

# Chapter 2

## Hydrogen Production by Cyanobacteria

Patrick C. Hallenbeck

**Keywords** Cyanobacteria • Heterocyst • Hydrogenases • Nitrogenase • Hydrogen production • Light conversion • Efficiency • Biophotolysis

### 2.1 Introduction

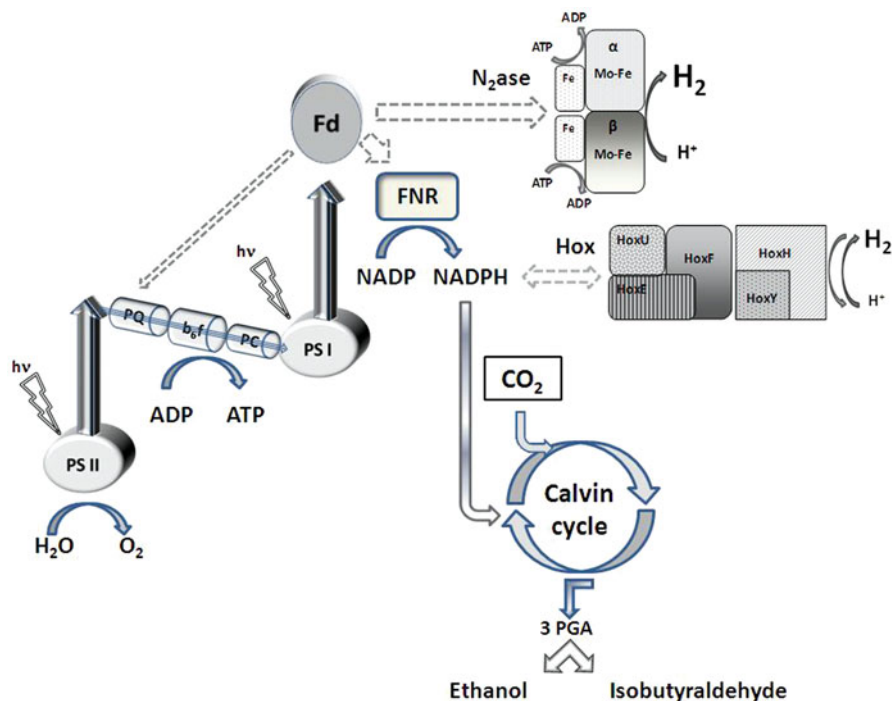
The cyanobacteria, formerly known as blue-green algae, are a diverse group of prokaryotes capable of carrying out oxygenic photosynthesis. They are a monophyletic group of Gram-negative bacteria consisting of both freshwater and marine species and grow and prosper in a wide variety of habitats; oceans, rivers and lakes, deserts, and Antarctic ice as well as forming a number of symbioses with different plants and fungi. Cyanobacteria can be either unicellular or filamentous (heterocystous and nonheterocystous), and are present as cells of varying sizes and a broad spectrum of morphologies. Diversity is also seen in the sizes of their genomes, with sequenced genomes ranging from 1.66 (*Prochlorococcus marinus*) to 9.2 Mbp (*Nostoc punctiforme*). These organisms continue to serve as models for the study of basic biological process, in particular photosynthesis and, since some filamentous species can differentiate various cell types, heterocysts, akinetes and hormogonia, cellular development, and intracellular communication. Nutritional requirements are minimal and are easily met by simple salt solutions, which might be provided by the dilution of various waste streams.

Many possible biotechnological processes using cyanobacteria have been proposed. They have been shown to produce a wide variety of bioactive compounds of possible pharmaceutical interest and may have some applications in wastewater treatment

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P.C. Hallenbeck (✉)

Département de microbiologie et immunologie, Université de Montréal,  
CP 6128, Succursale Centre-ville, Montréal, Québec H3C 3J7, Canada  
e-mail: patrick.hallenbeck@umontreal.ca



**Fig.2.1** Pathways for possible biofuels production by cyanobacteria. Shown is the typical Z scheme of photosynthesis operative in plants, algae, and cyanobacteria. Captured solar energy (photons) is used to split water, liberating oxygen, and to reduce ferredoxin. Electron passage through the membrane-bound electron transport chain (plastoquinone, cytochromes  $b_6$  and  $f$  and plastocyanin) that connects the two photosystems drives proton translocation and subsequent ATP synthesis. Reduced ferredoxin can participate in a number of metabolic reactions. It can reduce plastoquinone, driving cyclic photophosphorylation through photosystem I (PSI), thus generating additional ATP. Reduced ferredoxin can directly donate electrons to the nitrogenase system which can catalyze the ATP-dependent production of hydrogen ( $4ATP/H_2$ ). Additionally, reduced ferredoxin can reduce NADP to NADPH through the action of FNR. NADPH can drive hydrogen production by the bidirectional (reversible) Hox system or can drive  $CO_2$  fixation by the Calvin cycle. Pathways can be introduced to produce various biofuels or biofuel precursors from the metabolic intermediates, mainly 3-phosphoglycerate, produced by the Calvin cycle

(Abed et al. 2009; Rastogi and Sinha 2009). However, the only economically viable products currently being made from cyanobacteria are either the organisms themselves, grown commercially on relatively large scale in open ponds for use as nutritional supplements (Cyanotech, Earthrise), or, on a smaller scale, pigments are extracted and used in cell labeling for fluorescence-activated cell sorting (FACS). Thus, not only are cyanobacteria of potential interest in various biotechnological applications, this also suggests that useful by-products might be obtained from the biomass produced during their use in biofuels production.

Cyanobacteria are appealing since they are capable of the direct capture of solar energy and its conversion to useful chemical energy using water as a substrate (Fig. 2.1). This capacity could be used in a number of ways in biofuels production.

**Table 2.1** Comparison of hydrogen production by direct and indirect biophotolysis

Process	Possible advantages	Likely disadvantages
Direct biophotolysis	Direct conversion of solar energy to fuel, maximum efficiency	Large hydrogen-impermeable photobioreactors required
	Single-stage process, simpler facility, ease of operation	Possible generation of explosive hydrogen/oxygen mixtures
	Uses the existing metabolic machinery	Oxygen evolved in vicinity of oxygen-sensitive hydrogenase
Indirect biophotolysis	Separation of incompatible oxygen and hydrogen-evolving reactions	Possible energy loss in pumping between stages
	Possible reduced photobioreactor requirement for H <sub>2</sub> -producing stage	Energy loss in production and reuse of stored energy carrier

For one thing, their capacity to drive carbon dioxide fixation with photosynthetically derived energy, ATP and reductant, suggests that the newly recycled carbon could be converted to useful biofuels through the introduction of novel (to cyanobacteria) metabolic pathways (Angermayr et al. 2009). One cyanobacterial-derived fuel that is under active investigation and commercial development (Algenol Biofuels) is ethanol (Dexter and Fu 2009). Recently, high production rates of isobutyraldehyde, precursor of isobutanol among other things, have been demonstrated with metabolically engineered *Synechococcus elongatus* (Atsumi et al. 2009). As well, *Synechocystis* has been engineered to divert newly fixed carbon into isoprene synthesis (Lindberg et al. 2010). However, cyanobacteria have been mainly investigated for their capacity to convert captured solar energy to hydrogen. In this process, light energy captured by the two photosystems acting in concert is used to split water, producing oxygen, and generate a high-energy, low-potential reductant capable of reducing protons to hydrogen via a hydrogenase enzyme, a process that has been called biophotolysis. This is an inherently appealing and conceptually simple approach in which an abundant substrate, water, and a ubiquitous energy source, solar energy, are used to produce a highly diffusible and energy-dense fuel, hydrogen. Note that a true biophotolytic process should produce a 2:1 ratio of hydrogen to oxygen without the need for carbon dioxide fixation and other intermediate metabolic reactions. Indeed, carbon fixation in this case is a side reaction that would reduce hydrogen yields by using reductant that otherwise could go to proton reduction.

Biophotolysis by cyanobacteria has been under active investigation for over 35 years (Benemann and Weare 1974; Weissman and Benemann 1977; Miyamoto et al. 1979a, b), and several systems have been considered, at least conceptually, including direct biophotolysis and indirect biophotolysis (Table 2.1) (Benemann et al. 1980; Hallenbeck and Benemann 2002). In direct biophotolysis, the photosynthetically produced reductant, either ferredoxin or NADPH, directly reduces hydrogenase. Thus, in this process, hydrogen production is strictly light dependent. In indirect biophotolysis, water splitting photosynthesis and consequent ferredoxin

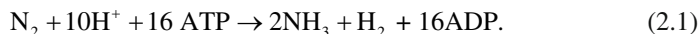
reduction are used to fix  $\text{CO}_2$  and the resulting reduced carbon compound can be used to drive hydrogen evolution in a separate reaction. Thus, hydrogen production can be separated in time and/or space from oxygen evolution potentially overcoming the problematic production of oxygen in the vicinity of a highly oxygen-sensitive hydrogenase.

## 2.2 Hydrogenase Enzymes in Cyanobacteria

Three different hydrogenase enzymes have been identified and studied in cyanobacteria; nitrogenase, a reversible bidirectional hydrogenase (Hox), and an uptake hydrogenase (Hup) (Fig. 2.2) (Ghirardi et al. 2007; Tamagnini et al. 2007).

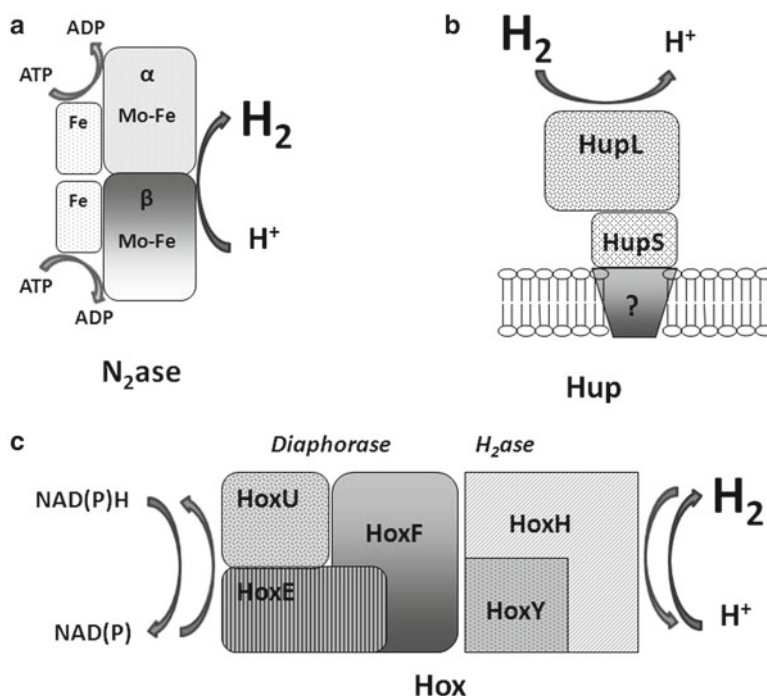
### 2.2.1 Nitrogenase

Nitrogenase (Fig. 2.2a) evolves hydrogen under two different conditions. First, while actively fixing  $\text{N}_2$ , 25% of the electron flux through the enzyme goes to proton reduction to hydrogen during an unavoidable side reaction (2.1).



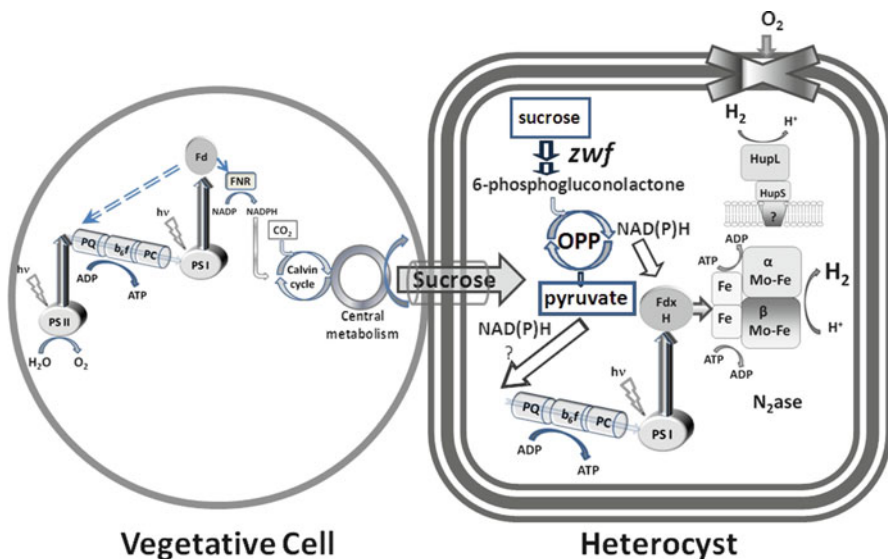
Actually, this is the case for the major nitrogenase, Mo–Fe hydrogenase, which has an active site formed by a complex iron–sulfur cluster containing molybdenum. Other forms of nitrogenase are known with slightly different active sites, a vanadium nitrogenase and an iron-only nitrogenase, that at first glance seem more suitable for hydrogen production since a larger percentage of the electron flux is devoted to hydrogen production during nitrogen fixation. However, total electron flux through these alternative nitrogenases is much lower making their application to hydrogen production a dubious proposition. In the absence of  $\text{N}_2$ , nitrogenase turnover continues unabated with total electron flux going to proton reduction to  $\text{H}_2$ ; thus hydrogen production under these conditions is much higher. The absence of nitrogen fixation also restricts growth, not a bad thing if one is interested in making a biofuel and not biomass. This strategy was applied early on (Benemann and Weare 1974) and is discussed in more detail below. There are a variety of diazotrophic (nitrogen fixing) cyanobacteria, including unicellular, filamentous, and filamentous heterocyst-containing types, and the physiology of nitrogen fixation differs somewhat among them.

The basic incompatibility of possessing an active oxygen-sensitive nitrogenase while carrying out oxygenic photosynthesis is combated by a number of different strategies. Unicellular cyanobacteria typically separate these incompatible metabolic reactions in time with active photosynthesis occurring during the daytime and



**Fig.2.2** Cyanobacterial enzymes participating in hydrogen metabolism. Cyanobacteria, depending upon the species, can contain up to three different enzymes capable of participating in hydrogen metabolism. **(a)** Nitrogenase produces hydrogen as a side reaction while fixing N<sub>2</sub> or, in the absence of other reducible substrates, continues to turn over at the same rate reducing protons to H<sub>2</sub>. Two specific protein components are required: Mo-Fe protein, a  $\alpha_2\beta_2$  complex which contains two highly oxygen-sensitive complex metallic clusters, the P cluster and Fe-MoCo, where substrate reduction occurs, and the Fe protein, a specific reductase for Mo-Fe protein. Note that hydrogen production requires ATP hydrolysis and that nitrogenase is only synthesized in response to fixed nitrogen deprivation. **(b)** The Hup hydrogenase is a membrane-associated nickel iron hydrogenase that is poised to carry out hydrogen oxidation. The exact site of electron donation to the membrane-bound respiratory electron transport chain is unknown. It is found in organisms that have nitrogenase and is coregulated with nitrogenase. It is thought to recover some of the energy lost through hydrogen evolution during nitrogen fixation. **(c)** The Hox system is also a nickel-iron hydrogenase, but is soluble or only very loosely membrane associated, and has a diaphorase module (Hox EFU) that interacts with pyridine dinucleotides (NAD, NADPH). It is reversible (bidirectional) and can catalyze proton reduction or hydrogen oxidation depending upon the state of cellular metabolism. It is responsible for hydrogen evolution during dark fermentation as well as a burst of hydrogen seen upon reillumination of dark-adapted cultures

with maximal nitrogenase expression during the night time. Often, there is a burst of activity immediately upon reillumination. On the other hand, heterocystous species are able to simultaneously carry out oxygenic photosynthesis and nitrogen fixation since they are composed of two different cell types: vegetative cells, where normal photosynthesis (oxygen evolution) and carbon fixation occur, and heterocysts,



**Fig. 2.3** Metabolic interactions during biophotolysis by heterocystous cyanobacterial cultures. Nitrogen deprivation causes some filamentous cyanobacteria to differentiate a specialized cell type, the heterocyst. The heterocyst has many morphological and metabolic differences with the normal vegetative cell enabling it to provide an anaerobic environment permitting nitrogenase to function in a milieu that is otherwise often supersaturated with oxygen. These modifications include the development of a specialized cell envelope that impedes the inward diffusion of oxygen and an active respiratory chain that quickly consumes any oxygen that does enter. Moreover, photosystem II (PSII) is absent; thus, there is no oxygen evolved from photosynthesis within the heterocyst. Since the key enzymes of the Calvin cycle (including Rubisco) are absent, reduced carbon compounds to support nitrogen fixation or hydrogen production must be imported from the neighboring vegetative cells which carry out oxygenic photosynthesis and  $\text{CO}_2$  fixation. Disaccharides, quite possibly sucrose, are imported through special channels connecting the two cell types. Genetic studies have shown that the oxidative pentose pathway (OPP), in particular glucose-6-phosphate dehydrogenase (*zwf*), is absolutely required for reductant generation and consequent reduction of *fdxH* which, in concert with ATP formed by cyclic photophosphorylation catalyzed by photosystem I (PSI), drives hydrogen production by nitrogenase

which lack PSII (no oxygen evolution) and have a series of adaptations that permit the establishment of an anaerobic environment in an aerobic milieu and thus allow nitrogenase activity (Fig. 2.3).

### 2.2.2 Reversible Hydrogenase

Many cyanobacteria possess another enzyme capable of hydrogen evolution, a soluble, or loosely membrane-associated, Ni-Fe hydrogenase, Hox, that is reversible or bidirectional (Fig. 2.2c) (Schwarz et al. 2010). This enzyme can apparently, depending upon metabolic conditions, either evolve hydrogen or oxidize

(consume) it. The presence of this enzyme was noticed over 30 years ago (Hallenbeck and Benemann 1978) and it was shown to be capable of producing hydrogen in the dark (Hallenbeck et al. 1981). The cyanobacterial Hox is a heteropentameric enzyme consisting of a hydrogenase module, HoxHY, and a diaphorase module, HoxEFU, carrying out the transfer of electrons between the hydrogenase module and NAD(P)H. Much has been made about the similarity of HoxEFU with the Complex I subunits NuoE, NuoF, and NuoG (NADH-dehydrogenase type I), especially since cyanobacteria appear to lack other Complex I homologs (Appel and Schulz 1986). However, *hox* minus strains have normal Complex I activity and these subunits instead play critical roles in dark hydrogen evolution driven either by endogenous reserves, such as polyhydroxybutyrate or glycogen, or exogenous-reduced carbon compounds (Hallenbeck et al. 1981; Troshina et al. 2002; Gutthann et al. 2007). Hydrogenase activity can also potentially serve to correctly poise the photosynthetic apparatus for action upon illumination. Indeed, dark-adapted cells often show a transient burst of hydrogen upon reillumination (Appel et al. 2000; Schwarz et al. 2010).

### 2.2.3 *Hup*, an “Uptake” Hydrogenase

Many cyanobacteria contain an [NiFe] hydrogenase, encoded by *hupSL*, that is poised to act in hydrogen oxidation, i.e., hydrogen “uptake.” Unlike some other organisms where this class of enzyme can serve a variety of functions, in cyanobacteria the major metabolic function of Hup appears to be to recycle hydrogen that is evolved as a side reaction during nitrogen fixation since it is only found in nitrogen-fixing species and its transcription is coupled to the nitrogen fixation process. Thus, in *Anabaena variabilis* for example, *hupSL* is transcribed under conditions of nitrogen depletion through the action of the cyanobacterial global nitrogen regulator NtcA (Weyman et al. 2008). In some cases, maximum induction of *hupSL* expression has been reported to occur in the presence of hydrogen (Axelsson and Lindblad 2002). The situation is further complicated in some species by the need for excision of an intervening sequence, a process that is tied to heterocyst development (Carrasco et al. 2005).

## 2.3 Hydrogen Production

### 2.3.1 $H_2$ Production by Heterocystous Cyanobacteria

The majority of the hydrogen evolution observed with heterocystous cyanobacteria is due to nitrogenase, which, as discussed above, continues to turn over in the absence of other reducible substrates, reducing protons to hydrogen in a relatively



slow reaction ( $6.4 \text{ s}^{-1}$ ) which also requires substantial energy input (2 ATP/e<sup>-</sup>; 4 ATP/H<sub>2</sub>). Purification and characterization of the cyanobacterial nitrogenase have shown that its properties are essentially the same as those from heterotrophic organisms (Hallenbeck et al. 1979). Some groups of cyanobacteria grow in filaments and are able, under conditions of nitrogen limitation, to differentiate specialized cells called heterocysts (Kumar et al. 2010; Mariscal et al. 2010). In effect, heterocysts are microbial nanofactories which provide a quasi-anaerobic microenvironment which allows the oxygen-sensitive nitrogenase to function and evolve hydrogen in what is otherwise an environment supersaturated with oxygen. Nitrogenase expression is restricted to the heterocyst under normal aerobic conditions (Murry et al. 1984). Several mechanisms combine to protect nitrogenase from oxygen damage. Heterocysts do not express an active photosystem II, so do not produce oxygen through water splitting. Gas diffusion into the heterocyst is impeded by a unique cell wall structure. In addition, heterocysts possess a very active respiratory system which, being membrane bound, can consume trace amounts of entering oxygen before it reaches nitrogenase in the cytoplasm. Nevertheless, some continual synthesis of nitrogenase is necessary to replace oxygen-damaged nitrogenase (Murry et al. 1983).

Solar energy capture and subsequent hydrogen evolution by these organisms can be demonstrated by a simple co-opting of this elaborate machinery developed early on soon after oxygenation of the earth's atmosphere. Of course, some hydrogen is naturally evolved during active nitrogen fixation since one H<sub>2</sub> is produced during the reduction of N<sub>2</sub> to NH<sub>4</sub><sup>+</sup>, and some of this is captured by the so-called uptake hydrogenase and recycled. However, nitrogenase turnover continues in the absence of exogenous substrates, reducing protons to hydrogen gas and hence hydrogen evolution is much more significant under an argon atmosphere. Depending upon culture conditions, very little of this hydrogen is recaptured or alternatively mutations in the uptake hydrogenase can be introduced.

However, the Calvin–Bassham cycle for carbon dioxide fixation is also absent in the heterocysts. Thus, heterocyst metabolism and, therefore, some of the energy required to drive hydrogen production by nitrogenase depend upon the import of fixed carbon from the neighboring vegetative cells through specialized interconnecting pore structures (Mariscal et al. 2010). The imported sugar, recently shown to be sucrose (Lopez-Igual et al. 2010), is metabolized in the heterocyst through the oxidative pentose pathway (Summers et al. 1995). Since hydrogen production in the heterocysts depends upon reductant produced by water-splitting photosynthesis in the adjoining vegetative cells, this is in fact indirect biophotolysis on a microscopic scale. As a consequence, the possible maximal theoretical conversion efficiencies are reduced. Nevertheless, this system is of interest due to its inherent robustness and has been studied for over three and a half decades (Benemann and Weare 1974). Early on, very reasonable conversion efficiencies, capable of being sustained for days to weeks, were achieved using nitrogen-limited cultures. In laboratory studies, where higher efficiencies can be expected, conversion of total incident light energy to free energy of hydrogen produced was shown to be 0.4% (Weissman and Benemann 1977), and, by the same measure, cultures incubated under natural



sunlight were shown to convert 0.1% of incident light to hydrogen (Miyamoto et al. 1979a, b). However, although there have been a number of studies carried out in the years since then, very little improvement in yields have been noted. For example, much more recent reports of conversion efficiencies under laboratory conditions found  $\approx 0.7\%$  (total incident) (Yoon et al. 2006; Sakurai and Masukawa 2007; Berberoglu et al. 2008) and under natural sunlight 0.03–0.1% (Sakurai and Masukawa 2007; Tsygankov et al. 2002). Thermophilic strains exist, allowing for hydrogen production in photobioreactors that would exhibit lower cooling requirements; yet these of course show the same low efficiencies (Miyamoto et al. 1979c, d). Theoretical efficiencies with this nitrogenase-based system are around 4.6%, so there is some room for improvement.

Theoretical efficiencies can be calculated on the basis of the number of photons required to produce hydrogen. Photon energy must be used to derive the two necessary substrates, high-energy electrons, and ATP needed for nitrogenase to reduce protons to hydrogen. The calculation must also account for the source of electrons since heterocysts do not derive them directly from water splitting. Instead, the degradation of imported sugars by the oxidative pentose pathway generates up to 12 NADPH ( $24e^-$ ) per hexose. Each sugar molecule was synthesized in the vegetative cell at a cost of 48 photons/hexose or 2 photons per  $e^-$  used in hydrogen production. Absorption of a photon by PSI raises the energy sufficiently to reduce ferredoxin and drive nitrogenase. Thus, the photon requirement for the necessary electrons for  $H_2$  production (2) is 6. However, the additional requirement of nitrogenase for ATP, 4 ATP/ $H_2$  (2 ATP/ $2e^-$ ), effectively doubles the photon requirement, thus halving the efficiency. One of the required ATP comes at no additional energetic cost since it is produced during the noncyclic functioning of PSI required for the two electrons, since the passage of each electron causes the Q cycle to pump  $2H^+$  and approximately  $4H^+$  are needed per ATP. Six additional photons are required to produce the three additional ATPs needed, since six rounds of cyclic photosynthesis by PSI are necessary to make the number of necessary protons 12. Hence, the minimum photon requirement is 12 photons per  $H_2$ , which translates to an efficiency of 4.6%.

Thus, since observed conversion efficiencies are lower than predicted, it is worthwhile considering what steps might be taken to improve the overall performance. This is especially important since light conversion efficiencies directly dictate photobioreactor footprint, so doubling efficiency, for example, halves the required surface area for the same amount of fuel production. A number of avenues might be explored. For example, part of the reduction in efficiency for microbial-based photosynthetic systems is typically thought to be due to inefficient use of total light energy at high light intensities. This is because the light-harvesting apparatus, consisting of reaction centers surrounded by antenna pigments, is optimized for effective light capture at the low light conditions normally experienced by these organisms. This means that at high light intensities more photons are captured than can be productively processed and consequently, the excess energy is wasted via fluorescence, thermal decay, etc. Therefore, reducing the size of the photosynthetic antennae through genetic modifications should make for more

efficient use of high light intensities by the culture as a whole. Another leverage point is the hydrogen-producing catalyst. As pointed out above, half of the photon requirement goes to providing the ATP required for nitrogenase action, thus substituting hydrogenase, which does not require ATP for proton reduction, for nitrogenase should have an energy-sparing effect. Another possible way to increase yields would be to increase heterocyst frequencies, although this may not have the desired effect since even in fairly long-term studies with these organisms the  $H_2/O_2$  ratio is close to the desired stoichiometry of two, suggesting nearly optimal coupling between oxygen-generating photosynthesis in the vegetative cells and hydrogen production by heterocysts.

### 2.3.2 $H_2$ Production by Nonheterocystous Cyanobacteria

Although the heterocyst/nitrogenase-based system is certainly the best studied, there are a number of other known cyanobacterial hydrogen-producing reactions that could potentially be exploited in practical large-scale hydrogen production. For example, some unicellular (nonheterocystous) cyanobacteria possess nitrogenase and in nature are able to fix nitrogen without the oxygen protection afforded by the heterocyst. However, this is usually possible since photosynthesis and nitrogen fixation are under circadian control in these organisms assuring that active transcription and activity of the photosynthetic apparatus take place during the daytime and conversely, oxygen inhibition of nitrogenase is avoided since its transcription and activity are maximal during the dark (night period). Nonetheless, light-driven hydrogen production due to the action of nitrogenase has recently been demonstrated in the unicellular cyanobacterium, *Cyanothece* (Min and Sherman 2010), although this was carried out under especially favorable conditions: low light intensities, presence of glycerol, argon sparging to remove evolved oxygen, unlikely to be applicable to a practical system. In addition, many species are capable of dark hydrogen evolution catalyzed by either nitrogenase or a hydrogenase (Kumazawa 2004; Troshina et al. 2002; Prabakaran et al. 2010). Many cyanobacteria also possess a soluble NADH-linked [NiFe] hydrogenase that is capable of hydrogen evolution, especially when cells are newly illuminated after a dark period (Schwarz et al. 2010). This is thought to act as an electron valve, returning the cell to the proper redox status as photosynthesis resumes in the light. However, this activity is short-lived as it is quickly inhibited with the resumption of oxygen evolution.

Indirect biophotolysis is another strategy that could possibly be used for hydrogen production by cyanobacteria. In this process, oxygen-evolving photosynthesis is used in one stage to fix carbon and store, thus producing reduced carbon compounds that can later be used in a second, anaerobic, hydrogen-producing stage. Thus, the oxygen-sensitive proton reduction reaction is separated in time and/or space from oxygen-producing photosynthesis. While indirect biophotolysis

has long been discussed, it was only recently demonstrated on an experimental level, where the nonheterocystous *Plectonema boryanum* was cycled multiple times through an aerobic, nitrogen-limited stage, during which glycogen was accumulated, followed by a second anaerobic, hydrogen-producing stage (Huesemann et al. 2010). In this case, hydrogen evolution is catalyzed by nitrogenase, whose activity is permitted by the anaerobic conditions of the second stage. However, as noted above, many cyanobacteria contain a reversible NAD-linked [NiFe] hydrogenase and thus it might be possible to develop a two-stage indirect biophotolysis system using this enzyme in either an anaerobic, light-driven second stage or in a dark fermentative process. Indeed, a suitably modified *Synechococcus*, a unicellular cyanobacterium, has been shown to convert biomass accumulated in a first-stage photosynthetic stage to hydrogen in a second-stage dark fermentation, where an [NiFe] hydrogenase is active with a 12% efficiency (i.e., 1.44 moles  $H_2$ /mole hexose) (McNeely et al. 2010). Obviously, to be practical, this yield would have to be greatly increased, not an easy prospect given the present metabolic limitations to dark hydrogen fermentations, which restrict yields to at most 4 moles  $H_2$ /mole hexose (see the chapter on dark fermentative hydrogen production).

## 2.4 Conclusion and the Way Forward

Thus, cyanobacteria have been shown to possess multiple hydrogen-producing enzymes and are capable of both dark- and light-driven hydrogen production in a variety of configurations. However, as noted above, there are serious limitations at present to developing any of these on a practical level. Although some further improvements might be possible by discovery of new species or manipulation of culture conditions (Ananyev et al. 2008; Burrows et al. 2009; Carrieri et al. 2008; Chen et al. 2008), these would not be game changers as any increases would likely be incremental. Development of low-cost photobioreactors suitable for the task (i.e., transparent, durable, hydrogen impermeable, etc.) requires advances in materials sciences. On the biological side, real improvements are probably realized only through a thorough understanding of the systems biology involved (Navarro et al. 2009) and the application of genetic engineering. Although some effort has gone into adapting cyanobacteria for some liquid biofuels (Angermayr et al. 2009; Atsumi et al. 2009; Lindberg et al. 2010; Lu 2010), relatively little has been done until recently to attempt to improve cyanobacterial hydrogen production by metabolic engineering. Very recently, the successful expression of an [FeFe] hydrogenase in a cyanobacterium was reported (Ducat et al. 2011) and a “Biobrick” strategy widely applicable to cyanobacteria was laid out (Huang et al. 2010). The near future may bring some real attempts to advance one or more of the cyanobacterial systems to the next level with some real gains in conversion efficiencies.

## References

- Abed RMM, Dobretsov S, Sudesh K (2009) Applications of cyanobacteria in biotechnology. *J Appl Microbiol* 106:1–12
- Ananyev G, Carrieri D, Dismukes GC (2008) Optimization of metabolic capacity and flux through environmental cues to maximize hydrogen production by the cyanobacterium “*Arthrospira* (*Spirulina*) *maxima*”. *Appl Environ Microbiol* 74:6102–6113
- Angermayr SA, Hellingwerf KJ, Lindblad P, de Mattos MJT (2009) Energy biotechnology with cyanobacteria. *Curr Opin Biotechnol* 20:257–263
- Appel J, Schulz R (1986) Sequence analysis of an operon of a NAD(P)-reducing nickel hydrogenase from the cyanobacterium *Synechocystis* sp. PCC 6803 gives additional evidence for direct coupling of the enzyme to NAD(P)H-dehydrogenase (complex I). *Biochim Biophys Acta* 1298:141–147
- Appel J, Phunpruch S, Steinmüller K, Schulz R (2000) The bidirectional hydrogenase of *Synechocystis* sp. PCC 6803 works as an electron valve during photosynthesis. *Arch Microbiol* 173:333–338
- Atsumi S, Higashide W, Liao JC (2009) Direct photosynthetic recycling of carbon dioxide to isobutyraldehyde. *Nat Biotechnol* 27:1177–1182
- Axelsson R, Lindblad P (2002) Transcriptional regulation of nostoc hydrogenases: effects of oxygen, hydrogen, and nickel. *Appl Environ Microbiol* 68:444–447. doi:10.1128/aem.68.1.444-447.2002
- Benemann JR, Miyamoto K, Hallenbeck PC (1980) Bioengineering aspects of biophotolysis. *Enzyme Microb Technol* 2:103–111
- Benemann JR, Weare NM (1974) Hydrogen evolution by nitrogen-fixing *Anabaena cylindrica* cultures. *Science* 184:1917–1918
- Berberoglu H, Jay J, Pilon L (2008) Effect of nutrient media on photobiological hydrogen production by *Anabaena variabilis* ATCC 29413. *International Journal of Hydrogen Energy* 33:1172–1184
- Burrows EH, Wong WK, Fern X, Chaplen FWR, Ely RL (2009) Optimization of pH and nitrogen for enhanced hydrogen production by *Synechocystis* sp. PCC 6803 via statistical and machine learning methods. *Biotechnol Prog* 25:1009–1017
- Carrasco CD, Holliday SD, Hansel A, Lindblad P, Golden JW (2005) Heterocyst-specific excision of the *Anabaena* sp. strain PCC 7120 hupL element requires xisC. *J Bacteriol* 187:6031–6038
- Carrieri D, Ananyev G, Costas AMG, Bryant DA, Dismukes GC (2008) Renewable hydrogen production by cyanobacteria: nickel requirements for optimal hydrogenase activity. *Int J Hydrogen Energy* 33:2014–2022
- Chen PC, Fan SH, Chiang CL, Lee CM (2008) Effect of growth conditions on the hydrogen production with cyanobacterium *Anabaena* sp. strain CH3. *Int J Hydrogen Energy* 33:1460–1464
- Dexter J, Fu P (2009) Metabolic engineering of cyanobacteria for ethanol production. *Energy Environ Sci* 2:857–864
- Ducat DC, Sachdeva G, Silver PA (2011) Rewiring hydrogenase-dependent redox circuits in cyanobacteria. *Proc Natl Acad Sci USA* 108(10):3941–3946
- Ghirardi ML, Posewitz MC, Maness PC, Dubini A, Yu JP, Seibert M (2007) Hydrogenases and hydrogen photoproduction in oxygenic photosynthetic organisms. *Annu Rev Plant Biol* 58:71–91
- Gutthann F, Egert M, Marques A et al (2007) Inhibition of respiration and nitrate assimilation enhances photohydrogen evolution under low oxygen concentrations in *Synechocystis* sp. PCC 6803. *Biochim Biophys Acta* 1767:161–169
- Hallenbeck PC, Benemann JR (1978) Characterization and partial purification of the reversible hydrogenase of *Anabaena cylindrica*. *FEBS Lett* 94:261–264
- Hallenbeck PC, Benemann JR (2002) Biological hydrogen production: fundamentals and limiting processes. *Int J Hydrogen Energy* 27:1185–1193
- Hallenbeck PC, Kochian LV, Benemann JR (1981) Hydrogen evolution catalyzed by hydrogenase in cultures of cyanobacteria. *Z Naturforsch C* 36:87–92

- Hallenbeck PC, Kostel PJ, Benemann JR (1979) Purification and properties of nitrogenase from the cyanobacterium, *Anabaena cylindrica*. *Eur J Biochem* 98:275–284
- Huang H-H, Camsund D, Lindblad P, Heidorn T (2010) Design and characterization of molecular tools for a synthetic biology approach towards developing cyanobacterial biotechnology. *Nucleic Acids Res* 38:2577–2593
- Huesemann MH, Hausmann TS, Carter BM, Gerschler JJ, Benemann JR (2010) Hydrogen generation through indirect biophotolysis in batch cultures of the nonheterocystous nitrogen-fixing cyanobacterium *Plectonema boryanum*. *Appl Biochem Biotechnol* 162(1):208–220
- Kumar K, Mella-Herrera RA, Golden JW (2010) Cyanobacterial Heterocysts. *Cold Spring Harb Perspect Biol* 2:a000315
- Kumazawa S (2004) Hydrogen production capability in unicellular cyanobacteria. *Plant Cell Physiol* 45:S23
- Lindberg P, Park S, Melis A (2010) Engineering a platform for photosynthetic isoprene production in cyanobacteria, using *Synechocystis* as the model organism. *Metabolic Engineering* 12, 70–79
- Lopez-Igual R, Flores E, Herrero A (2010) Inactivation of a Heterocyst-Specific Invertase Indicates a Principal Role of Sucrose Catabolism in Heterocysts of *Anabaena* sp. *J. Bacteriol.* 192, 5526–5533
- Lu X (2010) A perspective: photosynthetic production of fatty acid-based biofuels in genetically engineered cyanobacteria. *Biotechnol Adv* 28:742–746
- Mariscal V, Flores E (2010) Multicellularity in a Heterocyst-Forming Cyanobacterium: Pathways for Intercellular Communication Recent advances in phototrophic prokaryotes, in: Hallenbeck PC (ed) Springer, New York, pp 123–135
- McNeely K, Xu Y, Bennette N, Bryant DA, Dismukes GC (2010) Redirecting reductant flux into hydrogen production via metabolic engineering of fermentative carbon metabolism in a cyanobacterium. *Appl Environ Microbiol* 76(15):5032–5038
- Min H, Sherman LA (2010) Hydrogen production by the unicellular, Diazotrophic cyanobacterium *cyanosphaera* sp. Strain ATCC 51142 under Conditions of Continuous Light. *Appl. Environ. Microbiol.* 76, 4293–4301
- Miyamoto K, Hallenbeck PC, Benemann JR (1979a) Effects of nitrogen supply on hydrogen production by cultures of *Anabaena cylindrica*. *Biotechnol Bioeng* 21:1855–1860
- Miyamoto K, Hallenbeck PC, Benemann JR (1979b) Solar-energy conversion by nitrogen-limited cultures of *Anabaena cylindrica*. *J Ferment Technol* 57:287–293
- Miyamoto K, Hallenbeck PC, Benemann JR (1979c) Hydrogen production by the thermophilic alga *mastigocladus-laminosus* – effects of nitrogen, temperature, and inhibition of photosynthesis. *Appl Environ Microbiol* 38:440–446
- Miyamoto K, Hallenbeck PC, Benemann JR (1979d) Nitrogen-fixation by thermophilic blue-green-algae (cyanobacteria) – temperature characteristics and potential use in biophotolysis. *Appl Environ Microbiol* 37:454–458
- Murry MA, Hallenbeck PC, Benemann JR (1984) Immunochemical evidence that nitrogenase is restricted to the heterocysts in *Anabaena cylindrica*. *Arch Microbiol* 137:194–199
- Murry MA, Hallenbeck PC, Esteva D, Benemann JR (1983) Nitrogenase inactivation by oxygen and enzyme turnover in *Anabaena cylindrica*. *Can J Microbiol* 29:1286–1294
- Navarro E, Montagud A, de Cordoba PF, Urchueguia JF (2009) Metabolic flux analysis of the hydrogen production potential in *Synechocystis* sp. PCC6803. *Int J Hydrogen Energy* 34: 8828–8838
- Prabaharan D, Arun Kumar D, Uma L, Subramanian G (2010) Dark hydrogen production in nitrogen atmosphere: an approach for sustainability by marine cyanobacterium *Leptolyngbya valderiana* BDU 2004. *Int J Hydrogen Energy* 35:10725–10730. doi:10.1016/j.ijhydene.2010.03.007
- Rastogi RP, Sinha RP (2009) Biotechnological and industrial significance of cyanobacterial secondary metabolites. *Biotechnol Adv* 27:521–539
- Sakurai H, Masukawa H (2007) Promoting R & D in photobiological hydrogen production utilizing mariculture-raised cyanobacteria. *Marine Biotechnology* 9:128–145

- Schwarz S, Poss Z, Hoffmann D, Appel J (2010) Hydrogenases and hydrogen metabolism in photosynthetic prokaryotes. In: Hallenbeck PC (ed) Recent advances in phototrophic prokaryotes. Springer, New York, pp 305–348
- Summers ML, Wallis JG, Campbell EL, Meeks JC (1995) Genetic evidence of a major role for glucose-6-phosphate-dehydrogenase in nitrogen-fixation and dark growth of the cyanobacterium *Nostoc* sp. strain ATCC-29133. *J Bacteriol* 177(21):6184–6194
- Tamagnini P et al (2007) Cyanobacterial hydrogenases: diversity, regulation and applications. *FEMS Microbiol Rev* 31:692–720. doi:10.1111/j.1574-6976.2007.00085.x
- Troshina O, Serebryakova L, Sheremetieva M, Lindblad P (2002) Production of H<sub>2</sub> by the unicellular cyanobacterium *Gloeocapsa alpicola* CALU 743 during fermentation. *Int J Hydrogen Energy* 27:1283–1289
- Tsygankov AA, Fedorov AS, Kosourov SN, Rao KK (2002) Hydrogen production by cyanobacteria in an automated outdoor photobioreactor under aerobic conditions. *Biotechnology and Bioengineering* 80:777–783
- Weissman JC, Benemann JR (1977) Hydrogen production by nitrogen-fixing cultures of *Anabaena cylindrica*. *Appl Environ Microbiol* 33:123–131
- Weyman PD, Pratte B, Thiel T (2008) Transcription of hupSL in *Anabaena variabilis* ATCC 29413 is regulated by NtcA and not by hydrogen. *Appl Environ Microbiol* 74:2103–2110. doi:10.1128/aem.02855-07
- Yoon JH, Shin JH, Kim MS, Sim SJ, Park TH (2006) Evaluation of conversion efficiency of light to hydrogen energy by *Anabaena variabilis*. *International Journal of Hydrogen Energy* 31:721–727



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