Chapter 4
Perspectives on Algal Engineering for Enhanced Biofuel Production

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4.1 Introduction

Algae as photoautotrophs can trap the solar energy and convert it into usable form. Solar energy is the most abundant and ultimate energy source. The total amount of solar energy absorbed by the Earth's surface is 1.74×10^5 terawatts (TW) (Bhattacharya et al., 2005), which is a tremendous amount as compared to the world's energy consumption (~13 TW) (Walter et al., 2010). Therefore, conversion of solar energy to fuels may constitute the most sustainable way to solve the energy crisis.

The incessant use of petroleum over the last century has greatly polluted the environment. In the USA alone, 3780 million metric tonnes of CO_2 are emitted from stationary sources alone (DoE Report, USA). Thus, while debating the next generation of fuels, besides energy efficiency, carbon neutrality of the fuel must be taken into consideration. As solutions to handle the waste emissions are discussed, algae can provide a suitable solution. They have a unique metabolism of sequestering CO_2 that can be redirected into biofuel production through photosynthesis. Algal fuel production reactors can be stationed near waste emission sources that could feed algae its nutrition and in turn provide valuable biofuels.

With a 1.2 % growth in the world population every year, arable land for cultivation of food crop is another concern (Summary Report, FAO, UN, 2002). The use of energy crops such as sugarcane for production of ethanol is intensely debated. However, algae can be grown on deserted wasteland/marginal land saving the arable land for cultivation of food crops. Moreover, algae are better photosynthesizers that can harvest upto 10 % of the incident solar light as compared to the efficiency of the C3 and C4 plants that can harvest a maximum of only 4.5 to 6 % (Janssen et al.,

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2000). Cyanobacteria and red algae contain phycobilisomes which make them more robust in the efficient absorption of polychromatic visible and far red irradiation. Moreover, unlike corn ethanol that can require up to 15 gallons of fresh water to produce a single gallon of fuel (Wu et al., 2008), algal cultivation does not require fresh water. Contrarily, use of algae can help address issues of fresh water scarcity since they can thrive in salt water and under harsh conditions (Um and Kim, 2009).

Currently available alternatives that can be processed from algae include: biodiesel, high carbon fuels like isoprene, hydrogen and alcohols. The chapter will give an overall perspective of how pathways for each of these fuels have been genetically engineered or synthetically circuited *in vivo* to drive biofuel production from algae. Some of these fuels are already in various stages of development and commercialization. Particularly, significant progress has been made in applying synthetic biology strategies to optimize algal chassis for the development of these biofuels. Overall, this article attempts to incorporate the recent advances to optimize the algal chassis and the recent advances in the production of various biofuels.

4.2 Improving the Algal Chassis for Biofuel Production

Both cyanobacteria and green algae can hugely impact the biofuel industry. Proof of concept studies have been carried out by several research groups demonstrating their feasibility. However, for a scale-up of these processes and wide scale industrial applicability the present processes face several technical limitations such as low yield and inherent light saturation limitations. To resolve these technical issues, the algae chassis must be optimized for biofuel production. There are several key features that can be engineered while developing a suitable chassis. Autotrophic carbon reserves are the feedstock of all the biofuels that are produced from algae. However, the cell must intelligently decide how much it needs to partition between cell growth and other cellular processes. Therefore, one way towards improving the chassis would be to trick the cell to channel more energy towards the desired product as compared to cellular growth and maintenance. This can be achieved by:

(a) increasing the rate of carbon fixation; and
(b) biasing the metabolic flux towards biofuel production.

Suitable chassis engineering must incorporate the specific requirements of the targeted biofuel. Recent studies reveal that the production of certain biofuels is stressful for the cells. It is thus imperative to increase the tolerance of the algal species towards them. Integrated omics approach has laid a foundation to suggest the stress response that is induced when algae is used as chassis to develop biofuels (discussed more in Section 4.4).

4.2.1 Increasing the Rate of Carbon Fixation

The rate of carbon fixation can be improved by engineering factors that are involved in sequestering the carbon. These primarily include the key catalase ribulose-bisphosphate carboxylase-oxygenase (Rubisco) and other components of the carbon concentrating mechanism (CCM). It is well known that 50 % of the total photosynthesis on Earth is carried out by algae (Field et al., 1998). Yet, these organisms face several challenges in obtaining the CO_2 and converting them into fixed carbon via its photosynthetic enzymes. Firstly, Rubisco is not designed to scavenge low concentrations of CO_2. It is a sluggish enzyme with a low affinity (K_m) for CO_2. At atmospheric concentrations alone, Rubisco can function only at a quarter of its catalytic efficiency due to the low concentration of dissolved CO_2 and the relatively high concentration of O_2 which competes with CO_2 (Long et al., 2006).

Rubisco Was Not Always Inefficient
It is worth to note that in the Earth's evolutionary history, the ability to fix CO_2 was not always a problem. When Rubisco first evolved in cyanobacteria some 3.5 billion years ago, it did so in an environment where CO_2 levels were many folds higher than the present. Under those conditions CO_2 levels were saturating Rubisco which seemed to have been efficient in achieving relatively high rates of carboxylation. However, as oxygenic photosynthesis evolved and the levels of O_2 increased and that of CO_2 dropped, the catalytic efficiency of Rubisco faced a setback and reduction of CO_2 became a rate limiting step (Badger et al., 1998; Badger and Price, 2003). As an evolutionary response to the changed atmospheric conditions, two general strategies evolved: in eukaryotes (higher plants), there was an increase in the affinity of Rubisco towards CO_2 as compared to O_2 (Badger et al., 1998; Tcherkez et al., 2006) while in an alternate strategy in prokaryotes (cyanobacteria) the carbon concentration mechanism evolved to increase the supply of CO_2 to Rubisco although at a minor metabolic cost.

Secondly the diffusion of CO_2 in water is 10,000 times slower than the diffusion of CO_2 in air. Lastly, algae often experience significant fluctuations in inorganic carbon ($C_i = CO_2 + HCO_3^-$) availability due to change in the environment pH. At an acidic pH, the C_i is mainly in the form of CO_2, while at an alkaline pH, C_i is mostly in the form of HCO_3^- (Moroney and Ynalvez, 2007). Thus, to overcome the challenge of low CO_2 availability along with a slow enzyme algae have developed the CO_2 concentrating mechanisms (CCM) (Fig. 4.1).

It is a mechanism which enhances photosynthetic productivity in algal cells by augmenting the availability of inorganic carbon within the immediate surroundings

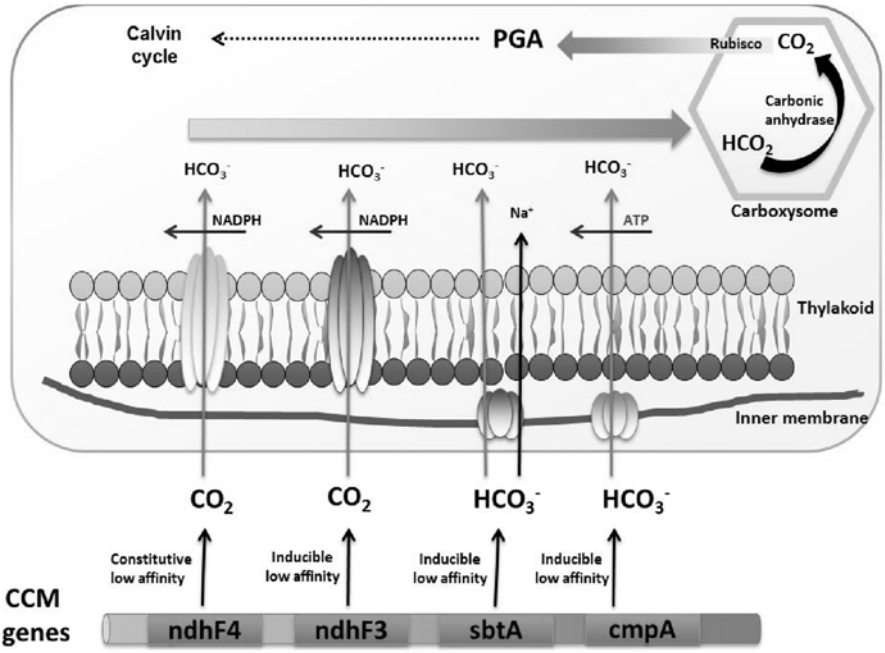

Fig. 4.1 Schematic of carbon concentrating mechanism in algae. NdhF3 and NdhF4, SbtA and CmpA have been putatively recognized as translocators located in the plasma membrane and the thylakoid membrane that pump HCO_3^- and CO_2 into the cytosol and thylakoid of a cyanobacterium. The internal gradient of HCO_3^- drive the inorganic carbon to the carboxysome. The carboxysomal carbonic anhydrase catalyzes the inter-conversion of HCO_3^- and CO_2 and, in so doing, increases the concentration of CO_2 around Rubisco, facilitating the carboxylation of ribulose 1,5-bisphosphate.

of the enzyme as compared to the atmospheric concentration. The components of CCM thus maybe the prime targets that could enhance the rate of carbon fixation.

Alternative pathways to Rubisco have been elucidated that fix approximately a quarter of the total fixed CO_2. This primarily includes the Phosphoenol pyruvate carboxylase (PepC) pathway. Besides the Calvin cycle, these pathways are also amenable to genetic engineering that could potentially increase the total amount/rate of CO_2 fixed. This is crucial to producing high yield biofuel. Below the recent updates on engineering of these pathways have been summarized.

4.2.1.1 Engineering the Rubisco Pathway

To date several studies have been conducted to increase the rate of photosynthetic efficacy by engineering Rubisco, the primary enzyme involved in the catalysis of CO_2 reduction. When Rubisco performs the carboxylation reaction, the first step of the photosynthetic carbon reduction, it uses CO_2 and ribulose-1,5-biphosphate

(RuBP) to produce two molecules of 3-phosphoglycerate (3PGA). One of these is recycled to regenerate ribulose 1,5-biphosphate whereas the other is diverted towards the carbon metabolism for biosynthesis of sugars, terpenoids and fatty acids. Rubisco are not the most efficient catalysts; in fact they are far from it. Owing to the low affinity of Rubisco towards CO_2, plants must devote up to one-half of their leaf-soluble protein to this enzyme to achieve acceptable rates of photosynthesis (Iwaki et al., 2006). To overcome this, the algal species evolved a carbon concentrating mechanism. In this design, Rubisco is localized in micro-cellular compartments called carboxysomes along with carbonic anhydrase. Carbonic anhydrase breakdown the accumulated cellular bicarbonate into CO_2 within the carboxysomes resulting in an elevated concentration of CO_2 around Rubisco. Other CCM components include the inorganic carbon transporters on plasma membrane as well as those on the thylakoids that uptake the CO_2 and bicarbonate. Since the CCM involves four modules: Rubisco, carbonic anhydrase, carboxysomes and Ci transporters, intuitively all the four features can be engineered to increase the rate of photosynthetic efficiency.

Rubisco, besides being a notoriously sluggish catalyst, cannot dis-criminate between its substrate CO_2 and O_2. Reaction with O_2 results in the formation of toxic phosphoglycolate that must be routed through the photorespiratory pathway which results in the loss of the previously fixed CO_2. Biotechnological advancements towards the improvement of Rubisco have proved challenging thus far. Attempts to increase the rate of catalysis has led to higher photorespiratory losses, while increasing selectivity towards the substrate CO_2 has led to partial loss in activity of the enzyme (Tcherkez et al., 2006). Earlier, Daniel et al. (1989) had accidentally obtained a mutant of *Anacystis nidulans* (now *Synechococcus*) that had high expressions and activity of Rubisco as compared to the wild type. Improved growth or photosynthetic rates could not be observed from these mutants. Experimental design revealed that though they used carbon dioxide saturating conditions, use of low intensity light appeared as a classic bottleneck in achieving high rate photosynthesis. In nature, variants of Rubisco are available, some intrinsically more efficient than their counterparts. Therefore, current strategies focus on replacing the sluggish native gene with a naturally more efficient counterpart. In one such study, Iwaki et al. (2006) expressed the Rubisco from *Allochromatium vinosum*, a purple sulphur bacterium in *Synechococcus elongates* sp. PCC 7942 which increased the CO_2 assimilation by almost 50 %. The study used two different strong promoters: psba from *Synechococcus elongatus* and psba2 from *Synechocystis* PCC 6803 (henceforth *Synechocystis*). However, the *A. vinosum* Rubisco was reported to be not fully incorporated into the carboxysomes.

Besides, heterologous expression, random mutagenesis and similar techniques including directed evolution and hybrid construction besides others have been in focus to increase the catalytic efficiency of Rubisco (Cirino and Frei, 2009). In the last decade, the technique of directed evolution has become a versatile tool for artificially mimicking the process of natural evolution (Bershtein and Tawfik, 2008). Directed evolution involves the creation of mutant library and then selecting the desirable phenotypes. This technique has been applied to improve both Rubisco and

Rubiscoactivase of various organisms (Mueller-Cajar and Whitney, 2008). Greene et al. (2007) considered the Rubisco from *Synechococcus* PCC7942 for directed evolution and examined it in *E. coli* hosts. They selected the strain with M262T mutation which conferred 26 % increase in carboxylation efficiency and increased Rubisco expression in *E. coli* by 5-folds. In another such study, trans-complementation of the *Rhodobacter capsulatus* (*R. capsulatus*) deletion mutant was carried out with a randomly mutagenized Rubisco from *Synechococcus elongatus* PCC 6301. The researchers screened for 5000 colonies. One of the selected engineered *R. capsulatous* strain carrying the randomly mutagenized single amino acid substitution showed higher growth rate compared to the strain carrying the wild type. Kinetic analysis of the engineered Rubisco showed a higher affinity to the substrate which was reasoned to provide growth advantages to the *R. capsulatus* strain (Smith and Tabita, 2003). It would be rather interesting to trans-complement the same mutagenized gene into its host *S. elongatus* to study if it would provide the same benefits (growth advantage) to the host strain.

So far it can be concluded that the extensive studies revolving around enhancing the catalytic efficiency of Rubisco have shown only modest success thus far. These results suggest that it may be possible that the Calvin cycle itself is not the major limiting factor for carbon fixation; instead it is possible that the downstream 'metabolic sinks' that utilize the product may be restricting carbon fixation to certain decree under specific environmental conditions (Stitt et al., 2010). It does appear that inspite of its sluggish catalytic action and non-specificity to substrate CO_2 it may be evolutionarily well optimized (Tcherkez et al., 2006). In view of this, it may perhaps be worth it to focus on the flux analysis and holistically look at the Calvin cycle in terms of multiple control points.

As mentioned earlier, carbon fixation in algae takes place in specialized compartments called 'carboxysomes' harbouring Rubisco and the carbonic anhydrase. Carbonic anhydrase deletion mutants with genetic disruption of the *icfA/ccaA* gene resulted in a high CO_2 requiring strain that fixed less than 5 % (v/v) CO_2 compared to the wild type (Fukuzawa et al., 1992). This indicates the crucial need of co-localizing carbonic anhydrase within the carboxysomes. In another interesting study, Lieman-Hurwitz et al. (2003) expressed the *ictB* gene involved in HCO_3^- from *Synechococcus* PCC 7942 in *Arabidopsis thaliana* and *Nicotianatabacum* to demonstrate faster photosynthetic rates in the engineered strain compared to the wild type under limiting but not saturating CO_2 concentrations. However, this did not alter the expression of Rubisco in the mutants. The authors suggest that the concentration of CO_2 in close proximity to Rubisco was nonetheless higher which could explain the increased photosynthetic rate. Similar expression studies in algae in a bid to improve the algal chassis may appear appealing. However, the expression of carbonic anhydrase is modulated by changes in the CcmM protein which is part of the structural shell of the beta-type carboxysomes. Transgeneic expression of the CcmM protein using a synthetic trc promoter led to higher expression of carbonic anhydrase in *S. elongatus* PCC 7942. The Rubisco activity still remained unchanged (Long et al., 2007). These results suggest that the amount of carbonic anhydrase is not limiting the activity of Rubisco*in vivo*. The study does suggest that carbonic

anhydrase and CcmM58 are co-regulated and overexpressing carbonic anhydrase alone might lead to increased levels of carbonic anhydrase in the cytosol, which is likely to be detrimental to the cells (Price and Badger, 1989). Thus for effective increase of the carbonic anhydrase in carboxysome, co-expression with CcmM may be required.

To create a high partial pressure of CO_2 within the carboxysomes, the flow of the substrate is facilitated by the presence of several CO_2/HCO_3^- transporters in the wall of the carboxysomes. This ensures that the oxygeneic photosynthesis is not plagued by photorespiration. Several research groups across are attempting to increase the number of inorganic transporters to see if they can further modulate the efficiency of the carbon fixation process by increasing the availability of the substrate in the cell and the carboxysomes in particular. To achieve this, four different transport systems for dissolved carbon uptake have been identified in cyanobacteria. The first two HCO_3^- transporters are single-gene systems and includes SbtA which is an inducible, high-affinity Na^+ dependent HCO_3^- transporter (Shibata et al., 2002; Price et al., 2004) that apparently acts as an Na^+/HCO_3^- symporter with a relatively low flux rate. In addition, BCT1 is a high-affinity HCO_3^- transporter (uniporter), inducible under dissolved inorganic carbon limitation, belonging to the ATPase family, and, unlike SbtA, is encoded by four genes (*cmpABCD*) to complete a functional transporter complex. NdhF3 and NdhF4 are the other two transporters that have been recognized as importers for CO_2 uptake. The transcripts of their genes were up-regulated under CO_2 atmosphere (McGinn et al., 2003). These transporters are being further evaluated for their role and possibility of transgenic over expressions.

The recent advent of synthetic biology has explored a whole new set of possibilities. Bonacci et al. (2012) demonstrated transgene expression of functional carboxysomes assembly and packaging in *E. coli.* Characterization of purified synthetic carboxysomes showed that they were well formed and were capable of CO_2 fixation *in vitro.* This shows the extent of modularity of expressing non-native systems in desirable hosts. This work has demonstrated that protein- protein interactions using scaffolds can be used to 'rewire' signal networks and improve the metabolic pathways. This could prove beneficial to improve the rate and yield of cellular chemical reactions while lowering toxicity to the host.

4.2.1.2 Engineering Synthetic Carbon Fixation Pathways

Other than the main Calvin cycle there are several alternative pathways available that can increase the rate of carbon fixation within the cells. Recently, Bar-Even et al. (2010), inspired by the alternate carbon fixation cycles prevalent in nature, devised indigenous synthetic pathways. These pathways were based on the repertoire of enzyme information already available in the databases. The pathways were accredited based on their physicochemical criteria such as kinetics, energetics and topology. Based on different modelling algorithms, their study concluded that the malonyl-CoA-oxaloacetate-glyoxylate (MOG) pathway involving phosphoenolpyruvate

carboxylase (PepC) was the most promising candidate for synthetic carbon fixation. In this pathway PepC is assisted by malic acid in the fixation of large amounts of CO_2 which is similar to that in C4 plants (Yang et al., 2002). However, instead of the final release of the CO_2, as occurs in the bundle sheath of the C4 plants, the metabolic bypasses of the pathway largely comprising eight or more enzymes would be used to convert malate to pyruvate without the loss of the fixed carbon (Bar-Even et al., 2010). This pathway appears as an attractive alternative because of the favourable properties of the carboxylation enzymes allowing much higher rates of carbon fixation as compared to the native Calvin cycle pathway.

To date there are limited reports about the effect of expression of these genes in prokaryotes and eukaryotes alike. In fact, prokaryotic PepC with the exception of that of *E. coli* were not well studied until recently when a deluge of information about them was available, mainly through the efforts of genome sequencing. In an attempt to study the effect of expression of *pepc* in plants, Chen et al. (2004) expressed the *pepc* from *Synechococcusvulcanus* in *Arabidopsis* using a strong constitutive promoter. In nature, most characterized PepC are found to be allosterically inhibited by the downstream product aspartate or malate. However, *Synechococcusvulcanus* harbours a unique enzyme almost insensitive to feedback inhibition at neutral pH. However, the *Arabidopsis* transgenic lines failed to respond along the expected lines of enhanced carbon metabolism. Instead, the T1 generation of plant transformants showed severe visible phenotypes such as leaf bleaching and were infertile when grown on soil. The growth inhibition of mutants was presumed to be primarily due to a decreased availability of phosphoenolpyruvate, one of the precursors for the shikimate pathway for the synthesis of aromatic amino acids and phenylpropanoids. However, it is still worth to attempt endogenous expression as well as transgeneic expression of these pathways in both cyanobacteria and green algae towards enhancing the rate of fixed carbon.

4.2.1.3 Improving the Light Harvesting Antennae

Another major problem of the algae when grown in reactors for the purpose of food or feed is the shading effect. For microalgae and photosynthetic bacteria, there exists a technical barrier caused by the 'cell-shading' phenomenon of these pigmented cells. The photosynthetic efficiency directly depends on the ability to harness the solar photons and store it in the form of fixed carbon. The algae have developed large harvesting complexes due to their evolution under low light regime. Presence of large antennas ensures maximum absorption of the incident light. However, at the same time, algae do not respond well to saturating light intensity. Under such conditions, most of the reaction centres are closed, and a mechanism that dissipates the excess absorbed light into heat is employed to digress part of the energy absorbed by the antennae, such that the excess energy does not reach the photosynthetic reaction centre. This prevents photo damage of the reaction centres. Considering the goal of maximizing fuel production from these organisms the incapability of harvesting saturating light intensity may limit the production efficiency

from large scale mass culture. Towards this, Mussgnug et al. (2007) applied the technique of RNAi technology to down regulate the entire LHC gene family simultaneously to reduce energy losses by fluorescence and heat. The mutant Stm3LR3 had significantly reduced levels of LHCI and LHCII mRNAs and proteins while chlorophyll and pigment synthesis was functional. The mutant showed higher photosynthetic quantum yield and a reduced sensitivity to photoinhibition, resulting in an increased efficiency of cell cultivation under elevated light conditions. Collectively, these properties offer three advantages in terms of algal bioreactor efficiency under natural high-light levels: (i) reduced fluorescence and LHC-dependent heat losses and thus increased photosynthetic efficiencies under high-light conditions; (ii) improved light penetration properties; and (iii) potentially reduced risk of oxidative photodamage to PSII.

Polle et al. (2003) developed chlorophyll antenna truncation of the green alga *Chlamydomonas reinhardtii* (henceforth *C. reinhardtii*), which showed higher photosynthetic efficiency and biofuel production capacity compared with the wild-type. The photosynthetic apparatus (PS) of cyanobacteria in general is similar to that described for higher plants; however, the PS in cyanobacteria have several peculiarities. In addition to the chlorophyll protein complex, the cyanobacterial PS possess phycobilisomes with the pigment proteins phycocyanin and phycoerythrin primarily attached to the PSII dimers. The presence of these pigments extends the spectrum of visible light usable for photosynthesis (Rascher et al., 2003). However, cyanobacteria also suffer from similar light limitations and, hence, another approach to enhance their biofuel potential may be to develop truncated PS. Using chemical mutagenesis, Nakajima et al. developed a phycocyanin-deficient PD1 mutant of *Synechocystis* PCC 6714 (Nakajima et al., 2001). This mutant showed 1.5-times the photosynthetic productivity of the wild type under high light intensity. However, the potential of this mutant as a biofuel producer has not been reported.

4.2.2 Biasing the Metabolic Flux Towards Biofuel Production

The synthesis of glycogen is catalyzed by glucose 1-phosphate adenyl-transferase (GlgC), glycogen synthase and 1,4-alpha glucan branching enzyme. GlgC catalyzes the primary step of conversion of Glucose 1-phosphate into ADP glucose. Since the storage metabolite glycogen is the major metabolic source for spending fixed carbon and reducing power, decoupling of the native metabolism from glycogen synthesis could redirect the carbon to other biosynthetic processes which may favour increased cell growth and higher growth dependent product yield. This was illustrated in the study of Li et al. (2014) who demonstrated the development of a GlgC mutant that could redirect its carbon and reducing power towards the production of a non-native isobutanol. Under high light the GlgC mutant showed growth impairment; however, induction of the isobutanol pathway in the same cell rescued the growth defects. The study strongly suggests that growth of GlgC mutant alone under high light faced redox imbalance due to accumulation of reduced

NADPH. Induction of the isobutanol pathways facilitated the cell in finding an alternate pathway to release the redox stress.

Another aspect that can be utilized in implementing biotechnological applications including the production of biofuels is the utilization of these solar harnessing photosynthetic cells as sugar producing factories. Endogenous production can be enhanced by increasing the rate of sugar transport out of the cell, knocking out the competing pathways and stimulating production by altering cultivation conditions. Xu et al. (2013) showed that by deleting the *glgAI* and *glgAII* genes responsible for converting ADP-glucose to glycogen amylose, *Synechococcus* PCC 7002 accumulated 1.8 fold more sugar than the wild type and these cells spontaneously excreted soluble sugars into the medium at high levels without the need for additional transporters. These cells producing soluble sugar in the medium can then directly be utilized by bacteria (mesophiles/thermophiles) co-cultivated in reactors to produce biofuels and other valuable compounds. This will also bypass the need of pretreatment costs considering instances where the stored glycogen/starch of algae has been used to cultivate bacteria (Nayak et al., 2014; Roy et al., 2014).

Another excellent example to diverting the metabolic flux towards the desired product was provided by Matthias Rögner's research group in Germany. They have shown how the affinity of the electron carrier ferredoxin (Fd) can be modulated towards the hydrogen production pathway instead of the Calvin-Benson cycle. The reductants obtained by splitting of water enter into the Calvin cycle via Ferredoxin NADPH Reductase (FNR). This is a diverging point where the reductants can either enter into the Calvin cycle or be accepted by hydrogenase (in green algae) to produce the biofuel hydrogen. They have shown for the first time in the model organism, *Synechocystis* that the bias of the ferredoxin can be decreased towards FNR (Rögner, 2013). This in turn makes the reduced ferredoxin available to donate the electrons to a transgenetically available non-native [FeFe] hydrogenase integrated into the genetic circuit of the strain. This increases the possibility of higher hydrogen production as compared to biomass generation. This is one of the most critical points in increasing the photosynthetic hydrogen production in *Synechocystis* and is due to the much higher affinity of Fd for FNR than for the [Fe–Fe]-hydrogenase from *C. reinhardtii* (K_m differs by a factor of 10–40). This may also be due to the much higher concentrations of FNR in the cell as compared to the non-native hydrogenase. Changing bias of ferredoxin in favour of hydrogenase was possible by decreasing the Fd affinity to FNR. Preliminary studies have shown that K_d values could be shifted by a factor of more than 10 as determined with isolated FNR from WT and directed mutants (Rögner, 2013). This would be a great step in the direction of obtaining higher rate of hydrogen production from cyanobacteria.

4.3 Genetic and Metabolic Engineering of Algae to Obtain High Yield

4.3.1 Alcohols

Among the alcohols that have been reportedly produced from cyanobacteria using either the native system or the non-native system are 2–4 carbon alcohols including ethanol, 1, 2 propanediol, 2,3-Butanediol, 1-butanol and isobutanol (Fig. 4.2).

Besides, fatty alcohols and *n*-carbon alcohols derived from fatty acids have also been reported. The first four aliphatic alcohols (namely methanol, ethanol, propanol and butanol) are of interest as fuels because they can be synthesized both chemically or biologically and they have properties that allow them to be used as fuel in internal combustion engines. Blends of ethanol with other fossil fuels have increasingly gained momentum in the transport industry.

The major pathway for ethanol synthesis is catalyzed by two enzymes, pyruvate decarboxylase (Pdc) and alcohol dehydrogenase (Adh). Pdc catalyzes the non-

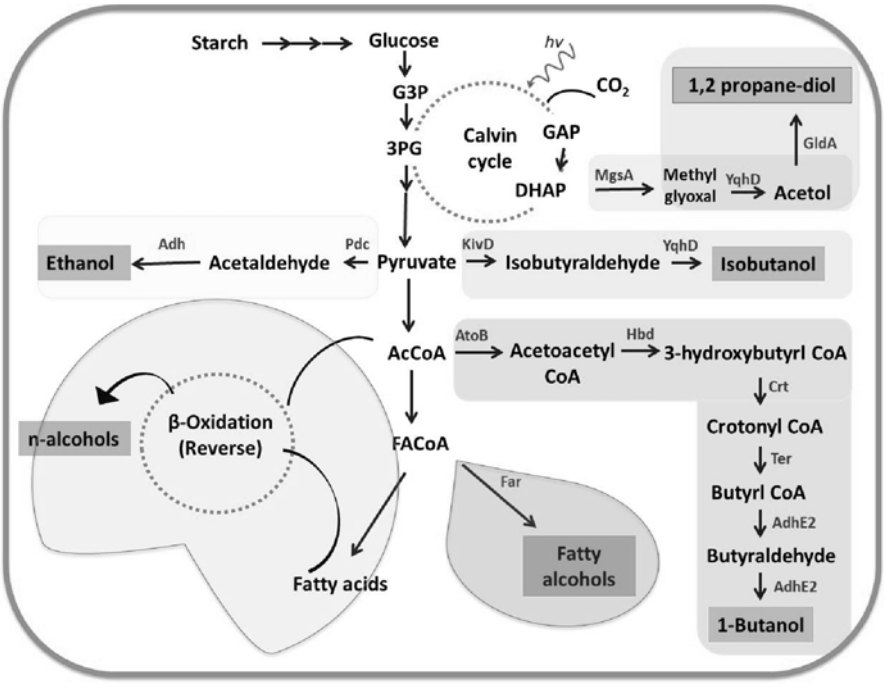

Fig. 4.2 Schematic of heterologous pathways that have been introduced into cyanobacteria for the production of various alcohols including 1,2 propanediol, isobutanol, 1-butanol, fatty alcohols, *n*-alcohols and ethanol. Abbreviations: G3P: Glucose-3-phosphate, 3PG: 3-phosphoglycerate, AcCoA: acetyl-CoA, FACoA: Fatty-acylCoA, GAP: Glyceraldehyde-3-phosphate, DHAP: Dihydroxyacetone-phosphate.

oxidative decarboxylation of pyruvate, which produces acetaldehyde and CO_2. Acetaldehyde is then converted to ethanol by Adh. This fermentation pathway plays a role in the regeneration of NAD^+ for glycolysis under anaerobic conditions in fungi, yeasts, and higher plants. Currently, the majority of alcohol is being produced by fermentation of starch or cellulose-derived sugars. Research groups have explored the possibility of utilizing the algal starch/glycogen as feedstock for bacterial ethanol production (Nguyen et al., 2009). However, recently researchers have come up with a more direct way of coupling photosynthetically fixed carbon to alcohol. Deng et al. demonstrated the production of ethanol from cyanobacterium *Synechococcus* sp. strain PCC 7942 as early as 1999. However, to date any significant improvements in yields have not been reported. The earlier report was a proof of concept design with high sensitivity to the oxygen due to splitting of water at PSII.

Recently, butanol has been explored for use as transport fuel. Butanol as fuel has advantage in the existing combustion engines since it has properties close to that of the combustion engines already in use. For a long time butanol production was reported by obligate anaerobes like *Clostridium*. Thus, the pathway is oxygen sensitive. The challenge then was to express the pathway in oxygenic autotrophs. This was successfully demonstrated by Lan and Liao (2012) where they reported the production of 1-butanol by a modified-CoA dependent pathway obtained from obligate and facultative bacteria into *Synechoccocus elongatus*. Five genes were integrated into the *Synechoccocus* genome including *hbd*, *crt*, *adhE2* from *C. acetobutylicum*, *ter* from *T. denticola*, and *atoB* from *E. coli*. The acetyl-CoA acetyltransferase (A to B) was selected to replace the acetoacetyl-CoA thiolase (Thl) from *Clostridium* due to its higher specific activity. The activities of these enzymes were determined by enzyme assays and it was found that the activities of ter and adh2 were lower than the other three enzymes in the pathway. This indicated that in cyanobacteria these enzymes were the rate limiting step. Addition of poly histidine tag increased the ter activity and thus the productivity of 1-butanol in cyanobacteria.

Another effort in the same direction was made by Oliver et al. (2013) towards the production of 2,3-butanediol production in *Synechococcus elongatus* PCC 7942. The operon comprising *alsS*, *alsD* and *adh* under the effect of plac promoter was inserted into the neutral site. They identified 2,3-butanediol as a biofuel with low host toxicity and designed an oxygen-insensitive, cofactor-matched biosynthetic pathway coupled with irreversible enzymatic steps to create a driving force toward the target. Similarly, very recently, Varman et al. (2013) demonstrated the production of isobutanol from *Synechocystis*. They improved the strain both by metabolic engineering as well as bioprocess optimization. They engineered a glucose tolerant *kivd* and *adhA* gene of the Ehrlich pathway. It is already well known that isobutanol is toxic to the cells and may also be degraded photochemically by hydroxyl radicals produced during the cultivation process. Therefore, a clever strategy to improve yields is to remove the product from the culture soon after its release into the medium. The study of Varman et al. demonstrated this with ease by simply placing a glass tube filled with oleyl alcohol placed within the cultivation flask. Due to the

high volatility, the produced isobutanol in the headspace of the cultivation flask would get trapped in the oleyl alcohol and could easily be separated. This depicted a non-intrusive way of downstream processing.

In yet another proof of concept Li and Liao (2013) demonstrated the production of 1,2-propanediol from *Synechococcus elongatus* PCC 7942 by introduction of the genes encoding methylglyoxal synthase (*mgsA*), glycerol dehydrogenase (*gldA*) and aldehyde reductase (*yqhD*) . However, a comparable amount of the toxic intermediate acetol was also produced. To overcome this issue, the NADH-specific secondary alcohol dehydrogenases was expressed which minimized the accumulation of the incomplete reduction product acetol.

Another category of alcohol the 'fatty alcohols' have recently been produced from the photoautotrophic organisms. They are also considered as suitable fuel additives due to favourable fuel properties. Generally fatty alcohols can be produced by two pathways: the first pathway involves the reduction of the fatty acyl-CoA (acyl-ACP) to fatty alcohols. The alternate pathway involves two distinct enzymes, a fatty aldehyde forming acyl-CoA reductase which catalyzes a two-electron reduction of fatty acyl-CoA to a fatty aldehyde intermediate, and a fatty aldehyde reductase which catalyzes the reduction of fatty aldehyde to fatty alcohol (Metz et al., 2000). Cyanobacteria do not contain endogenous fatty alcohol producing pathway. Yao et al. (2014) investigated the production of these alcohols by transgenetically expressing the fatty acyl-CoA reductase (FAR) from higher animal models like mouse, and plant models like *Jojoba* and *Arabidopsis*. They reported 9.73 ± 2.73 μg $OD_{730}^{-1} L^{-1}$ of fatty alcohol from *Synechocystis* strain expressing the *jojobaFar* gene.

Thus, several proof of concept studies have been reported in literature in the recent past, a boon made possible due to the advent of synthetic biology. Most precedents are recent and have occurred towards the end of the last decade. The issue now is how to move beyond the proof of concept study and identify the bottlenecks that are limiting further progress in this field. Expressing the pathway in optimal algal chassis would go a long way in realization of these fuels from phototrophic hosts.

Considering alcohol production, the general targets for improvements may include (1) tolerance of the developed strain towards the solvent produced. During fermentation alcohols are produced in the stationary phase. They are toxic due to their hydrophobic nature that increases the fluidity of the cell membranes. As a result the cell membrane permeability is compromised; transmembrane pH is affected resulting in energy shortage. Microbes in general react to the toxicity by producing heat shock proteins, and by activating efflux pumps for the solvents. This kind of toxicity leads to decreased productivity. Therefore, of foremost importance is to address the questions of how to make the cell more robust in terms of solvent production and prolonged cell viability. (2) Secondly, if these enzymes are linked to the photosynthetic chain the oxygen toxicity of these enzymes must be addressed due to the natural evolution of oxygen at PSII. (3) Competing pathways that require the same redox potential must be eliminated. In this case, native hydrogenase, lactate pathway may be deleted as they would dispose electrons/energy that could

otherwise be channelled towards alcohol production. Also, elimination of carbon sinks can increase productivity. Impairment of the glycogen synthesis pathway by deletion of *glgC* the key enzyme that converts glucose 1-phosphate to ADP-glucose has been suggested. This is an interesting approach since production of alcohol comes from the catabolism of glucose through the glycolysis cycle in the dark. However, GlgC steals away the glucose 1-phosphate and reconverts it into ADP-glucose that leads to glycogen synthesis. A non-native pathway linked to such an engineered strain could already have a high driving force to release the redox imbalance built due to the accumulation of Glucose 1-phosphate stress. (4) Next, optimizing pathways to drive the flux towards product formation must be considered. An interesting example of this was shown by James Liao's group where they constructed a non-native Clostridial pathway for 1-Butanol production in *E. coli* (Shen et al., 2011).

To improve the production beyond the known titer limits they identified the metabolic bottleneck in the pathway reversible on both thermodynamic and enzymatic grounds. Therefore, they envisaged the presence of an artificial driving force to improve product yields. The *Clostridium* 1-butanol pathway requires both NADH and reduced ferredoxin as cofactors (Bcd-EtfAB complex). If the pathway could be dependent on only NADH, then its cellular accumulation could be used as the driving force towards production of 1-butanol. Concurrently, the elimination of all other pathways utilizing NADH including ethanol, lactate and succinate would be desirable. Such a strain would not be able to survive under anaerobic conditions due to redox imbalance till it produced butanol as the fermentative redox stress buster. Therefore they replaced the Bcd-EtfAB complex with Ter (trans-enoyl-coenzyme A reductase) from *Treponema denticola* (Lan and Liao, 2012). This NADH-dependent enzyme could potentially facilitate tighter coupling of 1-butanol production with the NADH driving force without flavoproteins or ferredoxin.

4.3.2 Hydrogen

Hydrogen as a fuel has been shown to be attractive primarily because on combustion it produces water as the only "waste emission". Photoautotrophic organisms are considered as attractive alternatives because they can generate their own feed stock reserves and require only water and sunlight to produce hydrogen. The enzyme catalyzing the deceivingly simple reaction $2H^+ + 2e^- \; H_2$ are called hydrogenase. They are classified depending on the presence of the transition metals in the active site. The need for catalytic transition metals is explained by the significant increase in the acidity of molecular hydrogen when bound to them (Collman, 1996). Accordingly there are three phytogenetically unrelated class of these enzymes that include [NiFe], [FeFe] and a third class in which hydrogen uptake is coupled with methanopterin reduction. Most known hydrogenases are oxygen labile, and cyanobacteria and green algae are the only known organisms so far that are capable of both oxygenic photosynthesis along with hydrogen production. While

cyanobacteria contain the 'native hox operon' which is homologous to the [NiFe] hydrogenase for hydrogen production, the green algae *C. reinhardtii* hosts [FeFe] class of hydrogenase. Since they are the most promising hosts to produce this biofuel, a range of genetic engineering work including genetic knockouts, introduction of non-native genes and pathways and elimination of competing pathways utilizing the same reductant has been approached among others. However, to date these are only 'proof of concept' designs that are available and still research needs to go a long way before the system becomes commercially viable.

In algae, hydrogen production can occur through three pathways. Two of them, though essentially catalyzed by the same enzyme 'bidirectional hoxhydrogenase', differ in terms of source of reductants (Fig. 4.3), the third differs in terms of the enzyme catalyzing the reaction. In light, the source of reductants for the bidirectional hoxhydrogenase is obtained from the splitting of water at PSII that results in reduced NADPH via PSI, ferredoxin and ferredoxin:NADPHreductase (FNR). Notably, in green algae hosting [FeFe] hydrogenase the reduced Fd can directly act as an electron donor to it. This process is coupled to the oxygen evolving splitting of water at PSII and occurs only transiently when dark kept cells are exposed to light, the excess generated reductant is channelled out as molecular hydrogen. However, the rate of oxygen production is higher than the rate of consumption by respiration, hence the damage to the oxygen labile hydrogenase. In an alternate

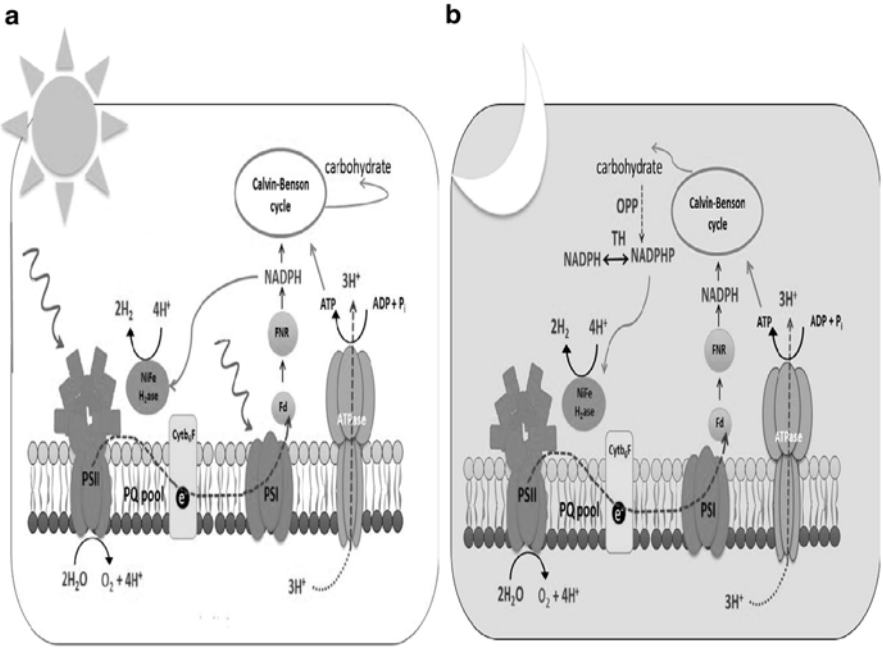

Fig. 4.3 Schematic of hydrogen production from cyanobacteria: (a) Under light the source of reductants comes from the splitting of water at PSII and (b) In dark the source of reductants is obtained from the catabolism of glycogen.

pathway, the hydrogen is produced in the dark where the source of the exogenous electrons is the stored carbohydrate. In the absence of light, catabolism of sugar occurs to feed the energy requirement of the cell and the reductant thus generated is employed by the hydrogenase, besides others. In the process of NADPH oxidation, the electrons are shuttled to the plastoquinone pool and subsequently used for hydrogen biosynthesis. Hydrogen production through this mode is prolonged.

The reversible bidirectional hoxhydrogenase is associated with the cytoplasmic membrane and likely functions as an electron acceptor from both NADH and H_2 (Boison et al., 1999). The reversible bidirectional hoxhydrogenase is a multimeric enzyme consisting of eight different subunits. Molecularly it is a [Ni–Fe]-hydrogenase of the $NAD(P)^+$ reducing type and consisting of a hydrogenase dimer coded by *hoxYH* gene. Maturation of reversible hydrogenases requires the action of several auxiliary proteins collectively termed as *hyp* (products of genes: *hypF*, *hypC*, *hypD*, *hypE*, *hypA* and *hypB*) (Casalot and Rousset, 2001). Unlike uptake hydrogenase, reversible hydrogenases are helpful in hydrogen production. While the uptake hydrogenase is present in all nitrogen-fixing strains screened so far, the bidirectional enzyme is distributed both among the nitrogen-fixing and the non-nitrogen-fixing cyanobacteria (although it is not a universal cyanobacterial enzyme).

The third pathway for hydrogen production is mediated by nitrogenase. Nitrogenase dependent production of molecular hydrogen occurs primarily in the heterocyst's nitrogen fixing cyanobacteria. The source of reductant for nitrogen fixation is obtained from the catabolism of sugar through the oxidative pentose phosphate (OPP) pathway in the vegetative cells. Hydrogen is produced as a by-product of nitrogen fixation and is an energy intensive process. Hydrogen production catalyzed by the nitrogenase occurs as a side reaction at a rate one-third to one-fourth that of nitrogen fixation, even in a 100 % nitrogen gas atmosphere. In close proximity of the nitrogenase is situated the uptake hydrogenase in the thylakoid membrane of the heterocyst where it transfers the electrons from the breakdown of hydrogen evolved from nitrogenase for the reduction of oxygen via the knallgas reaction. The enzyme itself consists of two subunits, a large subunit (hupL) which harbours the [NiFe] active site and a small subunit that is coded by (hupS) that acts as the molecular wire to transfer the electrons from the active site to an electron acceptor. The net hydrogen produced from such strains is lowered due to this counterproductive reaction. Higher hydrogen yields have been demonstrated by successful deletion of these enzymes. Previous studies have reported that hydrogen uptake-deficient mutants of *A. variabilis* AVM13 (ΔhupSL) (Happe et al., 2000), *N. punctiforme* NHM5 (ΔhupL) (Lindberg et al., 2002), *Anabaena* PCC 7120 AMC 414 (ΔxisC) (Carrasco et al., 2005), *Anabaena* PCC 7120 (ΔhupL, ΔhupL/ΔhoxH, ΔhupW) (Masukawa et al., 2002; Lindberg et al., 2012), *Nostoc* PCC 7422 (ΔhupL) (Yoshino et al., 2007) and *A. siamensis* TISTR 8012 (ΔhupS) (Khetkorn et al., 2012) have an ability to produce hydrogen at a significantly higher rate compared with the respective wild-types. Ekman et al. (2011) carried out whole scale proteomic analysis of one such deletion mutant developed by Lindberg et al. and found that the pathway for OPP was significantly up-regulated in the mutant suggesting

that the deletion of uptake hydrogenase may increase production of the substrate via the OPP pathway.

It is to be noted that hydrogenase are extremely oxygen labile proteins. With this understanding it is imperative that their expression is under anaerobic conditions. Development of anaerobic conditions in pilot scale is another challenge both technically and economically. But how does nature protect its oxygen labile enzyme? It has developed the micro-anaerobic environment in the heterocyst of filamentous cyanobacteria. This protects its oxygen labile nitrogenase and uptake hydrogenase. For those organisms in which heterocysts are not present, the expression of the enzymes are metabolically or cicardially controlled. Moreover, the metabolism within the heterocyst consists of only PSI, without the presence of the oxygen evolving PSII. The nitrogenase mediated nitrogen fixation is a reductive process, and gets an electron supply from the vegetative cells via ferredoxin. Thus, an electron transport system suitable for [FeFe]-hydrogenases may already be present in the heterocyst. Getting inspiration from nature, the best way forward to realize a hydrogen economy would be to express the enzymes in nature's special anaerobic compartments. Gärtner et al. (2012) attempted to achieve it by expression of the *Shewenella oneidensis* MR-I dimerichydrogenase in *Anabaena* sp. strain PCC 7120 using the heterocyst *hetR* promoter for heterocyst specificity. However, they could demonstrate the hydrogen production only in *in vitro* studies. The study showed that though the enzyme was functional the source of reductants *in vivo* was limited to the developed system. This was clear, since activity could be clearly observed on addition of a promiscuous electron donor (methyl viologen).

Recently, Ducat et al. (2011) showed the expression of *Clostridium acetobutylicum* hydrogenase in unicellular, non-nitrogen fixing cyanobacteria, *Synechococcus elongates* sp PCC 7942 along with its accessory genes. They demonstrated that the hydrogenase are functional *in vitro* and *in vivo*. Under anoxic light conditions obtained by addition of DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea), the mutants evolved 500-fold higher hydrogen as compared to the wild type. DCMU is a herbicide that inhibits photosynthesis by binding to the plastoquinone site of PSII thereby preventing the splitting of water and limiting the linear photosynthesis. However, the addition of DCMU has no effect on the activity of PSI.

Towards optimizing the cell for higher hydrogen production, several efforts have been made. Among these, consistent efforts have been made by the group of Matthias Rögner in Ruhr University, Bochum, Germany. The research group at Bochum is in the process of development of a suitable chassis for high rate hydrogen production using the model organism *Synechocystis* (Fig. 4.4).

The electrons released by splitting of water at PSII primarily govern the rate of hydrogen production. In *Synechocystis* the PSII to PSI ratio stands as 1:10. Moreover, the PSII reaction centre carries the phycobilisome (PBS) proteins that get over-saturated at high light intensities. For high rate hydrogen production this feature is a deterrent. In view of this, the German group was the first to show the development of olive mutant (mutant carrying deletion of phycocyanin-binding subunits) with reduced antennae size that was capable of 4-fold increase in linear electron transport. In a step further, 6-fold increase was obtained in the PAL mutant with

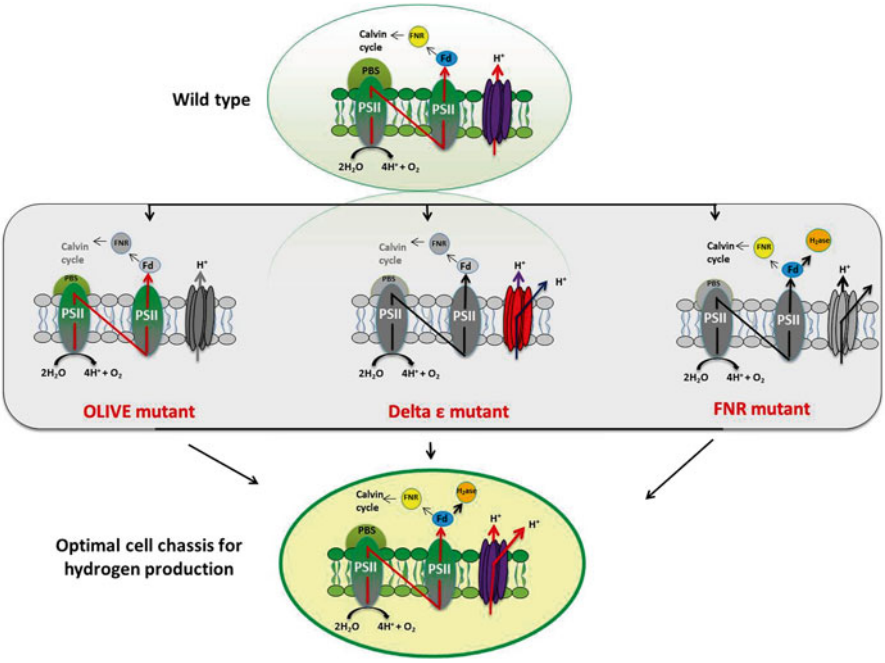

Fig. 4.4 Schematic for the development of an optimal chassis for hydrogen production. The optimized cell comprises (a) PAL mutant with completely deleted PBS (phycobilisome) genes, (b) Delta ε mutant with lower ATP synthesis rate as compared to the WT and (c) FNR mutant with decreased bias of the FerredoxinNADPH Reductase (FNR) towards ferredoxin (modified from Rögner, 2013).

completely deleted PBS genes (Bernat et al., 2009). Such truncated antennae mutants showed more tolerance to high light. The cellular metabolism of the phycobilisome mutant cells also saves considerable cellular energy currency. Considering the total protein pool, phycobilisomes constitute 63 % of all the soluble proteins in *Synechocystis* cells (Moal and Lagoutte, 2012).

In the next step towards the development of an optimal chassis they developed the delta ε mutant that had lower ATP synthesis rate compared to the wild type. This was developed with the hypothesis that the supply of electrons from water splitting is further limited by the tight coupling of luminal proton efflux with ATP synthesis. The partial uncoupling resulted in 2-fold higher linear electron transport (Imashimizu et al., 2011). Moreover, the impairment

did not affect growth or survival. This suggested the possibility of readjusting the cellular metabolism to the availability of NADPH and ATP required for carbon fixation and growth. It was postulated that the additional electrons available as a result of lower carbon fixation could be rechannelled towards biofuel production.

In yet another step towards chassis optimization, the group showed the decreased bias of FNR towards ferredoxin. Efforts are also ongoing to improve the green algae, *C. reinhardtii* chassis for hydrogen production. Towards this end, Melis

(2009) developed the antennae truncated mutants that showed higher rate of linear electron transport. Further, Reifschneider-Wegner et al. (2014) demonstrated that a codon-optimized native hydrogenase could be successfully expressed in the chloroplast. However, they observed a strong negative selection due to constitutive expression of the hydrogenase and which was resolved by using a vitamin induced gene expression system.

Thus efforts are ongoing towards the realization of a stable optimal hydrogen producing algae in an effort to replace a part of our current fossil fuel needs.

4.3.3 Biodiesel

Biodiesel is of significant importance because it has properties similar to that of petrol diesel and can be used with the current available infrastructure. Biodiesel is essentially fatty acids with long alkyl chains used by cells for energy storage and chemical production. It is a clean burning fuel made by the trans-esterification of the triacyl glycerides purified from photosynthetic plants or algae. Statistical analysis shows that in UK out of the 47 billion litres of transport fuel utilized, 53 % comprised petro-diesel (UK department of energy report, 2009). The world economy could greatly benefit if this could be achieved using sustainable sources. However, if this sustainability were to be achieved using plant oil such as rapeseed, it would require 17.5 Mha land, more than 50 % of the arable land area of UK (Scott et al., 2010). Algae may thus be the obvious alternative. Microalgae have the ability to accumulate oil in the form of triacylglycerides (TAGs) (Chisti, 2008). However, the lower yields limit their industrial application. An obvious choice would be to increase their oil content. Stress conditions are known to increase the oil content. Physiologically, this can be achieved by nutrient starvation such as nitrogen; however, this also decreases the biomass yields and hence lowers productivity. Therefore, indigenous studies around optimizing the lipid pathway and its recovery are required to increase productivity. The four obvious strategies would be (i) to increase the lipid accumulation, (ii) to decrease the lipid catabolism, (iii) to eliminate the competing pathways feeding on the same substrate and (iv) to optimize downstream processing.

Present efforts to obtain high yield biodiesel from photosynthetic microorganisms have been focused on the green algae, *C. reinhardtii* (Msanne et al., 2012). With the availability of the complete genomic information, many genes required for lipid and tri acycyl glycerol biosynthesis have been identified (Riekhof et al., 2005). Genome comparison and gene prediction analysis have shown that the pathways of fatty acid synthesis between plants and algae are largely conserved. The *in vivo* function of these fatty acids is to build membrane lipids and storage lipids. Acetyl CoA serves as the basic building block for fatty acid biosynthesis and in algae is derived mainly from glycolysis (Hu et al., 2008). The rate limiting step in this process is the formation of malonyl CoA from acetyl CoA catalyzed by acetyl CoA carboxylase. The malonyl group is then transferred to a small acyl carrier protein

(ACP) catalyzed by 3-keto acyly-ACP reductase and dehydration catalyzed by 3-hydroxylacyl-ACP dehydratase. Second round of reduction is catalyzed by enoyl-ACP reductase. These serial reactions result in the addition of two methylene carbons to the growing acyl chain. Fatty acids with a carbon chain length of 12–14 atoms are most suitable for the production of biodiesel. Most microalgae synthesize lipids with a chain length of 14–20 carbon atoms. The acyl ACP thiosterase enzyme is responsible for the length of the chain, and species specificity is typical for it.

To date most of the studies have focused on increasing the lipid biosynthesis by blocking competing pathways. Wang et al. (2009) reported that after 48 h of nitrogen starvation in the presence of acetate, the wild-type lipid body content has increased 15-fold. When starch biosynthesis was blocked in the *sta6* mutant, the lipid body content further increased to 30-fold, demonstrating that genetic manipulation can enhance lipid body production. However, on downside the cellular growth rate was much compromised. Thus to date strategies that have increased lipid production have resulted in decreased growth in the engineered strains. Maintaining high growth rates and high biomass accumulation is imperative for algal biofuel production on large economic scales, and engineering efforts that increase lipid content without decreasing growth or biomass can significantly reduce production cost and increase the economic viability of algal biofuels.

In *E. coli* the product of fatty acid biosynthesis is fatty-acyl-ACP (Acyl carrier protein), accumulation of which causes feedback inhibition of fatty acid biosynthesis. Lu et al. (2008) found that over expression of thioesterase 1 in *E. coli* bypass the feedback inhibition caused by product accumulation. Recently, researchers introduced a codon optimized acyl carrier protein thioesterase in *Synechocystis* with six different genetic mutations (Liu et al., 2011). These mutations resulted in weakened cell wall layers due to altered surface proteins and peptidoglycan layers allowing enhanced diffusion through phospholipid layers.

Downstream processing including (cell disruption, extraction and separation) is another area that is energy/cost intensive. In an indigenous study, lipase enzymes that degrade the membrane lipids and increase the cell wall permeability to release the oil were expressed using CO_2 inducible promoters. These promoters were previously tested by McGinn et al. (2003). They reported that aeration of illuminated cells with CO_2-free air for 30 min depleted the culture of CO_2 to near zero levels. Under these conditions the transcripts for three inducible inorganic carbon uptake systems *ndhF3*, *sbtA* and *cmpA* showed near maximal abundance after 15 min of CO_2 concentration reductions. Liu et al. (2011) used promoters of these genes to regulate the expression of lipase. For harvesting the culture was subjected to zero CO_2 environment for 30 min. This induced the lipolytic gene expression which permeabilized the cell wall for release of the molecules into the medium.

On the negative side, overexpression of lipid synthesis pathway genes affects microalgal proliferation. This can be overcome by use of an inducible promoter that can be activated once the microalgal cells have grown to a high density and have entered the stationary phase. There are several examples of inducible promoters in algae including copper-responsive elements in *C. reinhardtii* (Quinn and Merchant, 1995) and a nitrate-responsive promoter in diatoms (Poulsen and Kroger, 2005).

Inhibiting lipid catabolism may also cause problems with proliferation and biomass productivity since microalgae often rely on catabolic pathways to provide energy and precursors for cell division. Lately, it was shown that the targeted knockdown of lipase/phospholipase/acyltransferase increased lipid yields without affecting growth in the diatom *Thalassiosira pseudonana*. Further, antisense expressing knockdown strains 1A6 and 1B1 exhibited wild type like growth and increased lipid content under both continuous light and alternating light-dark conditions (Liu et al., 2011). Analyses of fatty acids, lipid classes, and membrane stability in the transgenic strains suggest a role for this enzyme in membrane lipid turnover and lipid homeostasis. These results demonstrate that targeted metabolic manipulations can be used to increase lipid accumulation in eukaryotic microalgae without compromising growth. Similar strategies in pathway engineering should be achievable also in cyanobacteria, where photosynthesis, rather than organic feedstock, would provide energy and carbon.

4.3.4 High Carbon Compounds

Alkanes with C4-C23 length possess higher energy density, hydrophobicity and compatibility with existing liquid fuel infrastructure and are the predominant constituents of gasoline, diesel and jet fuels (Peralta-Yahya et al., 2012). The capacity of cyanobacteria to produce alkanes was reported as early as the 1960's (Han et al., 1968). The pathway consists of an acyl–acyl carrier protein reductase and an aldehyde decarbonylase, which together converts intermediates of fatty acid metabolism to alkanes and alkenes (Fig. 4.5).

Heptadecane is the most common alkane found in algae. Heterologous expression of the alkane operon from cyanobacteria in *E. coli* led to the production and secretion of C13 to C17 mixtures of alkanes and alkenes (Schirmer et al., 2010).

Short and medium chain alkanes have the potential to be used directly as transportation fuel and have been reported to be secreted by a diverse group of organisms. Algae produce high amount of lipids. It is feasible to convert these lipids into desired alkanes via the formation of aldehydes. Aldehydes to alkanes is catalyzed by a decarboxylase enzyme (Li et al., 2012). Not all alkanes are produced naturally by algae. Ethylene synthesis via this route appears promising. It can be polymerized into gasoline, alcohol and diesel. It is currently produced exclusively from fossil fuels, and its production is the largest CO_2-emitting process in the chemical industry. Therefore, production of ethylene with simultaneous sequestration of CO_2 is no doubt a very attractive process. This feat was recently achieved by expression of the *efe* gene encoding an ethylene-forming enzyme from *Pseudomonas syringae* pv. In *Synechocystis* that led to continuous ethylene production. Interestingly, this gene was earlier used in other studies and was found to be unstable after 3 to 4 generations. Further investigations had revealed gene duplication of the sequence CTAT as the cause of gene inactivation. These sequences were found at three sites which were recognized as 'genetic hot spots'. Researchers introduced silent mutations at

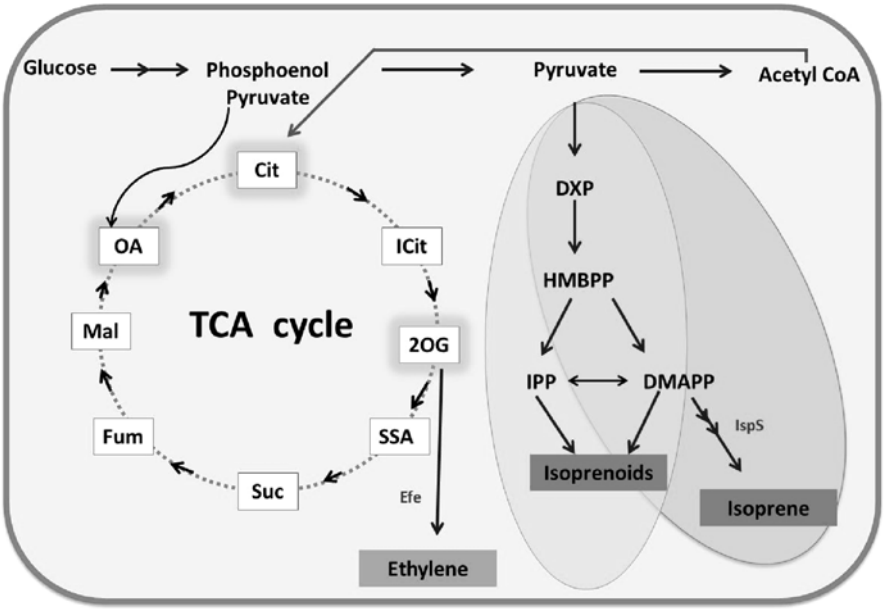

Fig. 4.5 Schematic of pathways in cyanobacteria for the production of various alkanes/alkenes. Abbreviations: Cit:Citrate, Icit:Isocitrate, 2OG: 2-Oxaloglycerate, SSA: Succinyl CoA, Suc: Succinate, Fum: Fumarate, Mal: Malate, OA: Oxaloacetate, DXP: 1-deoxyxylulose-5-phosphate, HMBPP: 1-hydroxy-2-methyl-2-butenyl-4-pyrophosphate, IPP: isopentenyl-pyrophosphate, DMAPP: dimethylallyl-pyrophosphate.

these sites and further codon optimized the sequence thereby resolving the stability issues and increasing yield. Up to 5.5 % of the fixed carbon was directed to ethylene synthesis, surpassing the published carbon-partition rate into the TCA cycle. Nitrogen and phosphorus enriched seawater can support both growth and ethylene production. Factors limiting ethylene production, including *efe* expression levels, light intensity and nutrient status, were identified and alleviated (Ungerer et al., 2012). Optimizing the expression of the alkane biosynthesis genes and enhancing the carbon flux through the fatty acid and alkane biosynthesis pathways can lead to the accumulation and/or secretion of notable amounts of alkanes. Further, it also becomes important to understand how to control the chain lengths of the produced alkane molecules.

Isoprenoids, e.g. the monoterpene pinene and the sesquiterpene farnesene, are considered precursors for future biodiesel or next-generation jet fuel. Cyanobacteria produce carotenoids and extending the carotenoid biosynthetic pathways by introduction of constructs for appropriate terpene synthases should allow the biosynthesis of selected mono and sesquiterpenes. Isoprenoids, also known as terpenoids, represent an incredibly diverse group of natural compounds, with more than 40,000 different molecules. In microalgae, isoprenoids are synthesized via the methylerythritol (MEP) pathway using glyceraldehydes-3-phosphate and pyruvate to generate

the basic building blocks of isoprenoid biosynthesis, isopentyl diphosphate (IPP), and dimethylallyl diphosphate (DMAPP). Molecules that could potentially work as gasoline substitutes, including isopentenol, have been produced by *E. coli* using isoprenoid biosynthesis pathways. Two enzymes from *Bacillus subtilis* that utilize IPP and DMAPP for the biosynthesis of isopentenol were overexpressed in *E. coli*, resulting in production of 112 mg/litre isopentenol (107, 200).

Recently, Lindberg et al. (2010) showed the feasibility of producing isoprene hydrocarbons from *Synechocystis* by expression of non-native isoprene synthase gene in *Synechocystis*. Codon usage further optimized the *ispS* gene expression which was driven by the photosynthesis *psbA2* promoter to obtain light driven isoprene accumulation. Further, Bentley in a follow up work from the same group described the improvement of the photosynthetic partitioning between isoprene and biomass in *Synechocystis*. Expression of the non-native Mevalonic acid pathway genes in *Synechocystsis* endowing a non-native pathway for carbon flux amplification to isopentenyl-diphosphate (IPP) and dimethylallyl-diphosphate (DMAPP) precursors of isoprene. Heterologous expression of the isoprene synthase in combination with the MVA pathway enzymes resulted in photosynthetic isoprene yield improvement by approximately 2.5-fold, compared with that measured in cyanobacteria transformed with the isoprene synthase gene only. These results suggest that the MVA pathway introduces a bypass in the flux of endogenous cellular substrate in *Synechocystis* to IPP and DMAPP, overcoming flux limitations of the native MEP pathway.

To make these biofuels more promising, the low tolerance of algae to alkanes must be overcome. In-depth studies have shown that the oxidative stress of the organisms increases upon production of these hydrocarbons suggesting that they are possibly the major protection mechanism against the production of these hydrocarbons. Expression of transporters to facilitate the secretion of the product into the culture medium may help develop more robust cells for the synthesis of such compounds.

4.4 Application of 'Omics Technologies'—Genomics, Transcriptomics and Proteomics

Omic technology adopts a holistic view of the molecules that make up a cell. They are aimed primarily at the universal detection of genes (genomics), mRNA (transcriptomics), proteins (proteomics) and metabolites (metabolomics). The aim of these technologies is that the complex interaction between the organisms can be understood more thoroughly. The study of metagenomes is important with respect to the discovery of more robust pathways in different organisms. For instance, the [FeFe] hydrogenase is a promising candidate; however, the enzyme is difficult to work with because it involves the issues of oxygen sensitivity. Genetic engineering approaches to resolve the issues have produced limited success so far. A way around

the issue would be to analyze the different metagenomes from complex ecosystems for different variants of this enzyme present in nature. In one such approach, Craig Venter's institute carried out large scale sequencing of millions of organisms from a myriad of locations to identify possible host candidates to improve hydrogen production.

Engineering algae into fuel factories may interfere with their overall cell homeostasis, which in turn might counteract sustainability and compromise product yields. Therefore, it is necessary to access the overall cellular response in terms of the production of the targeted molecule towards establishing more robust organisms. Cellular proteomics and transcriptomics find importance in holistic understanding of not only the wild type organisms, but also to study a comparative profile between the wild type and the engineered mutants. For example the deletion or over expression of a particular gene in a pathway may have several consequences. Pinto et al. (2012) compared the *Synechocystis* wild type proteome to that of the engineered strain where the hydrogen producing Hox operon had been deleted. The in-depth study showed that the deletion did not affect cell viability or any other pathways and that it could be used as another neutral site to express heterologous genes. Similar studies were carried out by Dienst et al. (2014) to study the cellular response towards the long-term production of ethanol from *Synechocystis*. Since the group observed specific physiological response to the long-term ethanol production such as a bleaching phenotype, slow growing biomass and down regulation of the light harvesting capacity, an omic study could indicate which stress factors were responsible. Future development of engineered strains could consider the effect of the same and engineer the associated responses. More such studies are encouraged so that the scientific community gets a holistic picture of the stress responses and other pathways that get affected by the introduction or deletion of non-native/native pathways. This knowledge will immensely contribute towards the development of more robust systems.

4.5 Conclusions

Algae are a phylogenetically diverse group of organisms and possess novel metabolic features that can be exploited for the production of sustainable biofuels. Several biotechnology companies have shown their interest in commercializing their products. Prominent among them are Algenol Biofuels that is currently commercializing algae-based ethanol produced by an engineered cyanobacteria which they claim can produce 10,000–12,000 gal/acre/year (Waltz, 2009). Exxon Mobil has developed expertise in free-fatty acid producing cyanobacteria and Joule unlimited has a patent for alkane producing cyanobacteria. These companies reflect the surging growth market for these biofuels as also their social acceptance. As new species are discovered and their genomes sequenced the possibility of implementation of a bio-based economy is highly probable. However, though many milestones have been accomplished there is still a long way to go. Towards this, advancement

of fundamental knowledge is crucial. A deeper insight into our current setbacks and technical limitations is encouraged. Future accomplishments must endow a high driving force (high enzyme activities and irreversible reaction), stable enzyme (active under oxygenic photosynthesis), low toxicity of accumulated products and an abundant source of carbon substrate. Genetic engineering and synthetic biology together have a power to reform the world. Recent advances and a surging biofuel market show that it can happen. A century earlier, the discovery of fossilized algae brought around the industrial revolution; a century later they demonstrate the capacity to spin the wheel yet again.

References

Badger, M.R. and Price, G.D. (2003). CO_2 concentrating mechanisms in cyanobacteria: Molecular components, their diversity and evolution. *Journal of Experimental Botany*, 54, 609–622.

Badger, M.R., Andrews, T.J., Whitney, S.M., Ludwig, M., Yellowlees, D.C., Leggat, W. and Price, G.D. (1998). The diversity and coevolution of rubisco, plastids, pyrenoids, and chloroplast-based CO_2-concentrating mechanisms in algae. *Canadian Journal of Botany*, 76, 1052–1071.

Bar-Even, A., Noor, E., Lewis, N.E. and Milo, R. (2010). Design and analysis of synthetic carbon fixation pathways. *Proceedings of the National Academy of Sciences, USA*, 107, 8889–8894.

Bernat, G., Waschewski, N. and Rögner, M. (2009). Towards efficient hydrogen production: The impact of antenna size and external factors on electron transport dynamics in *Synechocystis* PCC 6803. *Photosynthesis Research*, 99, 205–216.

Bershtein, S. and Tawfik, D.S. (2008). Advances in laboratory evolution of enzymes. *Current Opinion in Chemical Biology*, 12,151–158.

Bhattacharya, S., Schiavone, M., Nayak, A. and Bhattacharya, S.K. (2005). Biotechnological storage and utilization of entrapped solar energy. *Applied Biochemistry Biotechnology*, 120, 159–167.

Boison, G., Bothe, H., Hansel, A. and Lindblad, P. (1999). Evidence against a common use of the diaphorase subunits by the bidirectional hydrogenase and by the respiratory complex I in cyanobacteria. *FEMS Microbiology Letters,* **174,** 159–165.

Bonacci, W., Teng, P.K., Afonso, B., Niederholtmeyer, H., Grob, P., Silver, P.A. and Savage, D.F. (2012). Modularity of a carbon-fixing protein organelle. *Proceedings of the National Academy of Sciences of the United States of America*, 109, 478–483.

Carrasco, C.D., Holliday, S.D., Hansel, A., Lindblad, P. and Golden, J.W. (2005). Heterocyst-specific excision of the *Anabaena* sp. strain PCC 7120 hupL element requires xisC. *Journal of Bacteriology*, 187, 6031–6038.

Casalot, L. and Rousset, M. (2001). Maturation of the [NiFe] hydrogenases. *Trends in Microbiology*, 9, 228–237.

Chen, L.M., Li, K.Z., Miwa, T. and Izui, K. (2004). Overexpression of a cyanobacterial phosphoenolpyruvate carboxylase with diminished sensitivity to feedback inhibition in *Arabidopsis* changes amino acid metabolism. *Planta*, 219, 440–449.

Chisti, Y. (2008). Biodiesel from microalgae beats bioethanol. *Trends in Biotechnology*, 26, 126–131.

Cirino, P.C. and Frei, C.S. (2009). Combinatorial enzyme engineering. *In:* Sheldon, J., Park and Cochran, J.R. (eds) Protein Engineering and Design. CRC Press, BC, pp. 131–150.

Collman, J.P. (1996). Coupling H_2 to electron transfer. *Nature Structural Biology*, 3, 213–217.

Daniel, H., Torres-Ruiz, J.A. and McFadden, B.A. (1989). Amplified expression of ribulosebisphosphate carboxylase/oxygenase in pBR 322-transformants of *Anacystis nidulans. Archives of Microbiology*, 151, 59–64.

Deng, M.D. and Coleman, J.R. (1999). Ethanol synthesis by genetic engineering in cyanobacteria. *Applied and Environmental Microbiology*, 65, 523–528.

Department of Energy, USA. Source: http://www.netl.doe.gov/research/coal/carbon-storage/natcarb-atlas/co2-stationary-sources

Dienst, D., Georg, J., Abts, T., Jakorew, L., Kuchmina, E., Borner, T. et al. (2014). Transcriptomic response to prolonged ethanol production in the cyanobacterium *Synechocystis* sp. PCC6803. *Biotechnology for Biofuels*, 7, 7–21.

Ducat, D.C., Sachdeva, G. and Silver, P.A. (2011). Rewiring hydrogenase-dependent redox circuits in cyanobacteria. *Proceedings of the National Academy of Sciences of the United States of America*, 108, 3941–3946.

Ekman, M., Ow, S.Y., Holmqvist, M. and Lindblad, P. (2011). Metabolic adaptations in a H_2 producing heterocyst-forming cyanobacterium: Potentials and implications for biological engineering. *Journal of Proteomic Research*, 10, 1772–1784.

Field, C.B., Behrenfeld, M.J., Randerson, J.T. and Falkowski, P. (1998). Primary production of the biosphere: Integrating terrestrial and oceanic components. *Science*, 281, 237–240.

Fukuzawa, H., Suzuki, E., Komukai, Y. and Miyachi, S. (1992). A gene homologous to chloroplast carbonic-anhydrase (*icfa*) is essential to photosynthetic carbon-dioxide fixation by *Synechococcus* PCC7942. *Proceedings of the National Academy of Sciences of the United States of America*, 89, 4437–4441.

Gärtner, K., Lechno-Yossef, S., Cornish, A.J., Wolk, C.P. and Hegg, E.L. (2012). Expression of *Shewanella oneidensis* MR-1 [FeFe]-hydrogenase genes in *Anabaena* sp. strain PCC 7120. *Applied and Environmental Microbiology*, 78, 8579–8586.

Greene, D.N., Whitney, S.M. and Matsumura, I. (2007). Artificially evolved *Synechococcus* PCC 6301 Rubisco variants exhibit improvements in folding and catalytic efficiency. *Biochemistry Journal*, 404, 517–524.

Han, J., McCarthy, E.D., Hoeven, W.V., Calvin, M. and Bradley, W.H. (1968). Organic geochemical studies, a preliminary report on the distribution of aliphatic hydrocarbons in algae, in bacteria, and in a recent lake sediment. *Proceedings of the National Academy of Sciences, USA*, **59, 29–33.**

Happe, T., Schütz, K. and Bohme, H. (2000). Transcriptional and mutational analysis of the uptake hydrogenase of the filamentous cyanobacterium *Anabaena variabilis* ATCC 29413. *Journal of Bacteriology*, 182, 1624–1631.

Hu, Q., Sommerfeld, M., Jarvis, E., Ghirardi, M., Posewitz, M., Seibert, M. and Darzins, A. (2008). Microalgal triacylglycerols as feedstocks for biofuel production: Perspectives and advances. *The Plant Journal*, 54, 621–639.

Imashimizu, M., Bernát, G., Isato, K., Broekmans, M., Konno, H., Sunamura, E.I., Rögner, M. and Hisabori, T. (2011). Regulation of F_oF_1-ATPase from *Synechocystis* sp. PCC 6803 by the γ and ∈ subunits is significant for light/dark adaptation. *Journal of Biochemistry*, 286, 26595–26602.

Iwaki, T., Haranoh, K., Inoue, N., Kojima, K., Satoh, R., Nishino, T. and Wadano, A. (2006). Expression of foreign type I ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39) stimulates photosynthesis in cyanobacterium *Synechococcus* PCC 7942 cells. *Photosynthesis Research*, 88, 287–297.

Janssen, M., de Winter, M., Tramper, J., Mur, L. R., Snel, J. and Wijffels, R.H. (2000). Efficiency of light utilization of *Chlamydomonas reinhardtii* under medium-duration light/dark cycles. *Journal of Biotechnology*, 78, 123–137.

Khetkorn, W., Lindblad, P. and Inchroensakdi, A. (2012). Inactivation of uptake hydrogenase leads to enhanced and sustained hydrogen production with high nitrogenase activity under high light exposure in the cyanobacterium *Anabaena siamensis* TISTR 8012. *Journal of Biological Engineering*, 6, 19.

Lan, E.L. and Liao, J.C. (2012). ATP drives direct photosynthetic production of 1-butanol in cyanobacteria. *Proceedings of the National Academy of Sciences of the United States of America*, 109, 6018–6023.

Li, H. and Liao, J.C. (2013). Engineering a cyanobacterium as the catalyst for the photosynthetic conversion of CO_2 to 1,2-propanediol. *Microbial Cell Factories*, 12, 4.

Li, N., Chang, W.C., Warui, D.M., Booker, S.J., Krebs, C. and Bollinger, J.M. (2012). Evidence for Only Oxygenative Cleavage of Aldehydes to Alk(a/e)nes and Formate by Cyanobacterial Aldehyde Decarbonylases. *Biochemistry,* **51,** 7908–7916.

Li, X., Shen, C.R. and Liao, J.C. (2014). Isobutanol production as an alternative metabolic sink to rescue the growth deficiency of the glycogen mutant of *Synechococcus elongatus* PCC 7942. *Photosynthesis Research*, 120, 301–310.

Lieman-Hurwitz, J., Rachmilevitch, S., Mittler, R., Marcus, Y. and Kaplan, A. (2003). Enhanced photosynthesis and growth of transgenic plants that express ictB, a gene involved in HCO_3^- accumulation in cyanobacteria. *Plant Biotechnology Journal*, 1, 43–50.

Lindberg, P., Devine, E., Stensjö, K. and Lindblad, P. (2012). HupW protease specifically required for processing of the catalytic subunit of the uptake hydrogenase in the cyanobacterium *Nostoc* sp. strain PCC 7120. *Applied Environmental Microbiology*, 78, 273–276.

Lindberg, P., Park, S. and Melis, A. (2010). Engineering a platform for photosynthetic isoprene production in cyanobacteria, using *Synechocystis* as the model organism. *Metabolic Engineering*, 12, 70–79.

Lindberg, P., Schütz, K., Happe, T. and Lindblad, P. (2002). A hydrogen-producing, hydrogenase-free mutant strain of *Nostoc punctiforme* ATCC 29133. *International Journal Hydrogen Energy*, 27, 1291–1296.

Liu, X., Sheng, J. and Curtiss, R. (2011). Fatty acid production in genetically modified cyanobacteria. *Proceedings of the National Academy of Sciences of the United States of America*, 108, 6899–6904.

Long, B.M., Badger, M.R., Whitney, S.M. and Price, G.D. (2007). Analysis of carboxysomes from *Synechococcus* PCC 7942 reveals multiple Rubisco complexes with carboxysomal proteins CcmM and CcaA. *The Journal of Biological Chemistry*, 282, 29323–29335.

Long, S.P., Zhu, X.G., Naidu, S.L. and Ort, D.R. (2006). Can improvement in photosynthesis increase crop yields? *Plant Cell Environment*, 29, 315–330.

Lu, X., Vora, H. and Khosla, C. (2008). Overproduction of free fatty acids in *E. coli*: Implications for biodiesel production. Metabolic Engineering, 10, 333–339.

Masukawa, M., Mochimaru, M. and Sakurai, H. (2002). Disruption of the uptake hydrogenase gene, but not of the bidirectional hydrogenase gene, leads to enhanced photobiological hydrogen production by the nitrogen-fixing cyanobacterium *Anabaena* sp. PCC 7120. *Applied Microbiology Biotechnology*, 58, 618–624.

McGinn, P.J., Price, G.D., Maleszka, R. and Badger, M.R. (2003). Inorganic carbon limitation and light control the expression of transcripts related to the CO_2-concentrating mechanism in the cyanobacterium *Synechocystis* sp. strain PCC 6803. *Plant Physiology*, 132, 218–229.

Melis, A. (2009). Solar energy conversion efficiencies in photosynthesis: Minimizing the chlorophyll antennae to maximize efficiency. *Plant Science*, 177, 272–280.

Metz, J.G., Pollard, M.R., Anderson, L., Hayes, T.R. and Lassner, M.W. (2000). Purification of a *jojoba* embryo fatty acyl-coenzyme A reductase and expression of its cDNA in high erucic acid rapeseed. *Plant Physiology*, 122, 635–644.

Moal, G. and Lagoutte, B. (2012). Photo-induced electron transfer from Photosystem 1 to $NADP^+$: Characterization and tentative simulation of the *in vivo* environment. *Biochemistry Biophysics Acta*, 1817, 1635–1645.

Moroney, J. V. and Ynalvez, R.A. (2007). Proposed carbon dioxide concentrating mechanism in *Chlamydomonas reinhardtii. Eukaryotic Cell*, 6, 1251–1259.

Msanne, J., Xu, D., Konda, A.R., Casas-Mollano, J.A. and Awada, T. (2012). Metabolic and gene expression changes triggered by nitrogen deprivation in the photoautotrophically grown microalgae *Chlamydomonas reinhardtii* and *Coccomyxa* sp. C-169. *Phytochemistry*, 75, 50–59.

Mueller-Cajar, O. and Whitney, S.M. (2008). Directing the evolution of Rubisco and Rubiscoactivase: First impressions of a new tool for photosynthesis research. *Photosynthesis Research*, 98, 667–675.

Mussgnug, J.H., Thomas-Hall, S., Rupprecht, J., Foo, A., Klassen, V., McDowall, A., Schenk, P.M., Kruse, O. and Hankamer, B. (2007). Engineering photosynthetic light capture: Impacts on improved solar energy to biomass conversion. *Journal of Plant Biotechnology*, 5, 802–814.

Nakajima, Y., Fujiwara, S., Sawai, H., Imashimizu, M. and Tsuzuki, M. (2001). A phycocyanin-deficient mutant of *Synechocystis* PCC 6714 with a single-base substitution upstream of the cpc operon. *Plant Cell Physiology*, 42, 992–998.

Nayak, B.K., Roy, S. and Das, D. (2014). Biohydrogen production from algal biomass (Anabaena sp. PCC 7120) cultivated in airlift photobioreactor. *International Journal of Hydrogen Energy*, 39, 7553–7560.

Nguyen, M.T., Choi, S.P., Lee, J., Lee, J.H. and Sim, S.J. (2009). Hydrothermal acid pretreatment of *Chlamydomonas reinhardtii* biomass for ethanol production. *Journal of Microbiology and Biotechnology*, 19, 161–166.

Oliver, J.W.K., Machado, I.M.P., Yoneda, H. and Atsumi, S. (2013). Cyanobacterial conversion of carbon dioxide to 2,3-butanediol. *Proceedings of the National Academy of Sciences of the United States of America*, 110, 1249–1254.

Peralta-Yahya, P.P., Zhang, F.Z., Cardayre, S.B. and Keasling, J.D. (2012). Microbial engineering for the production of advanced biofuels. *Nature*, **488,** 320–328.

Pinto, F., van Elburg, K.A., Pacheco, C.C., Lopo, M., Noirel, J., Montagud, A. and Tamagnini, P. (2012). Construction of a chassis for hydrogen production: Physiological and molecular characterization of a *Synechocystis* sp. PCC 6803 mutant lacking a functional bidirectional hydrogenase. *Microbiology*, 158, 448–464.

Polle, J.E., Kanakagiri, S.D. and Melis, A. (2003). tla1, a DNA insertional transformant of the green alga *Chlamydomonas reinhardtii* with a truncated light-harvesting chlorophyll antenna size. *Planta*, 217, 49–59.

Poulsen, N. and Kroger, N. (2005). A new molecular tool for transgenic diatoms. *Journal of FEBS*, 272, 3413–3423.

Price, G.D. and Badger, M.R. (1989). Expression of human carbonic anhydrase in the cyanobacterium *Synechococcus* PCC 7942 creates a high-CO_2 requiring phenotype. *Plant Physiology*, 91, 505–513.

Price, G.D., Woodger, F.J., Badger, M.R., Howitt, S.M. and Tucker, L. (2004). Identification of a SulP-type bicarbonate transporter in marine cyanobacteria. *Proceedings of the National Academy of Sciences, USA*, 101, 18228–18233.

Quinn, J.M. and Merchant, S. (1995). Two copper-responsive elements associated with the *Chlamydomonas* Cyc6 gene function as targets for transcriptional activators. *Plant Cell*, 7, 623–638.

Rascher, U., Lakatos, M., Büdel, B. and Lüttge, U. (2003). Photosynthetic field capacity of cyanobacteria of a tropical inselberg of the Guiana Highlands. *European Journal of Phycology*, 38, 247–256.

Reifschneider-Wegner, K., Kanygin, A. and Redding, K.E. (2014). Expression of the [FeFe] hydrogenase in the chloroplast of *Chlamydomonas reinhardtii. International Journal of Hydrogen Energy*, 39, 3657–3665.

Riekhof, W.R., Sears, B.B. and Benning, C. (2005). Annotation of genes involved in glycerolipid biosynthesis in *Chlamydomonas reinhardtii*: Discovery of the betaine lipid synthase BTA1(Cr). *Eukaryotic Cell*, 4, 242–252.

Rögner, M. (2013). Metabolic engineering of cyanobacteria for the production of hydrogen from water. *Biochemical Society Transactions*, 4, 1254–1259.

Roy, S., Kumar, K., Ghosh, S. and Das, D. (2014). Thermophilic biohydrogen production using pretreated algal biomass as substrate. *Biomass and Bioenergy*, 61, 157–166.

Schirmer, A., Rude, M.A., Li, X.Z., Popova, E. and delCardayre, S.B. (2010). Microbial Biosynthesis of Alkanes. *Science,* **329,** 559–562.

Scott, S.A., Davey, M.P., Dennis, J.S., Horst, I., Howe, C.J., Lea-Smith, D.J. and Smith, A.J. (2010). Biodiesel from algae: Challenges and prospects. *Current Opinion in Biotechnology*, 21, 277–286.

Shen, C.R., Lan, E.I., Dekishima, Y., Baez, A., Cho, K.M. and Liao, J.C. (2011). Driving Forces Enable High-Titer Anaerobic 1-Butanol Synthesis in *Escherichia coli. Applied and Environmental Microbiology*, 77, 2905–2915.

Shibata, M., Katoh, H., Sonoda, M., Ohkawa, H., Shimoyama, M., Fukuzawa, H., Kaplan, A. and Ogawa, T. (2002). Genes essential to sodium-dependent bicarbonate transport in cyanobacteria—Function and phylogenetic analysis. *Journal of Biological Chemistry,* 277, 18658–18664.

Smith, S.A. and Tabita, F.R. (2003). Positive and negative selection of mutant forms of prokaryotic (cyanobacterial) ribulose-1,5-bisphosphate carboxylase/oxygenase. *Journal of Molecular Biology*, 331, 557–569.

Stitt, M., Sulpice, R. and Keurentjes, J. (2010). Metabolic networks: How to identify key components in the regulation of metabolism and growth. *Plant Physiology*, 152, 428–444.

Summary report: World agriculture: towards 2015/2013 (2002). Food and Agriculture Organization of the United Nation, Rome.

Tcherkez, G.G.B., Farquhar, G.D. and Andrews, T.J. (2006). Despite slow catalysis and confused substrate specificity, all ribulose bisphosphate carboxylases may be nearly perfectly optimized. *Proceedings of the National Academy of Sciences, USA*, 103, 7246–7251.

UK Department of Energy and Climate Change: Energy Trends March 2009, http://www.decc.gov.uk/en/content/cms/statistics/publications/publications.aspx.

Um, B.H. and Kim, Y.S. (2009). Review: A chance for Korea to advance algal-biodiesel technology. *Journal of Industrial and Engineering Chemistry*, 15, 1–7.

Ungerer, J., Tao, L., Davis, M., Ghirardi, M., Maness, P.C. and Yu, J. (2012). Sustained photosynthetic conversion of CO_2 to ethylene in recombinant cyanobacterium *Synechocystis* 6803. *Energy Environment Science*, 5, 8998–9006.

Varman, A.M., Xiao, Y., Pakrasi, H.B. and Tang, Y.J. (2013). Metabolic Engineering of *Synechocystis* sp. Strain PCC 6803 for Isobutanol Production. *Applied Environmental Microbiology*, 79, 908–914.

Walter, J.M., Greenfield, D. and Liphardt, J. (2010). Potential of light-harvesting proton pumps for bioenergy applications. *Current Opinion in Biotechnology*, 21, 265–270.

Waltz, E. (2009). Biotech's green gold? *Nature Biotechnology*, 27, 15–18.

Wang, Z.T., Ullrich, N., Joo, S., Waffenschmidt, S. and Goodenough, U. (2009). Algal lipid bodies: Stress induction, purification, and biochemical characterization in wild-type and starch-less *Chlamydomonas reinhardtii. Eukaryotic Cell*, 8, 1856–1868.

Wu, M., Mintz, M., Wang, M. and Arora, S. (2008). Consumptive Water Use in the Production of Bioethanol and Petroleum Gasoline. Argonne National Laboratory's work report.

Xu, Y., Guerra, L.T., Li, Z., Ludwig, M., Dismukes, G.C. and Bryant, D.A. (2013). Altered carbohydrate metabolism in glycogen synthase mutants of *Synechococcus* sp. strain PCC 7002: Cell factories for soluble sugars. *Metabolic Engineering*, 16, 56–67.

Yang, C., Hua, Q. and Shimizu, K. (2002). Metabolic flux analysis in *Synechocystis* using isotope distribution from 13C-labeled glucose. *Metabolic.Engineering*, 4, 202–216.

Yao, L., Qi, F., Tan, X. and Lu, X. (2014). Improved production of fatty alcohols in cyanobacteria by metabolic engineering. *Biotechnology for Biofuels*, 7, 94.

Yoshino, F., Ikeda, H., Masukawa, H. and Sakurai, H. (2007). High photobiological hydrogen production activity of a *Nostoc* sp. PCC 7422 uptake hydrogenase-deficient mutant with high nitrogenase activity. *Marine Biotechnology*, 9, 101–112.