Chapter 4

H_2 Production Using Cyanobacteria/Cyanobacterial Hydrogenases: From Classical to Synthetic Biology Approaches

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Summary

The simple nutritional requirements of cyanobacteria, the availability of molecular tools and genome sequences, as well as the recent genome scale-models of *Synechocystis* sp. PCC 6803 make these photosynthetic prokaryotes attractive platforms for the production of added-value

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compounds, namely hydrogen. Naturally, these organisms may contain up to three types of enzymes directly involved in hydrogen metabolism: one or more nitrogenases that evolve H_2 concomitantly with N_2 fixation, an uptake hydrogenase that recycles the H_2 released by the nitrogenase, and a bidirectional hydrogenase. Significant contributions from studies of genetic engineering, transcriptional regulation and maturation, and assessment of enzymatic activities in response to various environmental cues led to gaining further control of the mechanisms by which one can engineer cyanobacteria for H_2 production. In this chapter, the classical approaches of screening natural communities for improved activities, the manipulation of native enzymes and/or growth conditions, and the expression of heterologous hydrogenases into or from cyanobacteria will be summarized. Moreover, the recent synthetic biology approaches pursuing the use of cyanobacteria as photoautotrophic chassis, as well as the construction and characterization of synthetic parts and devices aiming at improving H_2 production will be reviewed. However, the generated knowledge and the molecular/synthetic tools developed in this field represent a valuable contribution not only to improve biohydrogen production but also to further drive the technology related to other biofuels and various industrial applications.

I. Introduction

Besides sharing the basic cellular features of other Bacteria, cyanobacteria possess unique and diagnostic characteristics. Distinctively, cyanobacteria are the only organisms ever to evolve coupled photosystems that harvest electrons from water and produce oxygen as a byproduct (Knoll 2008). They are photosynthetic prokaryotes typically possessing the ability to synthesize chlorophyll *a* (Whitton and Potts 2000) although four distinctive lineages produce alternative chlorophyll pigments (Swingley et al. 2008).

In terms of Earth history, cyanobacteria occupy a privileged position among organisms. As primary producers they play a significant role in Earth's carbon cycle; as nitrogen fixers, they also figure prominently in the nitrogen cycle (Knoll 2008). Moreover, they also loom large in our planet's redox history (Knoll 2008). One of the major changes on Earth, the introduction of oxygen into the atmosphere is widely accepted to be attributed to the photosynthetic activity of cyanobacteria. Cyanobacterial ecological plasticity is remarkable and their long evolutionary history is possibly related to their success in modern habitats. They are mostly found in aquatic, but also in many terrestrial environments (Whitton and Potts 2000). They can even be found growing near the limits for life in the dry deserts of Antarctica or in many thermal springs (Whitton and Potts 2000). Symbiotic interactions between cyanobacteria and other organisms are surprisingly diverse (Costa 2004): in these associations, cyanobacteria provide different hosts with fixed carbon, with the product of nitrogen fixation or with both products (Costa 2004). The secret that certainly contributes for the cyanobacterial easy adaptation to numerous ecological niches and makes them the driving force for shaping nearly every ecosystem on Earth is definitely their physiological flexibility.

Cyanobacteria are the only diazotrophs that perform oxygenic photosynthesis, and since the enzymatic complex carrying out nitrogen fixation – the nitrogenase – is extremely sensitive to oxygen, they present different strategies to cope with this incompatibility. Although these strategies are usually divided in spatial (N_2 fixation in specialized cells – the heterocysts) or temporal (N_2 fixation confined to the dark period) (Herrero et al. 2001), there is a range of mechanisms far more complex (for details see Berman-Frank et al. 2003). Nitrogen fixing cyanobacteria contain the so-called conventional nitrogenase with Mo and Fe in the active site, but some heterocystous strains

Abbreviations: OCD – Oxygen Consuming Device; SB – Synthetic Biology

e.g. *Anabaena variabilis* may also contain "alternative" nitrogenases (Thiel 1993; Bothe et al. 2010). Under Mo-deprived conditions the MoFe nitrogenase is replaced by a V nitrogenase, or in the absence of this element by a Fe only-nitrogenase (the existence of this enzyme has not yet been established in cyanobacteria). The reduction of atmospheric nitrogen to ammonium is always accompanied by the formation of molecular hydrogen as a byproduct. It has been reported that alternative nitrogenases are better H_2 producers compared with the MoFe nitrogenases but very little work has been performed on this subject. For an overview see Bothe et al. (2010).

Closely related to the ability to fix nitrogen, cyanobacteria possess a distinct H_2 metabolism. The H_2 produced by nitrogenase is rapidly consumed by an uptake hydrogenase, an enzyme that has been found in all nitrogen fixing cyanobacteria examined so far. In 2006, Ludwig et al. (2006) reported the existence of a N_2-fixing strain, *Synechococcus* sp. BG 043511, naturally lacking an uptake hydrogenase and this was believed to be the first exception to the rule. However, recent work carried out by Skizim and co-workers has shown that this strain, now referred to as *Cyanothece* sp. Miami BG 043511, does in fact possess not only the genes but also a functional hydrogenase (Skizim et al. 2012). Additionally, cyanobacteria may contain a bidirectional hydrogenase, an enzyme that is generally present in non-nitrogen fixing strains. However, it is absent in *Gloeobacter violaceus* PCC 7421, a cyanobacterium that possesses a number of unique characteristics such as the absence of thylakoids (Nakamura et al. 2003).

In summary, cyanobacteria may contain up to three types of enzymes directly involved in hydrogen metabolism: one or more nitrogenases that evolve H_2 concomitantly with dinitrogen fixation, an uptake hydrogenase that recycles the H_2 released by the nitrogenase(s), and a bidirectional hydrogenase. In these organisms, H_2 is naturally produced by the nitrogenase or in specific conditions (e.g. dark anaerobic) by the bidirectional hydrogenase. Therefore, given what is known in terms of H_2 metabolism, minimal nutritional requirements, and physiology, cyanobacteria emerge as prime candidates for H_2 production. In this chapter we focus on the efforts to manipulate their native enzymes and/or environmental conditions, as well as in the recent synthetic biology approaches to improve H_2 production.

II. Transcriptional Regulation and Maturation of Cyanobacterial Hydrogenases

Transcriptional regulation of cyanobacterial hydrogenases has deserved some attention over time, and significant advances have been accomplished in this field. In an attempt to assess the role of hydrogenases in cell fitness and physiological flexibility, several different strains have been grown (or subjected) to numerous environmental conditions, and the understanding of which cues trigger an up- or down-regulation of the hydrogenases genes transcription is fairly understood. It is not our intention to extensively review this topic here (for more details, the reader is suggested to see Oliveira and Lindblad 2009; Bothe et al. 2010). Nevertheless, given the fact that several contributions have been made in recent years it is worth referring in a bit more detail the work carried out on the characterization of transcription factors and transcription networks.

On one hand, the cyanobacterial uptake hydrogenase seems to be under the control of NtcA in unicellular (Oliveira et al. 2004), filamentous (Leitão et al. 2005; Ferreira et al. 2007) and heterocystous (Weyman et al. 2008; Holmqvist et al. 2009) strains. NtcA operates global nitrogen regulation in cyanobacteria, and these observations support the notion that part of the hydrogen metabolism is clearly related with nitrogen metabolism, namely overlapping with the process of nitrogen fixation.

On the other hand, cyanobacterial AbrB-like regulators have been shown to play a key role in the bidirectional hydrogenase genes transcription. Cyanobacterial AbrB-like proteins (also known as CalA and CalB, CyAbrB clade A and clade B, or AbrB1 and AbrB2) present a certain degree of sequence homol-

ogy to the *Bacillus subtilis* AbrB, which is a transition state regulator and involved in spore formation (Phillips and Strauch 2002). However, despite the sequence homology, the cyanobacterial AbrB-like proteins do not seem to be involved in the regulation of the same set of genes as in *B. subtilis*. Instead, the cyanobacterial AbrB-like regulator AbrB2 (CalB – Sll0822) was shown to have a broad range of regulatory points: (i) it works in parallel with NtcA to achieve flexible regulation of the nitrogen uptake system in *Synechocystis* sp. PCC 6803 (Ishii and Hihara 2008), (ii) AbrB2 is also directly involved in the modulation of low-CO_2-induced gene expression (Lieman-Hurwitz et al. 2009), and (iii) it regulates the promoter activity of functional antisense RNA of an operon that plays a crucial role in photoprotection of photosystem II under low carbon conditions (Eisenhut et al. 2012). Moreover, AbrB2 was demonstrated to interact with itself (Sato et al. 2007), suggesting to work as an oligomer, and most importantly to function as an autorepressor (Dutheil et al. 2012), inhibiting as well the bidirectional hydrogenase operon transcription in *Synechocystis* sp. PCC 6803 (Ishii and Hihara 2008; Dutheil et al. 2012). Furthermore, AbrB1 (CalA – Sll0359) has been equally implicated in the regulation of the hydrogenase operon in *Synechocystis* sp. PCC 6803, suggested to work as an activator (Oliveira and Lindblad 2008). The regulatory network operating on the fine tuning of the hydrogenase operon expression in *Synechocystis* sp. PCC 6803 becomes more complicated with three additional pieces of evidence: firstly, AbrB1 and AbrB2 have the capacity to interact with each other, as assessed by yeast two hybrid interactions (Sato et al. 2007) and his-tag pull down assays (Yamauchi et al. 2011), indicating that a regulatory balance may be achieved by protein-protein interactions; secondly, the AbrB-like proteins were shown to be modified on a post-translational level (Shalev-Malul et al. 2008), which introduces another level of possible regulation; and finally, a third transcription factor has also been shown to positively regulate the expression of this operon, namely LexA (Gutekunst et al. 2005), which can also be modified on a post-translational level (Oliveira and Lindblad 2011).

The action of AbrB-like proteins on the transcriptional regulation of the genes involved in hydrogen metabolism in cyanobacteria is not limited to the bidirectional hydrogenase. Recent work by Holmqvist and co-workers showed that AbrB1 binds specifically to the promoter region of *hupSL*, genes encoding the uptake hydrogenase in *Nostoc punctiforme* ATCC 29133 (Holmqvist et al. 2011), suggesting a possible active role in controlling their expression. *Anabaena* sp. PCC 7120 AbrB1 (Alr0946) was found to regulate negatively the hydrogenase accessory gene *hypC* (Agervald et al. 2010). Finally, in the filamentous non-heterocystous cyanobacterium *Lyngbya majuscula* CCAP 1446/4, NtcA was shown to interact specifically with the *hyp* genes promoter (Ferreira et al. 2007), indicating a possible co-regulation of *hyp* and *hup* genes via NtcA in response to various sources and amounts of nitrogen in the medium.

Hydrogenase accessory proteins, like HypC mentioned above are chaperones that assemble the various components of the hydrogenase NiFe active site and coordinate it with the enzyme's amino acid structure. A final maturation step consists of a proteolytic cleavage of the hydrogenase large subunit C-terminus, which can be regarded as a control checkpoint since it occurs only when all the active site components have been properly introduced. This step precedes the assembly between the hydrogenase small and large subunits, rendering a functional enzyme. The literature on this topic is quite scarce in respect to cyanobacteria and most of what is known regarding maturation of hydrogenases derives from work carried out in e.g. *Escherichia coli*. Considering that the hydrogenase accessory genes in *E. coli* share a high degree of homology with the cyanobacterial ones, it was generally assumed that the Hyp proteins in cyanobacteria would play a similar role. It was the work of Hoffmann and co-workers that finally set this question, when they were able to show convincingly that the hydrogenase accessory proteins in *Synechocystis* sp. PCC 6803 have a direct

involvement in the bidirectional hydrogenase maturation (Hoffmann et al. 2006). In filamentous heterocystous strains the only work available addressing the role of the hydrogenase accessory genes on the enzyme's maturation was done by Lindberg and co-workers, assessing the function of *hupW* in *Anabaena* sp. PCC 7120 (Lindberg et al. 2012). In this article, *hupW* is presented as the specific protease that completes the uptake hydrogenase maturation, leading to a hydrogen evolving phenotype (see below). In summary, the main driving forces for studying transcriptional regulation of cyanobacterial hydrogenases and the complex process of hydrogenase maturation have been (i) to improve the understanding of the physiological relevance of the hydrogen metabolism in cyanobacteria, (ii) to unravel novel overlapping points with other metabolic pathways, and (iii) to gain further control on the mechanisms by which one can engineer cyanobacteria for H_2 production.

III. Strategies to Improve Cyanobacterial H_2 Production

Over the years, several strategies have been adopted in an ultimate effort to improve cyanobacterial H_2 production. In the present review, we divide these efforts into "classical" and "synthetic biology" approaches. The first includes conventional work carried out in different domains of cell and molecular biology, ranging from thorough selections of cyanobacterial H_2 producers to simple genetic manipulations of key enzymes involved in the hydrogen metabolism. The latter represents a modern approach, where standardized and interchangeable modules are routinely used to assist rational metabolic engineering methodologies.

A. Classical Approaches

1. Screening for Strains/Enzymes with Improved Activities

To work with widely used and well characterized cyanobacterial model organisms has clear advantages regarding the study of the hydrogen metabolism. Genetic tools for their manipulation are usually well established (enabling the production of both knock-outs and knock-ins) and the amount of physiological and genetic data available supports a reasonably good understanding of the organism's overall metabolism. However, it is evident that cyanobacterial strains yet to be described may have different and more attractive performances regarding hydrogen evolution than the standard reference cyanobacteria. Therefore, serious efforts have been put together to screen both existing culture collections and environmental samples to search for more efficient H_2 producers. The work carried out by Allahverdiyeva et al. (2010) represents a good example of such approach: they describe a screen of 400 cyanobacterial isolates from the Baltic Sea and Finnish lakes and conclude that roughly half of the tested strains produce detectable amounts of H_2. Interestingly, ten of the evaluated strains evolved similar or up to four times as much hydrogen as uptake hydrogenase deletion mutants created in various laboratories and specifically engineered to evolve higher amounts of H_2 (Allahverdiyeva et al. 2010). These newly described strains represent excellent candidates for further characterization and future genetic engineering. Cyanobacterial strains isolated from diverse environments, including terrestrial, freshwater and marine intertidal settings, have also been the subject of recent studies aiming at quantitatively comparing their hydrogenase activities under non-nitrogen fixing conditions (Kothari et al. 2012). The authors selected these particular environments because most of the hydrogen evolving cyanobacteria described so far are originally from freshwater bodies, making their study unique in respect to origin, morphology, taxonomy and phylogeny. The main conclusions withdrawn from the work are that strains isolated from freshwater and intertidal settings had a high incidence of hydrogen production (concurrent with the presence of *hoxH*, encoding the bidirectional hydrogenase large subunit), while all terrestrial isolates were negative for both traits. Moreover, and most interestingly, some novel strains

displayed rates of hydrogen evolution several fold higher than those previously reported, making them potentially interesting for biohydrogen production (Kothari et al. 2012).

In addition, cyanobacterial strains that have been once the subject of studies regarding various metabolic processes (e.g. photosynthesis, and in particular nitrogen fixation) are now evaluated in terms of their H_2 evolution capacity. It is worth mentioning the work done with *Cyanothece* strains (Bandyopadhyay et al. 2010, 2011; Min and Sherman 2010a, b). *Cyanothece* is a genus of unicellular nitrogen-fixing cyanobacteria that have the remarkable capacity of performing both oxygenic photosynthesis (evolving molecular oxygen) and nitrogen-fixation (a highly oxygen sensitive reaction) in the same cell. The ability to fix N_2 and produce H_2 was assessed in six *Cyanothece* strains: ATCC 51142, PCC 7424, PCC 7425, PCC 7822, PCC 8801 and PCC 8802 (Bandyopadhyay et al. 2011). *Cyanothece* sp. ATCC 51142 showed the highest nitrogenase activity as well as H_2 production, followed by *Cyanothece* sp. PCC 8802 and PCC 8801. From all the strains evaluated in this work, *Cyanothece* sp. PCC 7425 was the only one unable to fix N_2 (and produce H_2) under aerobic conditions and with lower nitrogenase activity (Bandyopadhyay et al. 2011). More extensive work has been carried for *Cyanothece* sp. ATCC 51142. This cyanobacterium was shown to produce H_2 either through the bidirectional hydrogenase or the nitrogenase; in the latter case the yield was 30- to 60-fold higher (Min and Sherman 2010a). H_2 evolution by the hydrogenase was shown to be dependent on the electron transport by photosystem II whereas the activity of nitrogenase relies on photosystem I and respiration. Furthermore, it was demonstrated that H_2 production and N_2 fixation occurred at high rates even under continuous light conditions (when *hupSL* transcript levels are low). In addition, the results indicated that low-oxygen conditions (argon sparging) favor H_2 production and N_2 fixation by the nitrogenase (Min and Sherman 2010a). Also in 2010, Bandyopadhyay and co-workers reported that the same *Cyanothece* strain (ATCC 51142) could generate high levels of H_2 under N_2 fixing and natural aerobic conditions. The production yield was significantly enhanced in cultures supplemented with glycerol or high CO_2 concentrations, in which the excess of carbon source functions as a signal for enhanced nitrogenase activity (Bandyopadhyay et al. 2010). H_2 evolution was also evaluated in *Cyanothece* sp. Miami BG 043511 and production by the hydrogenase occurred in dark anoxic conditions, using the reducing power derived from fermentation, while H_2 production by the nitrogenase occurred in light, relying on photosystem I (Skizim et al. 2012). Further work performed by Min and Sherman (2010b) showed that in a *Cyanothece* sp. PCC 7822 deletion mutant (Δ*nifK*) defective in N_2 fixation, cells were unable to grow in absence of combined nitrogen and H_2 production was nearly abolished in air or low-oxygen conditions.

Cyanobacterial strains with outstanding physiological characteristics and selected for particular growth traits are also being target of thorough studies (Taton et al. 2012). The combination of their natural capacities and increased environmental fitness with the amenability to genetic modifications/manipulations represents an attractive and complementary way of moving forward towards a more sustainable cyanobacterial H_2 producer.

Technologies for high-throughput sequencing are developing fast and respective costs are dropping. Therefore, its application in metagenomics has been tremendous and it has enabled the acquisition of information at a rate never imagined before. Making use of this powerful technique, an alternative approach to screen strains for their natural ability to evolve molecular hydrogen has emerged, consisting instead of searching for novel hydrogenase genes, which may encode enzymes with a higher oxygen tolerance and/or a higher H_2 evolving activity. The focus is therefore concentrated on finding novel genes rather than on the organism itself, since maintaining and growing a microorganism under laboratory conditions may

proof difficult. The global ocean sampling metagenomic database (Venter et al. 2004; Rusch et al. 2007; Yooseph et al. 2007) represents one of the most adequate starting points for such quest. Barz and co-workers (2010) have taken the first steps into this matter by searching the database for all the described families of hydrogenases. In parallel, the authors have also investigated DNA isolated from samples taken from the North Atlantic, Mediterranean Sea, North Sea, Baltic Sea and two fresh water lakes for the presence of genes encoding the bidirectional hydrogenase. Surprisingly, this study shows that hydrogenases are quite abundant in marine environments and that marine surface waters could be a source of oxygen-resistant uptake hydrogenases. Furthermore, this approach helps clarifying the primary function of hydrogenases based on their ecological distribution (Barz et al. 2010). Certainly in a near future, new metagenomic studies looking into unexplored environments will be presented and novel hydrogenase genes will be uncovered with possible improved features. The challenging venture will be to take that genetic information into a cyanobacterium, wire the functional hydrogenase with the organism's metabolism and enhance the organism performance in terms of H_2 production rates and sustainability. As a matter of fact, similar approaches have been done in which cyanobacteria are used to express a heterologous hydrogenase (see below), the main difference here being that the foreign hydrogenases usually originate from well-known organisms, grown under laboratory conditions and with various limitations.

2. Tuning Physiological/Environmental Conditions

Another point that has attracted considerable attention over the years regarding the improvement of cyanobacterial H_2 production is the environment in which the cells are grown (or kept) in. When media compositions were initially established, maximization of the cells capacity to evolve molecular hydrogen was not top priority. Therefore, extensive work has been carried out to find the best conditions for higher and more sustainable production rates and total amounts. Ananyev et al. (2008) and Carrieri et al. (2010, 2011) in *Arthrospira* (*Spirulina*) *maxima* CS-328 and Burrows et al. (2008, 2009, 2011) in *Synechocystis* sp. PCC 6803 constitute a few examples of such work.

The effort that is being carried out in this field is in close association to the developments achieved in bioreactor design. It is not our goal to review what is being done in this area (for good and recent reviews on the topic, the reader is suggested to read Rupprecht et al. 2006; Kumar et al. 2011; Show et al. 2011), but it is impossible to miss out the multiple and alternative methods implemented in the most varied scales to optimize cyanobacterial H_2 production.

3. Knockout Mutants/Protein Engineering

Once the hydrogen metabolism became described in cyanobacteria and hydrogenase activities determined and characterized, identifying the genes coding for the hydrogenases structural and accessory proteins became the necessary following step. The availability of genetic tools to manipulate cyanobacteria turned out to be extremely useful to further characterize these genes and to determine their specific role in the hydrogen metabolism. The gene coding for the uptake hydrogenase large subunit has been a clear candidate for knockout constructions in various cyanobacterial strains, namely in *Anabaena variabilis* ATCC 29413 (Happe et al. 2000), *Nostoc punctiforme* ATCC 29133 (Lindberg et al. 2002), *Anabaena* sp. PCC 7120 (Masukawa et al. 2002), *Nostoc* sp. PCC 7422 (Yoshino et al. 2007), *Anabaena siamensis* TISTR 8012 (Khetkorn et al. 2012). In all these studies, the knockout organism was able to evolve significant higher amounts of H_2 than the respective wild-type, as a direct consequence of the organism's incapacity to recycle the molecular hydrogen released as byproduct of nitrogen fixation. Moreover, a proteomic study identified the specific metabolic processes

used by the *N. punctiforme* mutant NHM5 to maintain the high rate of nitrogen fixation despite the absence of the uptake hydrogenase (Ekman et al. 2011). Interestingly, all strains used in the studies mentioned above are invariably filamentous, heterocyst-forming cyanobacteria. In addition, a *hupW* (gene putatively coding for the uptake hydrogenase large subunit protease) deletion mutant in *Anabaena* sp. PCC 7120 was recently described (Lindberg et al. 2012). In this study, it was found that molecular hydrogen accumulates in comparable amounts as for the *hupL* knockout (Lindberg et al. 2012), suggesting that nitrogenase based H_2 evolution can also be attained by silencing the uptake hydrogenase maturation system.

It is quite apparent at this point that using nitrogenase as the H_2 producing enzyme in cyanobacteria represents a fairly common practice to accumulate desirable amounts of H_2. However, the nitrogenase is a very energy demanding enzymatic complex (it is estimated that 16 ATP molecules are necessary to fix one molecule of N_2 into two of NH_3) and H_2 production simply results as a byproduct (one molecule released per 16 ATP molecules). Therefore, engineering the cyanobacterial nitrogenase has deserved some focus with the clear goal of converting it into a more efficient H_2 producing device. Nevertheless, it should be noted that the nitrogenase is a quite complex multimeric enzymatic system, requiring metal coordination and incorporation into the protein's active site and insertion of FeS clusters that mediate the transfer of electrons between ferredoxin and the active site for N_2 reduction. Both of these processes depend on several well-known chaperones, but also on yet to be described mechanisms. Thus, the room for engineering cyanobacterial nitrogenases (or any other nitrogenase for that matter) is somewhat limited. Regardless of the difficulties, Weyman et al. (2010) created modifications on an alternative Mo-nitrogenase (Nif2) of *Anabaena variabilis*, which is expressed in vegetative cells grown with fructose under strictly anaerobic conditions. A V75I substitution in the α-subunit rendered an enzyme with greatly impaired acetylene reduction and reduced levels of $^{15}N_2$ fixation, but with a fourfold higher H_2 production in the presence of N_2 compared with the wild-type and similar H_2 production rates as the wild-type enzyme in an argon atmosphere (Weyman et al. 2010). The authors concluded that the amino acid substitution did not change the ability of the mutant enzyme to reduce substrates, but instead simply increased the selectivity for substrates. A more comprehensive study was carried out by Masukawa and co-workers, targeting the nitrogenase active site of *Anabaena* sp. PCC 7120. Their efforts were concentrated on six nitrogenase amino acid residues (Q193, H197, Y236, R284, S285, F388) predicted to be located within 5 Å of the metal MoFe active center, aiming at directing electron flow selectively towards proton reduction (Masukawa et al. 2010). The ability to fix N_2 was moderately or severely impaired in nearly all NifD variant strains examined, a necessary compromise if one aims to redistribute electrons toward proton reduction. In terms of H_2 accumulation during long-term incubations under physiologically relevant conditions, 11 selected *Anabaena* sp. PCC 7120 NifD variants (namely Q193S, Q193H, Q193L, Q193K, H197T, H197F, H197Q, Y236T, R284T, R284H and F388H) greatly exceeded the H_2 produced by the reference strains (ΔNifHΔHupL, ΔHupL, AnNifHΔHupL). Among the NifD variants, R284H presented the best performance, since the H_2 accumulated under N_2 after 1 week was 87 % of what was observed for the reference strains under an argon atmosphere (Masukawa et al. 2010). These encouraging results highlight the potential of using some of these nitrogenase variants as parental strains for additional engineering steps in a concerted effort to improve photobiological H_2 production. Still regarding the use of nitrogenase as the key factor for H_2 production in cyanobacteria, but moving away from the protein engineering approach, an alternative strategy has been attempted. This involved the deletion of the homocitrate synthase genes (*nifV1* and

nifV2) in *Anabaena* sp. PCC 7120 (Masukawa et al. 2007). The catalytic site of dinitrogenase normally binds the FeMo cofactor to which homocitrate is ligated. However, mutating *nifV* in various microorganisms resulted in significantly different nitrogenase *in vivo* activities. By creating Δ*nifV1*, Δ*nifV2* and Δ*nifV1*Δ*nifV2* mutants in the Δ*hupL Anabaena* sp. PCC 7120 strain diazotrophic growth rates were decreased moderately to severely depending on the mutant in comparison to the rates of the parental strain (Masukawa et al. 2007). The simple fact that Δ*nifV1*Δ*nifV2* mutant cells could grow under combined nitrogen depleted conditions indicates that the nitrogenase of this strain can fix N_2 in the absence of homocitrate. Interestingly, hydrogen production was found to be higher and more sustainable under air in the Δ*nifV1*mutant than in any other evaluated strain, suggesting this can be a useful strategy for improving photobiological H_2 production by cyanobacteria (Masukawa et al. 2007).

The potential of the cyanobacterial bidirectional hydrogenase to produce molecular hydrogen has also been extensively explored. *Synechocystis* sp. PCC 6803, as one of the most well studied cyanobacterial strains, has been a clear candidate for metabolic engineering. According to Gutthann et al. (2007), cells of *Synechocystis* sp. PCC 6803 growing under normal conditions have four main electron sinks competing with each other, namely the Calvin-cycle (and respective inorganic carbon concentrating mechanisms), nitrate, oxygen and protons. Gutthann's suggestion was brought up based on various observations and some follow below. The *ndhB* gene codes for a key subunit of all NDH-1 (Type I NADPH-dehydrogenase) complexes, and consequently a knockout mutant of this gene leads to a strain devoid of any of such complexes. A Δ*ndhB* mutant (M55) has been created (Ogawa 1991) and one of its most remarkable features has to do with its slow growth rates when compared to the wild-type. The phenotype can be explained by an impairment of the inorganic carbon concentrating mechanisms and by a reduced carbon fixation capacity (Ogawa 1991; Ohkawa et al. 2000). This M55 mutant has been evaluated in terms of H_2 production ability and it was found to evolve significant amounts of H_2 (Cournac et al. 2002, 2004). M55 is specifically impaired in NADPH oxidation and to avoid an over-reducing state of the cell it is believed that the bidirectional hydrogenase releases the electron excess by evolving H_2. Alternatively, Baebprasert et al. (2011) have targeted the nitrate assimilation pathway by creating *Synechocystis* sp. PCC 6803 strains lacking functional nitrate reductase (Δ*narB*) or nitrite reductase (Δ*nirA*) or both, and tested for their ability to produce hydrogen. In this work, it is reported that all engineered strains present a higher capacity of hydrogen production as well as higher hydrogenase activity than those of wild-type, and the best hydrogen producer was found to be the double mutant. The reduction of nitrate to ammonia before its incorporation to amino acids requires two sequential reactions carried out by nitrate reductase and nitrite reductase, demanding a large amount of reducing power. Therefore, by eliminating such electron sink the cell undergoes an unbalanced redox state which can be attenuated by the reduction of protons via bidirectional hydrogenase. Furthermore, Gutthann et al. (2007) made use of various oxidases deletion mutants (cytochrome *c* oxidase – *ctaI*, alternative cytochrome *c* oxidase – *ctaII*, quinol oxidase – *cyd*) that are deficient in extracting electrons from cytochrome *c* or from plastoquinone to reduce O_2 to water, to assess several aspects of the hydrogen metabolism in *Synechocystis* sp. PCC 6803. The study shows that the deletion of quinol oxidase in combination with one of the two cytochrome *c* oxidases causes a prolonged H_2 production phase in the light and a higher maximal amount, supporting their suggestion that O_2 is an important electron sink that competes with protons for reductant. All these pieces of evidence seem to support the idea that the bidirectional hydrogenase in the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 functions as a valve for low-potential

electrons generated during the light reaction of photosynthesis, thus preventing a slowing down of electron transport (Appel et al. 2000; Carrieri et al. 2011; Pinto et al. 2012). Therefore, understanding the physiological role of the cyanobacterial bidirectional hydrogenase will definitely lead to a more rational design of metabolic pathways, envisioning engineered strains with improved hydrogen production capacity.

Alternative approaches have been adopted to improve cyanobacterial hydrogen production via bidirectional hydrogenase, including genetic engineering of fermentative pathways. Within this line, McNeely et al. (2010) report their efforts on redirecting reductant into hydrogen production using the unicellular *Synechococcus* sp. PCC 7002. This cyanobacterium has a quite distinct dark anaerobic metabolism when compared to other cyanobacteria, since it is capable of yielding up to five fermentation products (McNeely et al. 2010). Lactate was found to be the product excreted in higher amounts, and therefore an *ldhA* (coding for D-lactate dehydrogenase) knockout mutant was created. Among several phenotypic traits, Δ*ldhA* cells presented an altered fermentative flux in comparison to wild-type cells, which reflected on an up to fivefold increase in hydrogen production (McNeely et al. 2010). In addition, the same lab has also focused on the pyruvate:ferredoxin oxidoreductase (NifJ) of *Synechococcus* sp. PCC 7002, which reduces ferredoxin during fermentation of pyruvate to acetyl-coenzyme A (McNeely et al. 2011). Interestingly, and even though Δ*nifJ* cells presented a higher *in vitro* hydrogenase activity, levels of H_2 produced were 1.3-fold lower than those accumulated by wild-type cells (McNeely et al. 2011). Regardless of the lower performance of Δ*nifJ* cells in terms of H_2 production, this study contributed significantly to a better understanding of the bidirectional hydrogenase role in *Synechococcus* sp. PCC 7002: it serves as a valve to reoxidize excess NADH formed during glycolysis, in addition to oxidizing reductant generated from ferredoxin, produced by the pyruvate:ferredoxin oxidoreductase (McNeely et al. 2011).

Despite all the efforts to find or produce an attractive and sustainable cyanobacterial H_2 producer, it became increasingly evident as new reports were available that different research groups with different laboratory routines grow these potentially interesting organisms in the most varied ways (e.g. light quality, intensity and regimen, media composition) and present H_2 evolution rates and amounts in multiple units and manners. Trying to compare activities and to assess which strain presents the best performance in terms of H_2 evolution represents therefore a really difficult challenge. Lopes Pinto et al. (2002) took on their hands the hard task to screen through the literature and present a uniform landscape of the cyanobacterial H_2 producers and respective H_2 evolving capacities. Alternatively, others opted to study various cyanobacterial strains (including both wild-types and H_2 evolving mutants), grown under the same culturing conditions and evaluated their H_2 evolving capacities, presenting a more comparable picture (Schütz et al. 2004).

4. Expression of Heterologous Hydrogenases in Cyanobacteria

The first work reporting the introduction and expression of a heterologous hydrogenase into a cyanobacterium, the unicellular non-N_2-fixing *Synechococcus elongatus* PCC 7942, was published in 2000 by Asada et al. These authors cloned the gene encoding the monomeric Fe hydrogenase I (*cpI*) from *Clostridium pasteurianum* downstream a strong native promoter and exchanged the Shine-Dalgarno sequence. After anaerobic adaption, the transformed *Synechococcus elongatus* PCC 7942 cells exhibited a significantly higher H_2 evolution (threefold increase) compared to the wild-type (Table 4.1).

Berto et al. (2011) reported to have expressed an active monomeric Fe hydrogenase, from the unicellular green alga *Chlamydomonas reinhardtii*, in another

Table 4.1. Expression of heterologous hydrogenases in cyanobacteria/use of cyanobacteria as a photoautothrophic chassis.

Cyanobacterial host	Fe-hydrogenase		NiFe-hydrogenase		Promoter	Site/plasmid	Protein expression	Protein activity	References
	Structural genes inserted	Accessory genes inserted	Structural genes inserted	Accessory genes inserted					
Synechococcus elongatus PCC 7942	*cpI* from *Clostridium pasteurianum*	None			Strong native promoter and exchanged Shine-Dalgarno sequence	Plasmid pKE4-9	Yes	Yes	Asada et al. (2000)
	hydA from *Clostridium acetobutylicum*	*hydEF*, *hydG* exogenous ferredoxins			P_{lac} – IPTG inducible (*hydA* and ferredoxins)	Neutral site NS3 (*hydA*)	Yes	Yes	Ducat et al. (2011b)
					P_{psbA1} – constitutive (*hydEF*, *hydG*)	Neutral site NS1 (*hydEF*, *hydG*)			
Synechococcus elongatus Δ*hoxYH* – strain PW416			*hynS and hynL* from *Alteromonas macleodii*	*orf1*, *cyt*, *orf2*, *hynD*, *hupH* and *hypCABDFE*	P_{trc} – IPTG inducible	Neutral site NS1	Yes	Yes	Weyman et al. (2011)
			hynSL from *Thiocapsa roseopersicina*	All accessory genes from *T. roseopersicina*		Neutral site NS2	Yes	No	
				+11 accessory genes from *A. macleodii*			Yes	Yes	
Synechocystis sp. PCC 6803	*hydA1* from *Chlamydomonas reinhardtii* (without the region encoding the N-terminal transit peptide)	None			P_{psbA2} – light inducible P_{ziaA} – Zn^{2+} inducible	Genome, between *sll1865* and *sll1864*	Yes	Yes	Berto et al. (2011)
Nostoc sp. PCC 7120	*hydA* and *hydB* (modified), from *Shewanella oneidensis* MR-1	S03922 *hydG,*S03924, *hydE, hydF*			P_{hetN} – heterocyst specific		Yes	Yes, in N_2 fixing conditions only	Gärtner et al. (2012)

unicellular non-N_2-fixing cyanobacterium *Synechocystis* sp. PCC 6803, in the absence of the native maturation system but already truncated at the N-terminal (Table 4.1). The authors suggested that the cyanobacterium hydrogenase accessory proteins could account for the folding/maturation of the algal hydrogenase, and that the low activity levels observed were consistent with what was expected for a heterologous enzyme working outside its specific environment (the enzyme is cloroplastidial in the alga) or that the cyanobacterial maturation machinery could impair the activity of the enzyme. To further prove their hypothesis they suggest expressing the *C. reinhardtii* hydrogenase in *Synechocystis* sp. PCC 6803 lacking the different *hyp* genes. In addition, *Synechocystis* sp. PCC 6803 mutant lacking the native hydrogenase could be used (see Pinto et al. 2012).

The genes encoding the oxygen tolerant and thermostable NiFe hydrogenase from *Alteromonas macleodii* Deep ecotype, as well as the HynSL from *Thiocapsa roseopersicina* were expressed in *Synechococcus elongatus* PCC 7942 hydrogenase knockout mutant (PW416 strain), using an IPTG-inducible promoter (Weyman et al. 2011). The *hynSL* genes from *A. macleodii*, together with 11 adjacent genes, were introduced into the previously identified neutral site 1, NS1 (Andersson et al. 2000). The *hynSL* genes of *T. roseopersicina* were introduced in neutral site 2 (NS2, Andersson et al. 2000) also via homologous recombination, together with different sets of accessory genes (Table 4.1). While the authors could demonstrate the presence of an active HynSL from *A. macleodii* in strain PW416, the one from *T. roseopersicina* was not active. However, this could be altered by adding the 11 accessory genes from *A. macleodii* (Weyman et al. 2011). Furthermore, the introduction of the bidirectional hydrogenase of *T. roseopersicina* (*hoxEFUYH*), that harbors similarities with the native cyanobacterial hydrogenase, was attempted. However, its co-expression with *T. roseopersicina* or *A. macleodii's* accessory genes was not sufficient to obtain an active enzyme in strain PW416 (Weyman et al. 2011).

Recently, and for the first time, a heterologous Fe hydrogenase, from *Shewanella oneidensis* MR-1, was successfully expressed in the heterocysts of a filamentous cyanobacterium *Anabaena* sp. PCC 7120 using the specific promoter P_{hetN} (Table 4.1). The spatial separation between photosynthesis and H_2 production overcomes the sensibility of the hydrogenase to oxygen by expressing it in a microaerobic compartment (Gärtner et al. 2012).

5. Expression of a Cyanobacterial Hydrogenase in Escherichia coli

The genes encoding the bidirectional hydrogenase (*hoxEFUYH*) from *Synechocystis* sp. PCC 6803 were cloned into *E. coli* and, the expression of the cyanobacterial hydrogenase lead to a threefold increase in H_2 production (Maeda et al. 2007). An optimization of the growth medium, replacing glucose by fructose, galactose or maltose resulted in 20 % increase in H_2 yield. Moreover, a time course evaluation of the H_2 production revealed that, after 18 h, there was over 41 times more H_2 production in the mutant compared with the wild-type. However, Maeda et al. (2007) also showed that in *E. coli* cells expressing the bidirectional hydrogenase from *Synechocystis* sp. PCC 6803, H_2 production is sustained by the native hydrogenase 3 while the HoxEFUYH inhibits H_2 uptake by hydrogenases 1 and 2.

B. Synthetic Biology Approaches

The emerging field of Synthetic Biology (SB) offers novel perspectives for the production of added-value compounds, namely biofuels. In this context, and due to their minimal nutritional requirements and metabolic plasticity, cyanobacteria could constitute exceptional photoautotrophic chassis for the production of hydrogen (Angermayr et al. 2009; Ducat et al. 2011a; Heidorn et al. 2011; Lindblad et al. 2012; Pinto et al. 2012). For this purpose, a wide toolbox containing well characterized standardized biological parts and devices should be developed.

In this section the recent advances in this field will be presented.

1. Cyanobacteria as Photoautotrophic Chassis

In SB applications parts, devices, and circuits must eventually be introduced into a host cell that is designated as chassis. The chassis should be based on a well-known organism with a sequenced genome, for which plenty of information is available (transcriptomic, proteomic and metabolomic) allowing the development of models to predict its behavior. Its genetic manipulation should also be easy and, preferably, the chassis should have a streamline genome making device implementation more effective and predictable (Etc Group 2007; O'Malley et al. 2008). The accomplishment of SB-based strategies for the production of biofuels, namely hydrogen, has been successfully explored using *Saccharomyces cerevisiae* and *E. coli* as chassis (Waks and Silver 2009; Agapakis et al. 2010). Still, there is a need to develop more/more predictable basic SB tools and to explore other chassis, namely photoautotrophic ones. In this context, cyanobacteria emerge as promising candidates due to their ability to use solar energy and CO_2 as energy and carbon sources respectively, thrive in different environments and metabolic plasticity. In addition, cyanobacteria have higher growth rates compared to plants and the molecular tools for their genetic manipulation are available, being easier to engineer than algae (Ducat et al. 2011a; Heidorn et al. 2011; Lindblad et al. 2012). The unicellular *Synechocystis* sp. PCC 6803 is the best studied cyanobacterial strain and its genome was the first to be sequenced among photosynthetic organisms (Kaneko et al. 1996). Moreover, the vast amount of data available allowed the construction and validation of genome-scale metabolic models – e.g. *i*Syn811 – that are powerful tools to develop a robust photoautotrophic chassis, and to predict changes when synthetic modules are introduced (Fu 2009; Montagud et al. 2010, 2011; Yoshikawa et al. 2011). Recently, a *Synechocystis* sp. PCC 6803 deletion mutant (Δ*hoxYH*), lacking an active bidirectional hydrogenase was produced and extensively characterized to be used as chassis for the introduction of heterologous hydrogenases/hydrogen producing devices (Pinto et al. 2012). The authors also developed vectors compatible with the BioBrick system that allow removing redundant genes and/or introducing synthetic parts into *Synechocystis* sp. PCC 6803 genome (Pinto et al. 2012). For the introduction of synthetic parts/devices, it is important to identify neutral sites i.e. genomic loci that can be disrupted without affecting cellular viability or causing any distinguishable phenotype (Clerico et al. 2007). The identification/validation of these neutral sites increases the chassis functionality since it permits the sequential integration of different/more complex devices into the genome. To date, four chromosomal loci have been used to introduce foreign DNA into *Synechocystis* sp. PCC 6803 (Williams 1988; Burnap et al. 1994; Aoki et al. 1995, 2011) but they have not been fully characterized. In another unicellular cyanobacterium *Synechococcus elongatus* sp. PCC 7942, three neutral sites (NS1, NS2 and NS3) have also been used for the integration of foreign DNA (Andersson et al. 2000; Clerico et al. 2007; Niederholtmeyer et al. 2010; Ducat et al. 2011b) but, similarly to *Synechocystis* sp. PCC 6803, they remain largely uncharacterized.

One of the current challenges in SB is the development of streamline genomes since lower genome complexity will facilitate chassis engineering, insulation of the introduced synthetic devices from the chassis regulatory network, and render systems behavior more predictable. A 15 % reduction of the *E. coli* genome led to unanticipated benefits such as higher electroporation efficiency and increased genome stability (Pósfai et al. 2006). In cyanobacteria, a blueprint for genome reduction of *Synechococcus elongatus* PCC 7942 has already been constructed (Delaye et al. 2011). In this case, the essential and non-essential genes were identified using a combination of methods:

unusual G+C content, unusual phylogenetic similarity and/or a small number of the highly iterated palindrome 1 (HIP1) plus unusual codon usage. This work will facilitate engineering *Synechococcus elongatus* PCC 7942, for which fewer tools are available but has higher growth rates and a smaller genome than *Synechocystis* sp. PCC 6803 (2.7 Mb compared to 3.6 Mb). At present, *Synechocystis* sp. PCC 6803 and *Synechococcus elongatus* PCC 7942 are among the best cyanobacterial candidates to be used as photoautotrophic chassis in SB applications.

2. Parts and Devices for H_2 Production

Synthetic Biology is also based on the premise that if one is able to learn how each modular component works and understands the interaction between parts, then is able to manipulate and recombine them (assembling them into devices and circuits) to design novel pathways with useful purposes. This implies the construction and characterization of standardized interchangeable biological parts with defined functions, that often need to be customized (e.g. codon optimized) to be fully functional in a given chassis. The development of standards and databases such as the BioBricks and the Registry of Biological Parts (Registry of Standard Biological Parts) facilitated the sharing of parts between researchers and led to the construction of numerous synthetic devices with applications in different areas such as pharmaceuticals and biofuels (Ro et al. 2006; Waks and Silver 2009; Agapakis et al. 2010). Although the quantitative control (predictable behaviour) of biological systems using a SB approach is possible, it still remains an iterative process. In different organisms, including cyanobacteria, an effort has been made in the characterization of promoters, ribosomal binding sites, terminators and regulatory elements, as well as in the development of BioBrick compatible plasmids (Shetty et al. 2008; Boyle and Silver 2009; Purnick and Weiss 2009; Huang et al. 2010). Still, there is the need to increase the repertoire of available parts, including orthogonal ones that should have the major advantage of insulating the synthetic devices and circuits from the genetic network of the chassis.

a. Hydrogen Producing Devices

Using a similar strategy as in the classical molecular biology approaches (see above), a gene encoding the monomeric Fe hydrogenase – HydA – from *Clostridium acetobutylicum* was expressed in the cyanobacterium *Synechococcus elongatus* PCC 7942 (Ducat et al. 2011b). The main difference here is that the authors made use of genes commercially synthesized, codon optimized, and acceptable for use in *E. coli* and other organisms, with all constructs in the BioBrick format (Ducat et al. 2011b). The genes encoding the hydrogenase maturation factors HydEF and HydG were cloned separately and placed under the *Synechococcus elongatus* PCC 7942 *psbA1* constitutive promoter, and then combined into a cassette that was subsequently integrated in the previously defined genomic neutral site NS1 (Clerico et al. 2007). The gene encoding HydA was inserted separately, downstream the IPTG-inducible promoter P_{lac}, and integrated into the neutral site NS3 (Niederholtmeyer et al. 2010) (see Table 4.1). Ducat et al. (2011b) demonstrated that the *in vivo* hydrogenase activity is connected to the light-dependent reactions of the electron transport chain, and that under anoxic conditions the heterologous enzyme is capable of supporting light-dependent hydrogen evolution at a rate 500-fold greater than that supported by the endogenous NiFe bidirectional hydrogenase. Moreover, to facilitate electron transfer to HydA and further increase hydrogen production, genes encoding heterologous ferredoxins (that constitute the strongest pair together with HydA) were inserted into *Synechococcus elongatus* PCC 7942 under an IPTG-inducible promoter (Ducat et al. 2011b). The expression of the *C. acetobutylicum* ferredoxin increased the rate of hydrogen evolution by twofold,

and additional experiments demonstrate that the addition of supplemental ferredoxins or optimization of ferredoxin-hydrogenase interactions can both increase the flux of electrons towards hydrogenase as well as rewire the redox pathway.

In another SB approach, a single synthetic operon containing 12 genes, five encoding the structural subunits (*hoxEFUYH*) and seven the maturation factors (*hypA$_1$B$_1$CDEF*, *hoxW*), was constructed to express the *Synechocystis* sp. PCC 6803 bidirectional hydrogenase in *E. coli* (Wells et al. 2011). The 11.7 kb sequence was codon optimized for the host and placed under the control of a T7 promoter, originating the pSynHox construct. The authors clearly demonstrated the production of hydrogen in an *E. coli* strain without the native hydrogenases, and it was also shown that the hydrogen output could be increased when formate production was abolished, reinforcing the hypothesis that hydrogen production is coupled with the NADH/NADPH pools. However, the relative low levels of the recombinant enzyme compared to the native *Synechocystis* sp. PCC 6803 host indicate that there is still room for improvement, e.g. coupling the hydrogenase with electron donors. Concerning the maturation machinery, the authors concluded that to express the bidirectional hydrogenase of *Synechocystis* sp. PCC 6803 in *E. coli*, only *hypA1* and *hoxW* are essential since the host can complement the deletion of all the other maturation factors.

b. Oxygen Consuming Devices

The photobiological production of H_2 is severely compromised by the presence of O_2 due to the sensitivity of the H_2-evolving enzymes: hydrogenases and nitrogenases (Tamagnini et al. 2007; Bothe et al. 2010). As oxygenic phototrophs, cyanobacteria have developed spatial- or time-based strategies to separate the O_2-evolving from the O_2-sensitive processes. In parallel with the search for O_2-tolerant enzymes, the development of synthetic Oxygen Consuming Devices (OCDs) capable of reducing intracellular oxygen is highly desirable. The DESHARKY bioinformatic tool (Rodrigo et al. 2008), was used to identify several proteins, native and heterologous to *Synechocystis* sp. PCC 6803, that were subsequently selected to produce OCDs. These OCDs can be introduced into a chassis together with efficient hydrogenases/hydrogen producing devices to improved H_2 production or used for other industrial applications that require low intracellular O_2 pressures.

IV. Conclusions and Future Perspectives

The use of photosynthetic organisms, such as algae and cyanobacteria, is a valuable option for H_2/biofuels production. However, several issues should be addressed to achieve economical relevant results. Some are considered in this chapter, but others like solar energy conversion efficiencies (see for e.g. Masukawa et al. 2012) and the development of adequate bioreactors are equally important. For cyanobacterial H_2 production both native and heterologous enzymes have been used. Nitrogenases have a high energy demand and hydrogen is only produced as a by-product of nitrogen fixation. Thus, the search or engineering of nitrogenases that preferably will function as “hydrogenases” is a possibility. Regarding approaches using hydrogenases, Fe enzymes are generally more active and have less complex maturation systems than the NiFe hydrogenases, consequently they have been preferably used for heterologous expression. A better understanding of the regulation and maturation processes of the NiFe hydrogenases, notably of the small subunits is also necessary. To different extents, all H_2 evolving enzymes are quite sensitive to oxygen and the search/engineering of oxygen tolerant enzymes is being actively pursued. In the Synthetic Biology field, cyanobacteria emerge as

prominent candidates to be used as photoautotrophic chassis for the accommodation of highly efficient hydrogenases/hydrogen producing devices. Protein fusions (e.g. hydrogenase and ferredoxin), the use of linkers and scaffolds would probably improve H_2 production. Nevertheless, efforts should be made to circumvent the impairment of H_2 production by oxygen. Development of synthetic oxygen consuming devices coupled with O_2 sensors, or compartmentalization of the process (in certain cells/cell types) are two of the possibilities. Interestingly, heterocystous cyanobacteria have microaerobic compartments – the heterocysts – where oxygen sensitive processes like N_2 fixation can occur. Moreover, the generation of genome scale models will help to re-direct the metabolism towards an efficient H_2 production.

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