyruvate concentrations or by knocking down PEP carboxylase (PC) to channel more PEP to pyruvate

and KEGG will then allow a view of where energy savings can be achieved or where problems may lie should too much intermediate be removed. KEGG may also allow the development of metabolic flux models and the identification of key bottlenecks. For example, in the case of bioethanol production, a key element is the diversion of the metabolic intermediate pyruvate to ethanol via acetaldehyde.

The enzyme pyruvate decarboxylase (*Pdc*) used to convert pyruvate to acetaldehyde (not encoded by cyanobacteria) has a requirement for two co-factors, thiamine diphosphate/pyrophosphate (ThDP) and Mg^{2+}. ThDP is also required for a number of other enzyme activities. Thus, one will be aware at an early stage that overexpression of a *pdc* gene to produce ethanol will give rise to a shortage of ThDP if not Mg^{2+}. Analysis of the *Synechocystis sp* PCC6803 genome and its KEGG database has in this case revealed that no ThDP transporters are present based on genome and KEGG data. Hence, a strategy of increasing expression of pathways to synthesise ThDP will be required or the provision of a heterologous ThDP transporter may need to be developed in addition to the production cassette containing the diverting metabolically engineered enzymes. It is this type of invaluable information that can be obtained from interrogation and analysis of model organism genome data, which

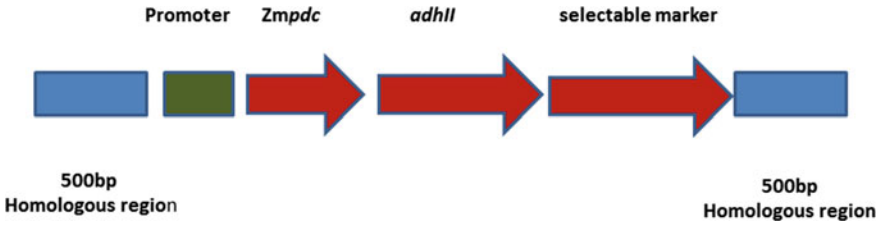

Fig. 3.2 Typical construction of an ethanol cassette utilised to divert flux to ethanol in *Synechocystis sp* PCC6803. The construct consists of two regions, typically of 500 bp with homology to the integration site within the host. The expression is controlled by a functioning cyanobacterial promoter which is linked to the *Zymomonas mobilis* pyruvate decarboxylase Zm*pdc* gene. This is linked to an alcohol dehydrogenase II gene (*adh*II) which is in turn linked to a selectable marker such as a kanamycin resistance determinant for host selection and maintenance of the cassette (Lopes da Silva et al. 2018)

can aid in developing systems for optimal biofuel production. Understanding limitations such as co-factor availability will be key to optimisation of cyanobacterial systems for any biofuel.

One of the first cyanobacterial species to be metabolically engineered to produce biofuel, ethanol, was *Synechococcus sp.* PCC7942 (formally called *Synechococcus elongatus* PCC7942) (Deng and Coleman 1999) by transforming the genes for pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase II (*adh*II) from *Zymomonas mobilis*, into *Synechococcus sp* PCC7942. *Zymomonas mobilis*, an obligate fermenter, is one of the few prokaryotes that produce large amounts of ethanol and whose genetic system has been extensively studied (Hoppner and Doelle 1983; Montenecourt 1985; Neale et al. 1986). These genes were expressed in the initial studies under the control of the cyanobacterial *rbc*LS promoter, alone and in combination with the *Escherichia coli lac* promoter. The reported yields of ethanol produced by the transformed strain reached 54 nmol. OD 730 nm unit^{-1} liter^{-1} day^{-1}(Deng and Coleman 1999).

This set the scene for further manipulation, which provided the initial proof of concept for photoautotrophic biofuel production. There followed a series of attempts to improve yields in *Synechococcus* PCC7942. Initially, (Woods et al. 2004) modified the promoter using the *p*L promoter of the coliphage lambda. This was followed by a number of industrial patents from Joule Unlimited and Algenol Biofuels (US patents US8163516B2 and WO2013098267A1), which focussed on different promoters to drive cassette expression and some construct modifications. Using the same *Zymomonas mobilis* genes for *pdc* and *adh*II Dexter and Fu (2009) reported expression in *Synechocystis* sp. PCC6803 under the control of the light-driven *psb*A2 promoter, reaching a production level of 5.2 mmol. OD 730 nm unit^{-1} litre^{-1} day^{-1}. Attempts to use other *pdc* genes with lower Km values, such as that from *Zymobacter palmae,* have also been undertaken but these have proven to be unsuccessful (Quinn et al. 2019).

Figure 3.2 illustrates the overall construction of a biofuel cassette for metabolic engineering ethanol production in *Synechocystis sp* PCC6803 for chromosomal integration. The homologous regions allow recombination with the appropriate neutral integration site. The cassette can have different promoter constructs to drive expression. The key genes are *pdc* and *adh* genes to convert pyruvate to ethanol via acetaldehyde and the final drug resistance gene allows selection and again can encode various determinants suitable for the model host.

Using two different engineered strains of *Synechocystis sp* PCC6803, one engineered with the ethanol cassette (*pdc* and *adh*) and co-cultured with another PCC6803 strain deleted in the production of glucose-1-phosphate adenylyltransferase and PHA-specific β-ketothiolase (Δ*apx* and Δ*gbk* unable to synthesise glycogen and PHB storage polymers), it was demonstrated that the deleted strain released various metabolites that were utilised by the ethanol producer (Velmurugan and Incharoensakdi 2020). This strategy resulted in an overall increased ethanol yield due to co-culture and may point to innovative co-culture strategies in the future.

In addition to ethanol, several other candidate biofuel products have been investigated in model cyanobacterial species. 1-Butanol is considered as a fuel substitute to displace gasoline which has an energy density of 27 MJ L^{-1}, whereas ethanol is 21 MJ L^{-1}. 1-Butanol can be synthesised by converting butyryl-CoA from acetyl-CoA where butyryl-CoA is then reduced to 1-butanol (Atsumi et al. 2008) To produce 1-butanol, a CoA-dependent 1-butanol pathway was transferred from *Clostridium* and expressed in *Synechococcus elongatus* PCC794 (Lan and Liao 2011). To overcome the need for *Clostridium* ferrodoxins, the *Clostridium acetobutylicum* butyryl-CoA reductase, which requires ferrodoxin, was replaced by a *Treponema denticola* trans-enyl-CoA reductase, which utilises NADH as a co-factor. In addition, the *Ato*B, encoding acetoacetyl CoA thiolase, was derived from *E.coli* as it possesses a higher specific activity and was used to generate a heterologous construct that was integrated in two parts into two separate neutral chromosomal sites (Lan and Liao 2011). An initial worry was the possibility that since these activities were derived from a strict anaerobe then enzyme activity might be oxygen-sensitive, however, this construct demonstrated 1-butanol production, suggesting no or little oxygen sensitivity.

Isobutanol is another biofuel candidate that has been investigated as it contains 98% of the energy content of gasoline, has a lower solubility than ethanol (potentially aiding recovery from metabolically engineered organisms), meaning that it can be blended perhaps more easily with gasoline (Miao et al. 2017). To produce isobutanol, an α-ketoisovalerate decarboxylase, termed *kivd,* from *Lactococcus lactis* was expressed in *Synechocystis sp* PCC6803 (Miao et al. 2017) and strains were shown to produce 3 mg L^{-1} OD 750^{-1} isobutanol in a 6-day growth period. Supplementation with isobutyraldehyde increased yields to 60.8 mg L^{-1} day^{-1}. Miao et al. (2017) demonstrated that overexpressing *kivd* via self-replicating vectors under the control of the strong P*trc* promoter resulted in even higher yields than chromosomal integration.

To aid construct development for metabolic engineering in cyanobacteria a standardised cloning and assembly system has been developed, termed CyanoGate. This has involved the development of a suite of parts and acceptor vectors to

generate knockouts, multigene expression and repression systems, for use via replicative vectors initially in *Synechococcus* and *Synechocystis* (Vasudevan et al. 2019). Such systems will aid with the development of synthetic biology approaches in cyanobacteria particularly towards biofuel production into the future.

Given that cyanobacteria have not evolved strategies to produce biofuels and therefore must be metabolically engineered to do so the process of biofuel production, optimisation and scale-up in cyanobacteria are not without their challenges. Such challenges can be considered as a mixture of biological, production and economic issues, which need to be considered in detail to ensure progress with biofuel production. Lessons have been learned from developments principally with ethanol production in model organisms but these lessons have implications for many of the biofuel candidates and merit further consideration and discussion.

3.3 Polyploidy and Cassette Stability

Many cyanobacteria are polyploid, possessing multiple chromosomal copies. Using real-time PCR, it has been shown that the 'Kasusa' strain of *Synechocystis* sp PCC6803 (one of the original *Synechocystis* sub-strains) (Pembroke et al. 2017) could have up to several hundred copies of its chromosome in exponential phase and an average of 58 copies in stationary phase (Griese et al. 2011). Ploidy appears to be a common trait in cyanobacteria, which has been proposed to be a response to life in a high UV environment with multiple copies aiding recombinational repair and chromosome maintenances. This high polyploid chromosomal number poses a number of challenges when using and manipulating polyploid hosts. The carbon, nitrogen and phosphate flux needed to maintain such a high chromosome copy number, which appears to vary with the growth phase is high and hence there is a diversion from using these resources for biofuel production. Copy number mutants with low ploidy have so far not been isolated but this could aid in enhancing biofuel production by lowering carbon flux to maintain chromosome number. A second key issue that emerges from the polyploid nature of cyanobacteria is related to genetically engineering them and maintaining the stability of any metabolically engineered cassette. In most cases, constructs are integrated directly into the host chromosome via recombination of the constructed cassette (Dexter and Fu 2009; Lopes da Silva et al. 2018; Pembroke et al. 2019). To develop stable and expressing recombinants, constructs have to undergo extensive selection. Initially, during selection, recombinant clones will be only partly segregated in the polyploid genome with some chromosomes possessing the insert, while others will not. This can be tracked using PCR primers across the integration site.

The absence of the cloned cassette will manifest with PCR amplicons of a size representing just the integration area, while an integrated cassette will give rise to larger PCR amplicons. In practice, during the early phases of uptake, there will be a mixture of amplicons that will stabilise as each chromosome integrates the cassette or the integrated chromosome is copied. Polyploidy also means that there are gene

dosage effects. An integrated cassette with a chromosome copy number of 58 can lead to high expression levels. This coupled to an inducible promoter, such as the *psb*A2 light-inducible promoter, can result in significant heterologous enzyme expression, during the light phase. If selection is not maintained, then the inserted cassette can be lost as there is a biological prerogative to remove the insert as usually its expression, in the case of a cassette to produce biofuel, puts a metabolic burden on the host. Thus, in the case of bioethanol, the cassette needs to be maintained and, even with selection, stability may be affected by mutagenesis to knock out the heterologous genes or delete them entirely.

The variation in polyploid copy numbers can also be problematic for the stability of introduced cassettes. As the chromosome copy number reduces during stationary and late stationary phases (Griese et al. 2011), mutations or deletions picked up at this stage may be advantageous as it may relieve the pressure of biofuel production and allow more rapid selection of non-producers, which by virtue of this relief will be faster growers and may lead to a population of non-biofuel producers. Thus, polyploidy can have major effects on engineered producers and needs further assessment.

3.4 Copy Number and Insert Site Selection

One of the strategies utilised to maximise the expression of constructs to divert flux to biofuels in cyanobacterial model species has been to utilise gene dosage as a tool. By inserting into the chromosome and by virtue of the polyploid nature of model cyanobacteria, there will be a gene dosage effect as the chromosome number increases. Initial studies on bioethanol production in model cyanobacteria were carried out using one copy of an ethanol cassette. The original bioethanol work (Deng and Coleman 1999) utilised replicative plasmids based on the multi-copy *Synechococcus* sp. plasmid pCB4 to generate a series of constructs, pCB4-LRpa and pCB4-LR (TF), utilising ampicillin resistance as a selectable marker. Later, (Dexter and Fu 2009) utilised an integration system in *Synechocystis,* which resulted in higher yields with reported greater stability. Based on such studies, there has thus been the tendency to utilise integration as the engineering technique, particularly with the model organism *Synechocystis sp* PCC6803. A natural progression towards increasing productivity would be to engineer strains with more than one copy of a cassette and, to this end, Gao et al. (2012b) reported the use of a two-cassette system where two copies of the ethanol cassette were integrated in *Synechocystis* sp PCC6803 with a yield of 5.50 g L^{-1} after 26-day growth (or 212 mg L^{-1} day^{-1}). This two-cassette strategy was also utilised towards pilot-scale production (Lopes da Silva et al. 2018), but thus far, a three-cassette model has proven difficult to isolate and with ploidy and gene dosage two copies may be close to the maximum that can be tolerated by an engineered organism (at least in the case of ethanol cassettes).

Indeed, the generation of modified engineered strains, with multiple inserts can be problematic. The modifications themselves can be time-consuming with multiple

rounds of transformation, cloning and selection particularly if marker-less clones are needed for production strains where the antibiotic selection may not be an option. In addition with multiple inserts, many different neutral sites may be required for insertion and there may be differential expression and stability at different integration sites. Another option would be to utilise native stable, multi-copy plasmids as integration sites. *Synechocystis sp* PCC6803 contains at least three small plasmids pCA2.4, pCB2.4 and pCC5.2 and in addition at least four large plasmids pSYSM (125 kb), pSYA (119 kb), pSYSG (45 kb) and pSYSX (106 kb) (Kaneko et al 2003). These appear to be highly stable and to have been maintained within the strain since the strain was originally isolated more than 50 years ago. Other cyanobacteria also appear to contain multiple plasmids. Genome sequencing of *Anabaena* sp. PCC7120 has revealed six plasmids ranging from 5.6 to 408 kb (Kaneko et al. 2003) suggesting that possessing native plasmids may be common in cyanobacteria. As a proof of concept, the yellow fluorescent protein gene (*yfp*) was cloned into the small *Synechocystis* sp. PCC6803 pCA2.4 plasmid (Armshaw et al. 2015). This plasmid, of 2.4 kb, is consistently maintained at seven times the chromosomal polyploid copy number and is extremely stable. When the comparison between chromosomal integration and pCA2.4 integration of this YFP cassette was determined it was demonstrated that a 20-fold higher fluorescence could be detected (Armshaw et al. 2015) upon the integration of yfp into pCA2.4. This level of heterologous protein production illustrates interesting possibilities for future integration of biofuel cassettes into multi-copy, stable native plasmids as a strategy towards enhanced gene dosage and production.

As integration strategies for biofuel cassettes are common when manipulating cyanobacteria, it is important to integrate at neutral sites (locations where integration does not impact cell viability or phenotype) (Ng et al. 2015; Pinto et al. 2015). A comprehensive analysis of such sites was carried out for *Synechocystis sp* PCC6803 by analysing insertion and deletion mutations at these sites (Pinto et al. 2015). This type of analysis is essential, as to build metabolic pathways in cyanobacteria many different locations may be necessary and key issues such as stability and expression differences at these sites will ultimately affect production. Potential neutral sites need to be identified and characterised for the stability of integrated cassette while analysing proteome changes that might result from the insertion can also aid choice. There is in addition the possibility that a potential neutral site might be part of a cis-regulatory sequence or be essential during a particular but limited growth phase. Thus, the identification of real neutral sites (Pinto et al. 2015), which show identical growth patterns to wild type may be complex but feasible. Hence, in the future, developing integration-cloning strategies for potentially faster growing, more productive, more resistant, more competitive biofuel strains lessons gained from strategies applied to model cyanobacteria can prove hugely informative.

With any gene expression effect, whether it be promoter-enhanced expression or increased gene dosage, one must reach a point of diminished return. A point, where any increase in a protein expressed from a cassette will not enhance production further. This point may be related to the maximum flux that can be reached and, beyond this point, no extra enzyme level will have a beneficial effect. Producing organisms will have a minimum metabolic intermediate need for maintenance of

cell structures, metabolism and other metabolic intermediates. Thus, the flux towards biofuel can only go so far before no further enhancement can occur and diversion towards biofuel production will result in a major decrease in biomass.

3.5 Promoter-Driven Expression and Vector Systems

One of the challenges to the expression of engineered biofuel cassettes has been finding suitable promoter systems to optimise expression which is seen as a key element of the manipulative toolbox. As many inducible systems that operate in *E.coli* do not function well or similarly, in cyanobacteria, native promoters need to be utilised. One of the early promoters investigated was the *psb*A2 light-driven promoter (Dexter and Fu 2009). Use of the *psb*A2 promoter was based on observations that expression patterns of chimeric genes containing the promoter regions of the *psb*A2, gene fused to the firefly luciferase (*luc*) reporter gene indicated that transcription of *psbA2/luc* transgenes was elevated, similarly to that of the endogenous *psbA* gene (Máté et al. 1998). The *psb*A2 promoter has a number of unique characteristics (Asayama et al. 2002; Shibato et al. 2002) involving cis-acting sequences, which are involved in circadian expression and light-driven promotion of fused genes. Thus, this promoter-driven system has been popular in many studies (Dexter and Fu 2009; Gao et al. 2012b; Lopes da Silva et al. 2018).

A systematic analysis of promoters and ribosome binding sites has been carried out specifically for *Synechocystis* (Englund et al. 2016). Comparison with metal inducible, light-inducible and constitutive promoters revealed that the P*nrs*B could be induced some 40 folds by nickel or cobalt addition up to the level of the strong light-inducible promoter *psb*A2 (Englund et al. 2016). Inducible promoters such as P*nrs*B may have an application in decoupling growth from production. On other occasions, obtaining large quantities of enzyme activity may be the goal and, in such cases, a strong promoter will be required. Using the promoter for the *cpc*B gene, P*cpc*560, functional proteins were produced at a level of up to 15% of total soluble protein in *Synechocystis* sp PCC6803, a level comparable to that produced in *E. coli* (Zhou et al. 2014).

There is somewhat of a scarcity of well-characterised replicative vectors for cyanobacterial model systems (Huang et al. 2010; Taton et al. 2014). This has led to the development of new, more functionally designed vector systems with model organism functionality considerations being to the fore. Three plasmids pSEVA251 (KM^R) pSEVA351 (CM^R) and pSEVA451 (SP/SM^R) have been developed to add to the toolbox of existing integrative systems (Ferreira et al. 2018). These vectors now carry a range of promoters based on PT7opol and P*trc* giving up to 41-fold enhanced expression and containing repression systems. Detailed analysis of these vector systems demonstrated that the presence of the plasmid does not lead to an evident phenotype effect on *Synechocystis* growth, with the majority of the cells able to retain the replicative plasmid even in the absence of selective pressure (Ferreira et al. 2018).

Self-replicating shuttle vectors have also been developed based on *pANS* of *Synechococcus elongates* PCC7942. This vector constructs involved the introduction of a heterologous toxin–antitoxin cassette into the shuttle vector for stable plasmid maintenance in the absence of antibiotic selection (Chen et al. 2016). The vector was shown to be stable in *Anabaena* and in cured *Synechococcus* cells and in *E.coli.* It was shown to give rise to ten copies per cell and reporter genes were expressed some 2.5 folds compared to chromosomal integration. Such shuttle systems will add to the toolbox to aid construction in well-developed backgrounds, such as *E.coli,* and allow rapid shuttle into a variety of cyanobacterial strains.

There are sometimes alternative needs and requirements for controlled expression of biofuel cassettes such as decoupling production from growth where it may be useful to allow biomass production firstly and only induce biofuel production when the biomass resources are available. This is analogous to how ethanol is produced in the yeast *Saccharomyces* with ethanol production triggered on shift to anaerobic metabolism.

Several P*trc* riboswitches have been characterised in cyanobacteria and tightly regulated expression shown to be possible using theophylline as the switch inducer (Nakahira et al. 2013). Using a riboswitch technique in *Synechocystis sp* PCC6803, where the ethanol cassette was fused to a riboswitch, has allowed the decoupling of ethanol production from biomass (Armshaw et al. 2018). Here biomass is allowed until ethanol production is induced by theophylline at set points in the exponential or stationary phases; however, there was no increase in overall ethanol productivity reported via such constructs although higher biomass yields were reported. Thus, the toolkit to obtain time-dependent, via an inducible system, or high-level expression, via a strong promoter is available and will be key elements for future genetic manipulation strategies in cyanobacteria.

3.6 Knockouts, Rerouting Carbon Flux and Flux Analysis

Rerouting carbon flux has been utilised in attempts to improve carbon flow to biofuel products in many cases (Dexter et al. 2015; Hendry et al. 2017). The principle underlying this being that if there are two competing pathways, then knocking out one or more may alter the flux towards the preferred product (Fig. 3.1). In cyanobacterial cells, there are many examples of storage compounds such as glycogen or PHA, which are used for energy storage during high levels of photosynthesis, effectively acting as a carbon sink. Theoretically, manipulating or deleting such pathways should alter the flux to product (Dexter et al. 2015). Using ^{13}C metabolic flux analysis, the rerouting of carbon was examined in a glycogen synthase-deficient mutant (*glg*A-I *glg*A-II) strain of *Synechococcus* sp. PCC 7002 (Hendry et al. 2017). Normally, between 10 and 20% of the fixed carbon is stored in the form of glycogen in many cyanobacterial strains during balanced photoautotrophic growth (Hendry et al. 2017). In the *glg*A-I and *glg*A-II mutants, a redistribution of carbon flux occurs, some to other storage compounds such as glucosyl glycerol and sucrose while the rest partitions

to other metabolic networks such as glycolysis and the TCA cycle. In this respect, Monshupanee et al (2019) disrupted the γ-aminobutyric acid (GABA) shunt, one of the metabolic pathways for completing the TCA cycle in *Synechocystis*, by inactivating the glutamate decarboxylase (*gdc*) gene. This resulted in an increase in pyruvate levels (1.23 folds) and a 2.5-fold increase in poly(3-hydroxybutyrate) (PHB) production while reducing TCA cycle intermediates. Such a knockout is potentially one of many strategies that could divert flux to pyruvate and hence ethanol in a cassette containing host.

Changing the carbon sink by genetically engineering alternative pathways can also have a major effect on the flux as it redirects the sink within the host organism. Two engineered strains of *Synechocystis* sp. PCC 6803 with altered carbon sink capacity were assayed for their photosynthetic and CO_2 concentrating properties (Holland et al. 2016). A comparison of knocking out and adding a sink via analysis of a Δ*glg*C mutant, where a carbon sink was removed (unable to synthesise glycogen as a storage compound) and strain JU547 (engineered to produce ethylene, a new sink) revealed that the Δ*glg*C mutant displayed a diminished photochemical efficiency, a more reduced NADPH pool, delayed initiation of the Calvin–Benson–Bassham cycle, impairment of linear and cyclic electron flows and a reduced PQ pool, and an undefined dissipative mechanism to spill excess energy. In the case of JU547, more oxidised PQ and NADPH pools were observed with increased rates of cyclic electron flow and enhanced demand for inorganic carbon was observed as suggested by increased expression of the bicarbonate transporter, *Sbt*A (Holland et al. 2016). This study identified that subtle changes in pathways and flux can affect many areas of photosynthetic metabolism, which can ultimately affect the production of metabolically engineered products.

Since cofactors, such as NADPH, are essential for *Adh*II activities (catalyses the reversible oxidation of alcohols to aldehydes or ketones in a NAD(P)-dependent manner) one can see that changes that alter one sink may have unplanned consequences on another pathway and ultimately affect the production of metabolically engineered strains as with ethanol in this case. It has been shown that *Zymomonas Adh*II utilises NAD(H), while the native *Synechocystis Adh*A utilises NADP(H). A number of *adh*II genes have been trialled in conjunction with the *Zymomonas mobilis pdc,* but it has been shown that the *Adh*A (slr1192) of *Synechocystis*, with its NADP(H) co-factor preference, is the most efficient (Gao et al. 2012b). In practice in many engineered hosts both activities, the *Zymomonas* and native, are present (Gao et al. 2012b; Pembroke et al. 2017) however for maximal activity and coupling with the *Pdc* activity any reduction in the NADP(H) pools will affect production.

Flux distribution studies can provide a quantitative view of the way carbon is partitioned in cyanobacterial hosts and much information can be obtained by flux balance analysis, which requires flux values (Baroukh et al. 2015) and metabolic flux analysis data, generally based on isotopic labelling. Such data allow the construction of useful metabolic models (Mueller et al. 2013), which can aid in determining strategies for useful knockout or pathway enhancement protocols. How such adjustments might affect biofuel production or diversion of key intermediates can also be examined by modelling strategies such as Minimisation of Metabolic Adjustments

(MOMA). MOMA strategises that any perturbation will result in minimal adjustment (Segre et al. 2002) and such models have been applied to biofuel producers to predict knockout strategies towards higher yields (Hendry et al. 2016). While modelling can provide useful data, the availability of real data for metabolic flux analysis based on isotopic labelling (Hendry et al. 2017) will in future dramatically aid knockout and enhancement strategies.

In generating designer microbes for biofuel production, Angermayr et al. (2014) has proposed a number of 'design' principles for model organisms such as *Synechocystis sp* PCC6803. Although they focused on the increased production of lactic acid, the principles are valid to other cyanobacterial production systems. The principles include increased expression of the product forming enzymes, co-expression of heterologous pyruvate kinase to increase flux to pyruvate, knockdown of PEP carboxylase to decrease flux to competing pathways (Fig. 3.1) and optimising the production enzymes via mutagenesis to improve the kinetics or co-factor affinity (Angermayr et al. 2014). Many of these principles hold fast for ethanol production and indeed other potential biofuel candidates where pyruvate is also the key cellular intermediate.

3.7 Increasing Carbon Uptake

As engineered strains of cyanobacteria are pushed to produce biofuel products, there is an inevitable reduction of biomass at a fixed CO_2 uptake. Therefore, a potential strategy to increase flux to biofuel is to manipulate the CO_2 uptake systems. Photosynthetic organisms have evolved different forms of Carbon Concentrating Mechanisms (CCM's) to aid RuBisCO in capturing CO_2 from the aqueous/gaseous environment (Badger et al. 2002). The CCM in cyanobacteria is one of the most effective concentrating mechanisms known, able to concentrate carbon up to 1000 folds within the cell. Many cyanobacteria, including *Synechocystis* sp. PCC6803, use a number of scavenging systems to concentrate bicarbonate and CO_2. CCMs in model cyanobacteria studied thus far involve six functional elements: (1) Passive or energised entry of dissolved inorganic carbon, (2) Increase in HCO_3 concentration in the cell, (3) Entry into carboxysomes, (4) Providing saturation of CO_2 near RuBisCO, (5) Fixation of CO_2 and (6) Prevention of CO_2 leakage from the carboxysomes (Kaplan and Reinhold 2002). To effect carbon uptake and concentration, *Synechocystis* sp. PCC6803 has been shown to contain a number of transporters (Shibata et al. 2001, 2002) including three bicarbonate transporters:

(a) A high-affinity inducible *Bct*1 bicarbonate transporter (slr0040–44)
(b) An inducible medium affinity sodium-dependent *Sbt*A bicarbonate transporter (slr1512)
(c) A medium-affinity *Bic*A transporter (sll0834).

The *Bct*1 and *Sbt*A transporters are regulated by the *Ccm*R transcription factor, which senses intercellular levels of α-ketoglutarate and NADP (Daley et al. 2012),

while *Bic*A is constitutively expressed (Badger et al. 2002). In addition, there are also a number of CO_2 uptake systems termed NDH-I_3 and NDH-I_4 (multiple variants may also exist) that involve proton translocating NAD(P)H-dependent oxidoreductases, which have a multi-subunit composition, 6 in the case of *Synechocystis* (Battchikova et al. 2010). Although many of the systems are multi-gene, in principle increased levels of CO_2 could be achieved by cloning or overexpressing one or more of these carbon concentrating systems. Strains of the model organism *Synechocystis sp* PCC6803 have been engineered by installing extra bicarbonate transporters via the introduction of inducible copies of the single gene encoding *Bic*A (Kamennaya et al. 2015). When cultured under atmospheric pressure, the strain expressing *Bic*A grew almost twice as fast and accumulated twice as much biomass as the wild type. Interestingly, an accumulation of increased sugar-rich exopolymeric material was also detected in these strains. This indicated that carbon flux could be redirected in a similar manner to enhance biofuel production and such strategies may offer potential in the future for many biofuel candidates.

3.8 Cellular Tolerance to the Engineered Biofuel

Given that model organisms do not naturally produce more than minute quantities of bioethanol during fermentative growth in the dark, it is of interest to examine the response of engineered strains to ethanol or other biofuels (Kumar et al. 2019). Almost all engineered products and biofuel candidates elicit some form of stress or tolerance response (Nicolaou et al. 2010). Commercial production of biofuels such as ethanol at industrial levels may require production strains to produce up to 20% (v/v) ethanol to be economic. Current production levels are still very far from even 1% (v/v) but the production needed at scale illustrates what potentially is required (Dexter et al. 2015; Pembroke et al. 2017). In a comparison of ethanol tolerance between nine different cyanobacterial strains, it was demonstrated that the growth inhibition GI_{50} values ranged from 3 g L^{-1} (0.4% v/v) to 28 g L^{-1} (3.5% v/v) (Kämäräinen et al. 2018). In this study, the most tolerant strains were *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7002, with both model organisms showing little effect on growth below ethanol concentrations 9.2 g L^{-1} (1.2% V/V).

Currently, the highest reported ethanol yields are 5.5 g L^{-1} (Gao et al. 2012b) and 7.1 g L^{-1} (Dehring et al. 2012, a US Patent). Both of these studies were from idealised photobioreactor conditions operating in full light under sterile culture conditions, which could be far from potential industrial conditions. Thus, while current yields appear far from the GI_{50} levels, strategies that would improve yields would eventually lead to some form of cellular toxicity. Qiao et al. (2012) demonstrated that the addition of ethanol at 12 g L^{-1} resulted in a 50% growth reduction of *Synechocystis sp* PCC6803. To analyse the effects of ethanol toxicity on model strains of *Synechocystis,* both transcriptomic and proteomic analyses have been carried out (Wang et al. 2012; Qiao et al. 2012; Dienst et al. 2014; Borirak et al. 2015). A comprehensive transcriptomic analysis, using an RNA-seq library was carried out by supplementing

cultures of *Synechocystis sp* PCC6803 with 1.25–2.5% (v/v) of ethanol and sampling between 24 and 72 h post-ethanol addition (Wang et al. 2012) and 1.5% was reported to cause a 50% reduction in growth rate and visible aggregation. Some 274 genes were reported to be up-regulated, associated with photosynthesis, the Calvin cycle, ribosomal subunits, energy metabolism and ATP synthesis.

These transcriptomic observations were similar to proteomic data (Qiao et al. 2012) with both studies reporting up-regulation of a number of unknown transcripts and proteins such as *slr*0144, *slr*0373 and *slr*1470 suggesting some potentially, as yet uncharacterised, response mechanisms. The individual transcripts and proteins up and down-regulated suggest that the effected organism may be triggering the oxidative stress response, enhancing transport mechanisms (12 transporters were up-regulated). Equally, proteins associated with membrane modification were expressed suggesting that membrane modification may be a key tolerance mechanism. Squalene hopene cyclase (*slr*2089) was one such protein with overexpressed activity observed (Wang et al. 2012; Qiao et al. 2012) which has been proposed to be involved in strengthening the membrane in response to ethanol. Cell envelope proteins and genes were also up-regulated as were genes involved in PHA storage and some ten signal transduction activities were also up-regulated. Some 1874 genes were down-regulated of which over 60% were of unknown function, indicating a comprehensive response elicited to ethanol.

In an allied study, Dienst et al. (2014) examined the transcriptomic response via high-density microarrays to the continuous production of ethanol from an integrated ethanol cassette at current low production levels (0.03% v/v). This study confirmed that the production of ethanol in this engineered strain resulted in a 40% reduction of biomass. Although the ethanol environment might be expected to be less toxic than adding 1.5% as in the previous addition studies (Wang et al. 2012; Qiao et al. 2012), there was still a significant response. The *adh*A gene encoding the *Synechocystis-Adh*II enzyme was up-regulated suggesting this may have some sort of detoxifying role. An ABC transporter (*slr*1897) was up-regulated as were a number of ribosomal proteins suggesting an initial limited response by protein synthesis, metabolic conversion and perhaps some transport phenomenon may initially be induced. Several genes were downregulated, but there was no evidence of induction of the stress response (Dienst et al. 2014). A similar study (Borirak et al. 2015) carried out a quantitative proteomic study of ethanol producers examining the proteome in a similar way to the transcriptomic study previously reported (Dienst et al. 2014).

Even with low ethanol levels, some 267 proteins were up or downregulated. These included upregulation of carbon fixation, presumably to compensate for the flux away from biomass to ethanol, and evidence of oxidative stress induction was reported. Interestingly, the enzyme phosphor-methyl-pyrimidine synthase (*thi*C), involved in ThDP biosynthesis, was also up-regulated suggesting that even at early stages in production the Pdc co-factor, ThDP, may be in limited supply and could affect overall flux to ethanol (Borirak et al. 2015). Such transcriptomic and proteomic studies may offer strategies to respond to the apparent ethanol toxicity observed. Some interventions such as cloning ThDP transporters to prevent ThDP co-factor limitation, or overexpressing squalene hopene cyclase (*slr* 2089) to increase membrane solidity

may be options going forward but others such as induction of stress responses may need the evolution or use of more tolerant model organisms.

Tolerance to other biofuel candidates has also been examined. Isobutanol tolerant strains of *Synechocystis sp* PCC6803 were obtained by long-term laboratory evolution using media containing 2 g L^{-1} (Matsusako et al. 2017). Mutant strains capable of growing at 5 g L^{-1} were isolated and genetic analysis revealed they had accumulated multiple mutations in *slr*1044, (*mcp*A) encoding a methyl-accepting chemotaxis protein which may affect aggregation, *slr*0369 (*env*D), an efflux transporter allowed growth in 5 g L^{-1} isobutanol or *slr*0322 (hik43), a histidine kinase sensor regulator again controlling cell aggregation. These mutations generally demonstrated stress resistance not only to isobutanol but also to other alcohols including ethanol when examined (Matsusako et al. 2017). This observation of synergism may allow data generated on tolerance evolution to be utilised amongst several biofuel production systems as resistant strains to isobutanol also demonstrated increased isobutanol production when containing production cassettes (Matsusako et al. 2017). In an attempt to observe the effects of these isobutanol resistance mutations alone, strains were constructed with deletions in *mcpA*, *hik43* and *envD* and, while no mutation alone gave high-level resistance, it was the synergistic effect of the combination of these mutations that gave rise to the evolved resistance (Matsusako et al. 2017).

Tolerance to n-butanol, which limits growth in cyanobacterial production strains, was also examined via transcriptome (RNA-seq) sequencing (Anfelt et al. 2013) in *Synechocystis sp* PCC6803. Some 80 transcripts were differentially expressed by the addition of 40 mg L^{-1} of n-butanol, while some 280 were differentially expressed at 1 g L^{-1}. Analysis of data suggested that issues with membrane function, impaired photosynthesis, electron transport, reduced biosynthesis and accumulation of reactive oxygen species were all inferred from the transcriptome data (Anfelt et al. 2013). Using the transcriptome data a number of proteins were overexpressed as informed by the differentially expressed transcriptome, one of which *Hsp*A, a small heat shock protein, improved tolerance to butanol. However, the picture can be complex. Comparative quantitative proteomic analysis of the response to n-butanol led to the identification of 303 differentially regulated proteins in metabolically engineered *Synechocystis* sp PCC6803 (Tian et al. 2013) with data analysis concluding that *Synechocystis* employed multiple and synergistic resistance mechanisms in dealing with butanol stress most notably induction of heat shock proteins, cell membrane modification and transporters. Such studies indicate that transcriptome and proteome data can be useful in informing strategies for enhancing tolerance and production.

3.9 Mutation or Deletion of Production Cassettes

Genetic instability in cyanobacteria is considered somewhat of ‘an elephant in the room’ (Jones 2014). When model cyanobacteria are manipulated with heterologous DNA for biofuel production this puts a metabolic burden on the host. With the use of selective pressure, the construct can be maintained and its presence as part of the

polyploid genome means that its removal by the organism is not simple. However, even though there are multiple copies, clones that put a burden on the host are prime targets for an adaptive mutation to remove the burden. Because biofuel production diverts metabolic intermediates away from biomass it leads to slower growth rates in the producer. Therefore, mutations in the construct will alleviate this burden, remove the bottleneck to growth and allow the development of more competitive faster growing (non-producing) mutants. Although there are few publications in the literature (Jones 2014), it is recognised by those who work in the area that genetic instability exists and is indeed common. It is not unusual to see strains emerge suddenly which show very low production rates and examination reveals that constructs that have taken time to construct, transform and select have suddenly undergone mutation. The literature on the mutation of constructs in cyanobacteria is sparse.

Takahama et al. (2003) reported instability in engineered strains of *Synechococcus elongatus* PCC 7942 modified for ethylene synthesis while similar instability was observed in engineered mannitol-producing *Synechococcus sp.* PCC 7002 strain (Jacobsen and Frigaard 2014). Angermayr et al. (2012) reported revertants in a lactate-engineered construct of *Synechocystis sp* PCC6803, while Kusakabe et al. (2013) reported mutations in *ato*B one of the genes central to a construct synthesising isopropanol in *Synechococcus sp.* PCC 7942. We have also observed mutation accumulation in the *pdc* gene of pUL004 a construct utilised to produce ethanol (Lopes da Silva et al. 2018), although at a relatively low rate. Jones (2014) has suggested that mutations may be inducible in cyanobacteria that undergo stress and indeed other organisms also undergo mutation as a result of stress (Sleight and Sauro 2013). However, the added stress both metabolically and through solvent interaction with cell structures and components during biofuel production may force the selection to negate this stress and ultimately effect production. Thus, stability and instability of biofuel constructs will need to be examined as part of the toolkit for generating industrial-scale biofuel producers moving forward.

3.10 Growth and Product Recovery at the Pilot or Industrial Scale

In the drive towards industrial production of biofuels from cyanobacteria at scale, consideration will be needed as to the growth and production strategies. A key determinant of this strategy will be the cost of the biofuel product itself. Currently, ethanol has a market price of around $0.40 per litre (US Grains Council https://grains.org/) and there are similar constraints on other biofuel candidates. Market price will be the main driving force as to Capital Expenditure (CapEX) on any commercial cyanobacterial biofuel enterprise. At the current price for ethanol, large-scale photobioreactors (used to optimise growth and production at laboratory scale) are not an option. Current production rates at laboratory scale are generally carried out

Fig. 3.3 Tubular reactor system for growing metabolically engineered *Synechocystis sp* PCC6803 viewed at various stages of cyanobacterial growth. The darkening colour indicates growth with time

in optimised bioreactors, in a 24-hr cycle with maintenance of optimal conditions of CO_2 supply, mixing, aeration and pH control. As it is generally assumed that such conditions will not be feasible at scale, other growth systems need to be developed or utilised. With relatively volatile biofuels such as ethanol, open pond systems will also pose problems, as there may be evaporative loss and even in high light environments photosynthetic growth can only be maintained during daylight. Hence, some form of a tubular reactor system that will retain the producing organism and the biofuel product will be sensible and in an environment that maximises daylight for photosynthesis. During the recent EU-funded DEMA project (Direct Ethanol from MicroAlgae) http://www.dema-etoh.eu/en/ the production partner A4f in Portugal utilised tubular reactors (Fig. 3.3).

CO_2 can be supplied by air aeration, while optimisation of tube design can allow mixing and retention of the ethanol produced (Lopes et al. 2019). The nature of the culture conditions at scale is important to consider. Such a tubular system does not give rise to axenic (pure) culture but can be optimised to maintain monoculture for many hours of production with optimised inoculum strategies. To keep costs on track the media will not be sterile, although the inoculum may be. If maintenance of sterile conditions were to be essential then the CapEX and OpEx would be uneconomic due to the energy costs of sterilisation of both media and reactor. Under such non-sterile culturing conditions, the production strain would have to be competitive with other contaminants. However, the burden of biofuel production on growth rate would limit this particularly for model strains not optimised for growth.

With the need for faster growing more productive cyanobacterial hosts, *Synechococcus* UTEX 2973 has been described (Yu et al. 2015; Ungerer et al. 2018) with photoautotrophic growth reported comparable to industrial yeast strains. This strain, a relative of PCC7942, but which grows some two times faster and can be genetically manipulated has been shown to possess a number of single-nucleotide polymorphisms compared to PCC7942 which may cast light on factors that increase its biomass productivity and growth rate (Ungerer et al. 2018).

The use of ultrasonic intensification (periodic ultrasonic treatment during the fermentation process) can also result in a more effective homogenisation of biomass and faster energy and mass transfer to biomass over short time periods, which can result in enhanced microbial growth during fermentation processes (Naveena et al. 2015). Such short ultrasonic pulses have been proposed to increase ethanol yields

during the production phase in engineered *Synechocystis sp* PCC6803 (Naveena et al. 2016) and may offer a strategy to manipulate the growth conditions for product release with ethanol and other biofuel products.

The presence of produced biofuel during the production cycle may itself lower production levels perhaps due to feedback loops or more likely due to biofuel stress. During isobutanol production, biomass productivity and isobutanol increased some 1.2 and 2.5 folds, respectively, by removal of the produced isobutanol using a solvent trap (Varman et al. 2013a). The addition of such removal systems may have implications for the production of other biofuels also including ethanol, particularly as contaminants in an open process could lead to product loss. The presence of contaminants could also affect the growth of the biofuel producer by metabolising micronutrients faster, shading the light source or as mentioned even utilising the produced biofuel, which could be a major consideration in the case of ethanol. Another production issue would be the availability of light. Industrial-scale culture would need adequate light supply meaning that only certain global locations may be optimal for natural light supply over a long day period. However, sunlight is just one element, an adequate supply of water to allow culture is also a prerequisite that may impact site location of a production scale plant.

Many of the proof of concept laboratory studies have been carried out under optimal photobioreactor conditions with 24-hour light regimes. Under industrial conditions, only diurnal lighting would exist unless supplemented during the night (by LEDs, e.g. which would add energy cost), hence data from idealised photobioreactors needs to be seen as only an indicator of potential productivity. Temperature maintenance is also an issue, should the temperature spike this could affect growth and limit production or indeed lead to an evaporative loss. Again, to limit CapEx, cooling systems would need to be passive in such situations. Maintenance of such systems can also impact operational expenditure (OpEx). Inevitably, reactor systems will need to be cleaned of biofilm formers and light blockers and reactor leakages maintained. Cleaning will be essential to ensure that future cultures can be maintained as much as possible as a monoculture during the production phase. Contamination from previous culturing will obviously have an effect on productivity and yield. However, adding any complexity or cost to such a production system when the value of the product is $0.40 a litre, in the case of ethanol, will increase OpEx. Thus, the type of biofuel and the profit margin associated with it may have a major impact on production strategies, particularly where the price of the product is low.

Another key issue that is often overlooked at the production stage is being able to rapidly monitor biofuel production levels to monitor operational parameters. In the case of ethanol, or indeed other biofuel candidates, monitoring can be somewhat cumbersome requiring HPLC or enzymatic analysis of product streams. Recently, online systems have been reported (Memon et al. 2017), which may aid the drive towards industrial production in the case of ethanol but which will be needed to monitor the production of other biofuel candidates.

Once optimal production levels are reached, there will then be the need to recover product which will be the case for any biologically produced cyanobacterial biofuel.

In the case of ethanol and other volatile biofuel candidates, the most obvious technique would be evaporation, however, calculation of capital input and energy costs make this unsuitable (Lopes et al. 2019).

Other technological scenarios include controlled natural evaporation and incorporation of collection systems into the reactor design or indeed membrane separation techniques such as pervaporation (Wee et al. 2008). This latter technique involves permeation through specially designed membranes and evaporation to the vapour phase. Such techniques allow the concentration of the product from production broths to commercial levels in an energy-efficient manner. Pervaporation however relies on sufficient initial concentrations of biofuel products in the production stream. Here, improvements in biological manipulations will help. The higher the biofuel concentration the more efficient and cost-effective will be the purification strategy (Kour et al. 2019). Thus, there are a number of challenges to the production cycle that need to be considered. Strategies utilised for the production of ethanol in commercial situations such as in the US companies Joule Unlimited and Algenol are generally not in the public domain. However, they have undoubtedly encountered many of the issues discussed here. Some may have been overcome, while others may still be proving a challenge. Only when commercial production occurs might one get a glimpse of the potential solutions.

3.11 Techno-Economic Evaluation of Biofuel Production from Cyanobacteria

A techno-economic assessment for the direct production of ethanol using metabolically engineered *Synechocystis* sp. PCC6803 has recently been published (Lopes et al. 2019). A number of scenarios and variations on the process were analysed for a 1000 L day^{-1} ethanol plant. This study highlighted issues with overall CapEx, OpEx and capital return on investment as discussed earlier. Because of the current cost structure for ethanol and other low-cost biofuels, a number of biorefinery strategies have also been proposed for the co-production of biofuels and other bioproducts that might make a biofuel process economic. However, a drawback has been the need for further processing of the products such as esterification or fermentation (Trivedi et al. 2015; Moncada et al. 2015; Chew et al. 2017; Moreno-Garcia et al. 2017) or follow on separation of bioproducts which adds to CapEx and OpEx. The current consensus based on process modelling appears to be that ethanol production is currently only economically feasible as a co-product in a biorefinery-based scenario at current cyanobacterial production rates (Lopes et al. 2019) and this may indeed also be the situation for many other biofuel candidates at present.

3.12 Future Perspectives on Cyanobacteria as Engineered Biofuel Producers

The adage *"much done much more to do"* currently applies to biofuel production in cyanobacteria. There are many challenges both biological and technical that need to be addressed and overcome. Strategies utilised with model organisms and engineered model biofuels and lessons learned from these proofs of concept strategies will aid future developments not only in the case of ethanol but also in the case of other biofuels that will pose their own particular challenges. Future biofuels may be gaseous, such as hydrogen, rather than miscible in an aqueous solution such as ethanol. Others may be immiscible and prove easier to purify.

Many of the challenges are not necessarily biological in nature but may be aided by tailoring the biology of the producer to the eventual process. Issues such as developing competitive producers perhaps using thermophilic cyanobacteria could be used to aid monoculture, while faster-growing cyanobacterial species with better partition characteristics could aid flux to biofuel products. More tolerant strains may be less affected by the product. Strains that have less need for co-factor synthesis may generate higher yields. More stable, more productive cassettes in better production backgrounds may generate higher yields and indeed all of these options need to be explored further.

Finally, techno-economic studies (Lopes et al. 2019) suggest that there may be an economic prerogative to co-produce the biofuel product with another high value, non-biofuel product to make the overall production cycle economic. Thus far, such options have been little explored as only model cyanobacteria have been utilised thus far and those do not currently produce other high-value co-products. Modelling such systems and estimating flux patterns and mutation strategies may be useful in developing optimal candidate strains and indeed a co-production strategy may be the key to seeing progress in this exciting though challenging area.

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