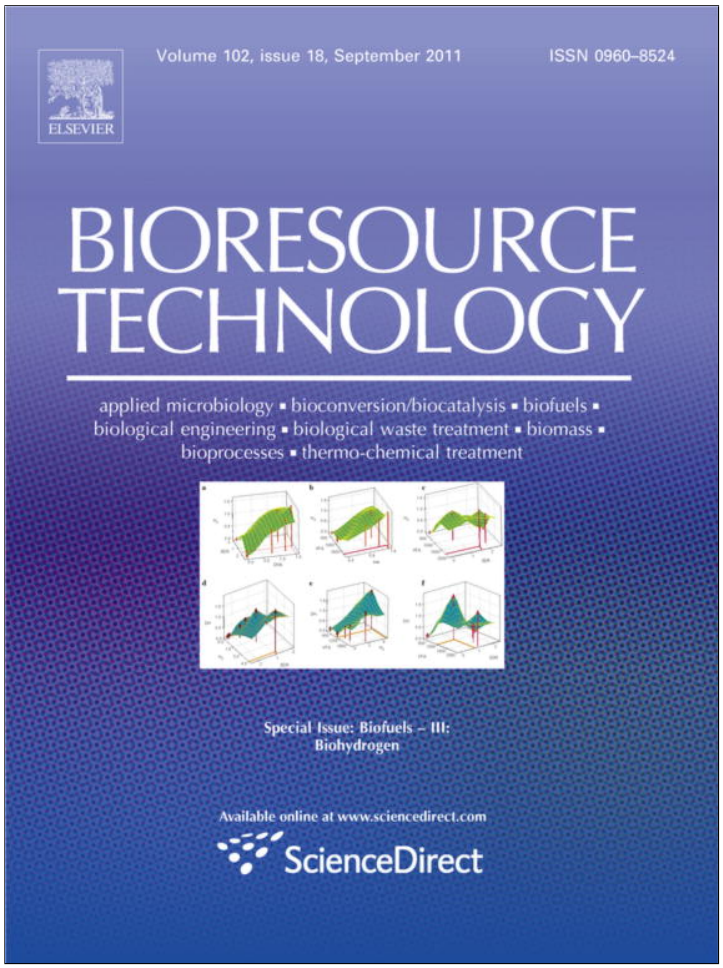

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Biochemical and genetic engineering strategies to enhance hydrogen production in photosynthetic algae and cyanobacteria

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ABSTRACT

As an energy carrier, hydrogen gas is a promising substitute to carbonaceous fuels owing to its superb conversion efficiency, non-polluting nature, and high energy content. At present, hydrogen is predominately synthesized via chemical reformation of fossil fuels. While various biological methods have been extensively explored, none of them is justified as economically feasible. A sustainable platform for biological production of hydrogen will certainly impact the biofuel market. Among a selection of biological systems, algae and cyanobacteria have garnered major interests as potential cell factories for hydrogen production. In conjunction with photosynthesis, these organisms utilize inexpensive inorganic substrates and solar energy for simultaneous biosynthesis and hydrogen evolution. However, the hydrogen yield associated with these organisms remains far too low to compete with the existing chemical systems. This article reviews recent advances of biochemical, bioprocess, and genetic engineering strategies in circumventing technological limitations to hopefully improve the applicative potential of these photosynthetic hydrogen production systems.

1. Introduction

While hydrogen represents the most abundant element in the universe, comprising approximately 75% of all matter by weight, molecular hydrogen (H_2) exists in only trace amounts within the Earth's atmosphere. As a gaseous and carbon-free fuel, hydrogen can be combusted with water as the sole oxidation product and therefore is considered a clean form of energy. It has a high energy content since, compared to traditional hydrocarbon fuels, roughly three times more energy, equivalent to 122 kJ/g, can be generated through the combustion of hydrogen (Balat, 2008). Currently, 96% of the global hydrogen supply is generated from non-renewable, chemical-based processes such as petroleum refining, coal gasification, and steam reforming of natural gas (Balat, 2008). However, the mounting environmental and socioeconomic concerns associated with the consumption of diminishing petroleum resources has led to the investigation of biological systems for the production of renewable hydrogen.

A wide range of fermentative and photosynthetic organisms have been explored as cell factories for hydrogen production. Within these biological systems, hydrogen production acts as a valve to maintain redox poise by relieving the cell of excess reducing equivalents. Biological hydrogen production processes include dark fermentation, photofermentation, and biophotolysis (Table 1). Dark fermentation, typically performed by chemoheterotrophic species of *Clostridium* and *Enterobacter*, involves the light-independent anaerobic fermentation of carbohydrates or other organic substrates. Through this process, protons accept electrons generated from substrate catabolism to form hydrogen. Hydrogen can also be produced fermentatively in a light-dependent process, termed photofermentation, by non-oxygenic photosynthetic bacteria, such as purple non-sulfur and green sulfur bacteria. Rather than carbohydrates, photofermentation involves the use of alternative reduced compounds, such as organic acids and hydrogen sulfide, as electron donors. Additionally, solar energy is required in order to photoheterotrophically convert the substrate compounds to CO_2 and hydrogen as the only fermentation products. Biophotolysis, a third mode of biohydrogen production, is unique to photoautotrophic organisms, with green algae and cyanobacteria exhibiting the greatest propensity for hydrogen production. Light is required in this process for the functioning of oxygenic photosynthesis, in which water, rather than carbohydrates or organic

Abbreviations: ATP, adenosine-5′-triphosphate; Chl, chlorophyll; Cyt b_6f complex, cytochrome b_6f complex; Fd, ferredoxin; FNR, ferredoxin-NAD(P)$^+$ reductase; LHC, light-harvesting complex; NAD(P)H, reduced nicotinamide adenine dinucleotide phosphate; NPQR, NAD(P)-plastoquinone oxidoreductase; PC, plastocyanin; PFR1, pyruvate ferredoxin oxidoreductase (PFR1); PQ, plastoquinone; PSI, photosystem I complex; PSII, photosystem II complex.

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Table 1
Different biological processes leading to hydrogen production.

Process	General reaction	Feedstock	Electron donor	Prevalent metabolism	Enzyme(s)	Organisms
Dark fermentation	$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 4H_2 + 2CO_2$	Organic compounds (carbohydrates)	Organic compounds	Chemoheterotrophic	[FeFe]-hydrogenase	*Enterobacter*
	$C_6H_{12}O_6 \rightarrow C_3H_7COOH + 2H_2 + 2CO_2$			Chemoheterotrophic	[FeFe]-hydrogenase	*Clostridium*
Photofermentation	$C_xH_yO_z + (2x - z)H_2O \xrightarrow{light} (y/2 + 2x - 2)H_2 + xCO_2$	Organic acids (PNS) S-compounds (GS)	Organic compounds	Photoheterotrophic	Catabolic enzymes	PNS and GS bacteria
	$N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16P_i$				Nitrogenase	PNS and GS bacteria
Biophotolysis (water splitting)	$2H_2O \xrightarrow{light} 2H_2 + O_2$	Inorganic CO_2	H_2O	Photoautotrophic	[FeFe]-hydrogenase	Green algae
					[NiFe]-hydrogenase	Cyanobacteria
Biophotolysis (N_2 fixation)[a]	$N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16P_i$	Inorganic CO_2	Organic compounds and H_2O	Photoautotrophic	Nitrogenase	Cyanobacteria

[a] Occurs under nitrogen-limiting conditions following accumulation of glycogen via photosynthesis.

acids, serves as the sole electron donor. Through the splitting of water, protons are generated and subsequently reduced to molecular hydrogen.

The need for a sustainable source of hydrogen has been delineated through the concept of a global 'hydrogen economy', in which many of the intrinsic problems resulting from the consumption of petroleum resources are alleviated or even eradicated. The advent of biomass-based technologies has led to a profound interest in the biological production of hydrogen accentuated by green and economic processes. The future success of an industrial biological process for hydrogen production is strictly dependent upon the ability to compete economically with current petrochemical methods. However, the paramount problem of feedstock cost, including transportation, handling, and pretreatment of raw materials, has blighted the large-scale production of many biofuels (Pfromm et al., 2010), with feedstock being the single greatest cost contributor, representing up to 95% of total production costs (Table 2). This salient limitation gravely jeopardizes the feasibility of large-scale hydrogen bioprocesses based upon dark fermentation and photofermentation, both of which entail consumption of costly carbonaceous feedstocks. As a result, biophotolysis, relying upon free solar energy and atmospheric CO_2 as the feedstock, is ostensibly identified as a feasible avenue due to a significant reduction in feedstock costs.

Nevertheless, biophotolysis might not still be able to afford a comprehensive solution to sustainable hydrogen production, at least in the near future, due to several existing technological limitations such as expensive photobioreactor systems and inherently inefficient photosynthesis and hydrogen evolution yields of biological systems. This review intends to conduct a technical evaluation on photosynthetic hydrogen production in green algae and cyanobacteria by specifically targeting biophotolysis to hopefully foresee its technological future. First, metabolic pathways and reaction mechanisms associated with hydrogen production in green algae and cyanobacteria are documented and compared. Second, potential biological and biochemical factors limiting hydrogen production in photosynthetic organisms are presented. Third, novel biochemical and genetic strategies recently developed to alleviate these technical limitations are reviewed. Readers are directed toward recent reviews of algal and cyanobacterial photobiological hydrogen production (Allakhverdiev et al., 2010; Ghirardi and Mohanty, 2010; McKinlay and Harwood, 2010).

2. Photosynthesis and hydrogen evolution

Photosynthesis, either oxygenic or anoxygenic, can be generalized as the conversion of light into chemical energy through a series of reduction–oxidation (redox) reactions. Light is first absorbed by pigment molecules associated with the photosystem protein complex, leading to the formation of electrons (out of electron donors) that are shuttled through an electron transport chain for generating ATP and NAD(P)H. These energy-rich intermediate compounds are then consumed to drive a selection of reductions. The most important one is known as carbon fixation through which CO_2 is reduced to synthesize glucose and its polymeric forms. Other examples include the reduction of nitrite ions or

Table 2
Contribution of the feedstock cost for the production of common biofuels.

Biofuel	Process	Feedstock	Percentage of total cost (%)	References
Biodiesel	Transesterification	Soybean oil	75–95	Tao and Aden (2009)
Butanol	Fermentation	Corn	56–79	Pfromm et al. (2010)
				Qureshi and Blaschek (2000)
Ethanol	Fermentation	Corn	60–85	Pfromm et al. (2010)
				Tao and Aden (2009)
	Fermentation	Lignocellulose	35.5	Piccolo and Bezzo (2009)
Hydrogen	Dark fermentation	Sugars	30–40	Wang et al. (2008)
	Pyrolysis	Agricultural biomass	36–62	Sarkar and Kumar (2010)
	Biophotolysis	Atmospheric CO_2	~0	Kruse et al. (2005)

molecular nitrogen to form ammonia (known as nitrogen fixation) and the reduction of sulfite ions to form hydrogen sulfide (Lawlor, 1993). Oxygenic photosynthesis, carried out by eukaryotic plants, algae, and cyanobacteria, involves the use of water as an electron donor, leading to the generation of molecular oxygen. Some ancient strains of cyanobacteria, however, can shift between oxygenic photosynthesis and anoxygenic bacterial-type photosynthesis (e.g. using hydrogen sulfide as an electron donor), suggesting an evolutionary continuum between aerobic phototrophic plants and algae and anaerobic phototrophic bacteria (Lawlor, 1993). This review focuses exclusively on oxygenic photosynthesis, which is specifically associated with hydrogen evolution in algae and cyanobacteria.

2.1. Oxygenic photosynthetic electron transport chain

Although the photosynthetic electron transfer and phosphorylation mechanisms are remarkably conserved between algae and cyanobacteria, the type, compartmentalization, and localization of the photosynthetic apparatus are rather different (Fig. 1a and b). The cyanophyta house the light harvesting complexes and accessory pigments within a proteinous structure called a phycobilisome. This feature is shared with the eukaryotic cryptomonods and rhodophyta in red algae, but is replaced by the Chl *b*-containing light harvesting protein complex II in green algae and higher plants. Furthermore, these light-harvesting complexes infuse the cytoplasm of cyanobacteria in varying arrangements, but are found within the chloroplast organelle in green algae and higher plants (Lee, 2008).

Oxygenic photosynthesis (Fig. 1c) begins with capture of photons by pigment molecules in the antenna complexes, resulting in the transfer of energy to the reaction center of PSII, P680. P680 is composed of dimeric Chl molecules that are brought to a state of excitation upon the energy transfer. The excited P680 (termed P680*) subsequently transfers electrons to its neighboring molecule called pheophytin. Pheophytin is also housed within the PSII complex and is structurally similar to Chl, but lacks the central magnesium ion. Following the electron transfer, the oxidized P680* is rapidly reduced to its neutral state using the electrons gained through the splitting of water. Water-splitting occurs as a result of charge separation orchestrated by four manganese atoms within the PSII complex. Protons released from this reaction and PQ are used to maintain the chemiosmotic potential to generate ATP via the ATP synthase complex. The electron from the reduced pheophytin is ferried in sequence through three electron acceptors, i.e. PQ, Cyt b_6f complex, and PC, to reduce a set of the oxidized PSI Chl molecules, termed P700. Upon the capture of light, the excited P700 (termed P700*) passes the electron through a series of intermediate stages within the PSI complex, i.e. A_0 (Chl *a*), A_1 (vitamin K), and FSA/FSB/FSX (collectively known as the iron–sulfur containing protein complexes), to reduce Fd, the central hub of electron flow in photosynthetic organisms. The reduced Fd can in turn donate electrons to FNR to reduce $NADP^+$ to NADPH, which, along with ATP, is consumed in the Benson–Calvin cycle to fix CO_2 to first form a monosugar of glucose, then starch reserves in eukaryotic plants and algae or glycogen stores in cyanobacteria. The reduced Fd also acts as a key branch point for shuttling electrons into several pathways other than CO_2 fixation, such as nitrite reduction, sulfite reduction, cyclic electron transfer around PSI, and, most importantly in this review, hydrogen evolution via hydrogenases (Lawlor, 1993).

2.2. Algal and cyanobacterial hydrogen production pathways

While the hydrogen production pathways are noticeably different in algae and cyanobacteria, both organisms share a fundamental commonality that hydrogen is a secondary metabolite produced to balance the organisms' redox energetics. Generally, in photosynthetic organisms, the hydrogen yield is appreciably higher when photosynthesis ceases and the stored sugar (or other carbohydrates) is catabolized. Under illuminated and anaerobic conditions, certain algal species also evolve hydrogen, but with a much lower yield, to facilitate a basal level of metabolism through the photosynthetic production of ATP. On the other hand, some cyanobacterial species evolve hydrogen as a byproduct of nitrogen fixation mediated by nitrogenase.

In green algae, there are two light-dependent pathways, and possibly one light-independent fermentative pathway for hydrogen evolution mediated by either [Fe]- or [FeFe]-hydrogenases, both of which are unidirectional. In all three algal pathways, the reduced Fd acts as a key station to supply electrons to the hydrogenase via the irreversible reaction: $2H^+ + 2e^- \rightarrow H_2$. Given that the first two pathways are light-dependent, the electron transport chain is used to shuttle electrons (gained through the oxidation of various compounds) for the reduction of Fd. In the first pathway, water is the source of electrons and is photosynthetically oxidized via the catalytic activity of PSII. In the second pathway, however, electrons are gained through the catabolism of endogenous carbohydrate stores (i.e. the glycolysis pathway and citric acid cycle) or other organic macromolecules such as lipids. The catabolism of these compounds generates NAD(P)H molecules, which are subsequently oxidized by NPQR to liberate electrons (in addition to protons and $NAD(P)^+$). The electrons are fed to the electron transport chain medially at the level of PQ. Lastly, the analysis of algal cultures placed under dark anoxic conditions has revealed a putative third pathway for hydrogen evolution. Under dark anoxia, algae degrade its endogenous starch reservoirs to sustain a basal level of metabolism, generating fermentative end products such as formate, acetate, ethanol, and possibly hydrogen. Since the electron transport chain is inactive during dark periods, pyruvate provides the electrons to reduce Fd, a step mediated by PFR1 (Seibert et al., 2008).

The cyanophyta hydrogen production pathways are noticeably different due to the presence of two different [NiFe]-hydrogenases, i.e. bidirectional [NiFe]-hydrogenases and uptake [NiFe]-hydrogenases, and a [MoFe]-nitrogenase found exclusively in nitrogen-fixing cyanobacteria (Note that some strains are also known to contain a vanadium atom, instead of molybdenum, in the prosthetic group) (Shestakov and Mikheeva, 2006). The cyanobacteria have three different pathways for hydrogen production. Two of them use water and organic compounds, respectively, as the electron donor, releasing electrons that are supplied to bidirectional [NiFe]-hydrogenase for hydrogen evolution. It should, however, be noted that the organic compounds (i.e. glycogen) that are catabolized for hydrogen production are formed through CO_2 fixation, with the electrons supplied by the splitting of water. Therefore in this case, water is the indirect electron donor for hydrogen evolution. Owing to the bidirectional nature of the cyanobacterial [NiFe]-hydrogenases, hydrogen can be either produced or consumed via the reversible reaction: $2H^+ + 2e^- \leftrightarrow H_2$. Bidirectional [NiFe]-hydrogenases are thought to be associated with the cytoplasmic membrane and accept electrons from both NAD(P)H and H_2. Studies from a small number of cyanobacterial mutant stains suggests that one of the two hydrogen evolution pathways mediated by bidirectional [NiFe]-hydrogenase is possibly coupled to the photosynthetic electron transport chain. This putative pathway, which ensues upon illumination, is different from the algal pathway as it does not solely rely on reduced Fd as an electron donor. Here, electrons are believed to be directly shuttled to bidirectional [NiFe]-hydrogenase at the level of NPQR (located near the photosynthetic membrane, between PSII and PQ). Electrons that are not diverted via NPQR continue along the electron transport

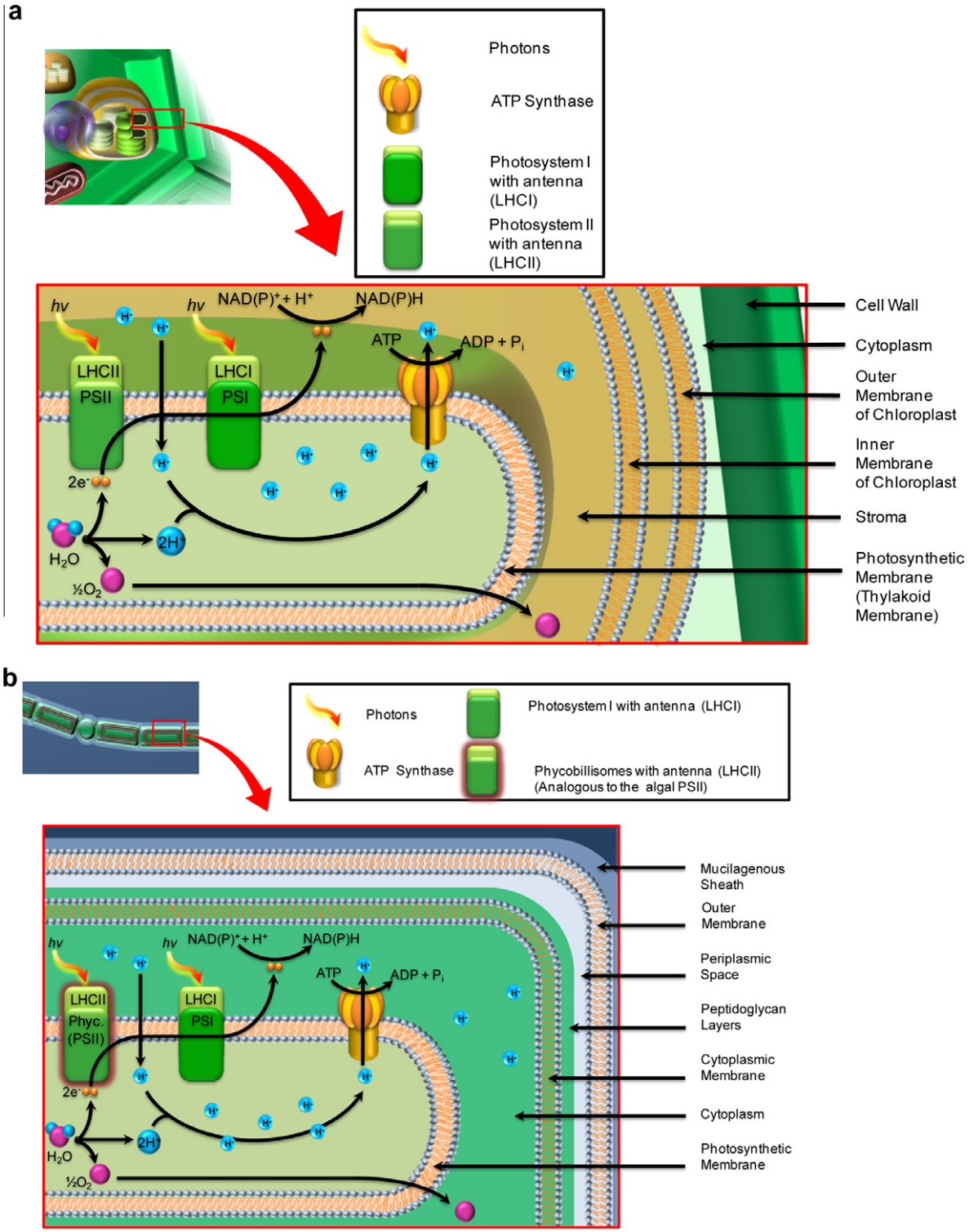

Fig. 1. Compartmentalization of the photosynthetic apparatuses in (a) algae and (b) cyanobacteria. (c) Light reactions of photosynthesis showing the electron transport chain embedded within the chloroplast thylakoid membrane (in green algae) or the photosynthetic membrane (in cyanobacteria, both diazotrophic and non-diazotrophic). Solar energy is captured by light harvesting complexes (LHCI/LHCII) at photosystem I and II (PSI/PSII). PSII is a protein complex found in plants, algae and cyanobacteria, and it mediates the light-dependent oxidation of water to yield protons, electrons, and oxygen. The resulting electrons are shuttled through a linear electron transport chain to the terminal electron acceptor of ferredoxin (Fd) via plastoquinone (PQ), cytochrome b_6f complex (Cyt b_6f), plastocyanin (PC), and PSI. Fd acts as a central hub of the electron flow, donating electrons to a number of biochemical pathways, including (i) Fd-NAD(P)$^+$ oxidoreductase (FNR) for the generation of NAD(P)H subsequently consumed in the Calvin–Benson cycle, (ii) oxygen for the regeneration of water via the Mehler reaction, (iii) hydrogenase (H_2ase) and nitrogenase (N_2ase) for the reduction of protons to hydrogen. In addition, electrons can be shuttled back to the PQ pool via PSI, i.e. cyclic electron transfer. Both linear and cyclic electron transfers lead to the generation of a proton motive force within the lumen, which can be partially released through ATP synthase to allow the concurrent production of ATP, used in the Calvin–Benson cycle, and the transfer of protons to either the chloroplast stroma (algae) or the cytoplasm (cyanobacteria).

chain through various electron acceptor intermediates (i.e. PQ, Cyt b_6f, PC, and PSI) for the reduction of Fd. While the majority of electrons gained by Fd are siphoned into other more essential assimilatory pathways (e.g. CO_2 fixation), a small number of them are

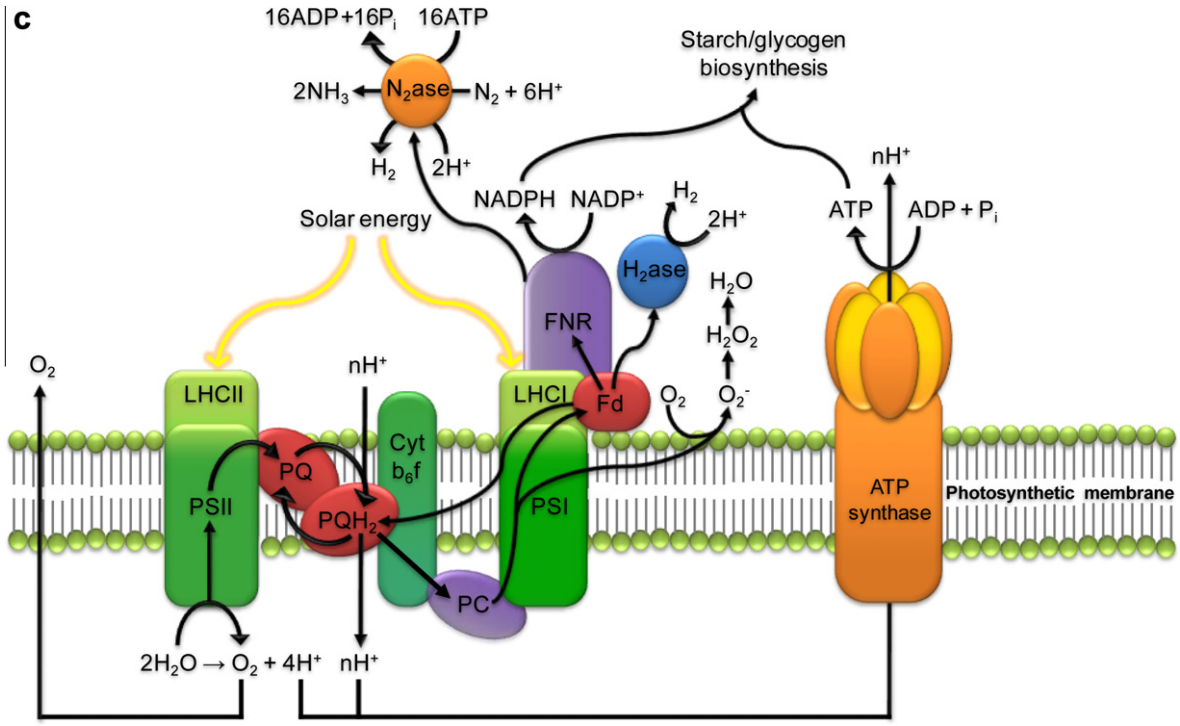

Fig. 1 (*continued*)

relayed back to NPQR through cyclic electron flow. At the onset of dark anoxia when the electron transport chain is nonfunctional, the second hydrogen production pathway can become active. This pathway is the most widely accepted hydrogen production pathway for cyanobacteria, and it is analogous to the aforementioned putative fermentation pathway for hydrogen production in green algae; where NAD(P)H generated through the catabolism of endogenous glycogen stores is oxidized by NPQR to yield the electrons required for hydrogen evolution (Appel and Schulz, 1998). The third hydrogen production pathway is found only in nitrogen-fixing cyanobacteria, in which nitrogenase fixes atmospheric nitrogen to form ammonia and hydrogen: $N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16P_i$. However, this pathway is energetically expensive since 2 ATP molecules are required for every electron being transferred. The electrons and ATP molecules fed to nitrogenase are obtained from either the electron transport chain associated with photosynthesis or the catabolism of carbohydrates. The electrons gained from these oxidations are first relayed to NPQR or FNR. NPQR will donate these electrons to the electron transport chain at the level of PQ, whilst FNR can directly ferry the electrons to nitrogenase. Furthermore, the spent reducing power for hydrogen evolution during nitrogen fixation can be regained by hydrogen consumption via uptake [NiFe]-hydrogenase. The electrons gained from hydrogen uptake are recycled back into the photosynthetic electron transport chain via the PQ pool and can be used by cytochrome c oxidase for the reduction of O_2 to water (i.e. Mehler reaction) or transferred back to nitrogenase via PSI and a heterocyst-specific Fd (Fig. 2) (Appel and Schulz, 1998).

2.3. Inherent limitations associated with photosynthetic systems

Production of hydrogen in algae and cyanobacteria can take forth through either direct or indirect biophotolysis. In direct biophotolysis, electrons required for hydrogen production are gained from the oxidation of water (mediated by PSII). Indirect biophotolysis generally refers to a hydrogen production scheme where the photosynthetic electron transport chain is partially or fully inactive. Thus, electrons required for hydrogen production are garnered through the catabolism of endogenous starch and lipids (Mathews and Wang, 2009). Sustained and enhanced hydrogen production relies on the effective proton reduction via hydrogen-evolving enzymes. However, this ostensibly simple reaction does not normally occur to its full extent in biological systems due to the following reasons. First, the proton is a poor electron acceptor, whereas hydrogen can be a favorable electron donor under many physiological conditions. Second, the hydrogen-evolving enzymes, in particular hydrogenases, are inherently incompetent: i.e. slow in turnover rate, complex in structure, difficult to attain in active form, and subject to inactivation by oxygen, a natural product of biophotolysis. Third, photosynthesis and hydrogen production are hardly coupled and typically do not occur simultaneously, as the electrons generated from biophotolysis are not often shuttled to the hydrogen-evolving enzymes directly for hydrogen production, but rather toward the pathway for carbon fixation. Such uncoupling complicates the practical operation of the hydrogen production bioprocess. Fourth, while hydrogen is relatively nontoxic compared to other organic metabolites, such as acids and alcohols, its physiological impact to cells is rather unpredictable knowing that the energy evolution associated with hydrogen production could represent a metabolic burden. Moreover, hydrogen is not normally considered a metabolite, but an energy form; making it difficult to implement typical metabolic engineering strategies (Mathews and Wang, 2009). As a result, developing novel strategies to improve hydrogen production in photosynthetic biological systems relies on the in-depth understanding of genetic aspects associated with these limitations.

3. Biophotolysis by green algae

The pioneering work of Hans Gaffron with algae in the early 1940s demonstrated that, under certain conditions, green algae are capable of photoproducing appreciable quantities of hydrogen gas (Gaffron, 1940). It was determined that light-dependent

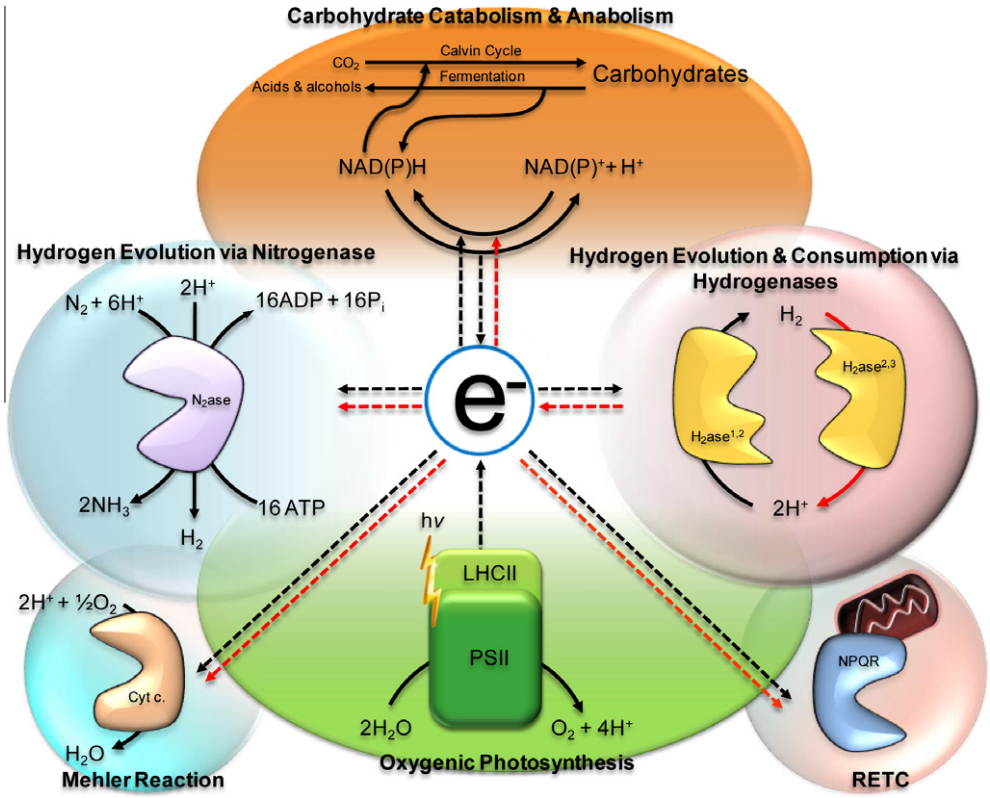

Fig. 2. An overview of possible electron flows (dotted arrows) associated with hydrogen production in algae and cyanobacteria. Hydrogen production can be mediated via [FeFe]-hydrogenases (H_2ase^1) in algae and bidirectional [NiFe]-hydrogenase (H_2ase^2) and nitrogenases (N_2ase) in cyanobacteria. The electrons required for hydrogen production are gained either through the photosynthetic oxidation of water or the catabolism of endogenous carbohydrates. Solid and dotted arrows depict chemical reactions and electron flows, respectively. Note that, in cyanobacteria, electrons can be also generated through hydrogen consumption (arrows in red) via the bidirectional [NiFe]-hydrogenase (H_2ase^2) and uptake [NiFe]-hydrogenase (H_2ase^3). Red arrows represent the chemical reaction and electron flows associated with hydrogen consumption, whereas black arrows represent those associated with photosynthesis and carbohydrate catabolism. In addition to hydrogen evolution, electrons can be siphoned to cytochrome c oxidase (cyt. c) for the generation of water called the Mehler reaction, the respiratory electron transport chain (RETC) at the level of NPQR, or can be used to generate NAD(P)H for carbohydrate synthesis (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

hydrogen production resulted only when cells were pre-incubated anaerobically in the absence of light, a requirement for induction of [FeFe]-hydrogenase gene expression. Following reillumination, hydrogenase is able to catalyze high level reduction of protons generated from the oxidation of water. Two distinct hydrogenase-encoding genes, *hydA1* and *hydA2*, have been located within the genome of the model green alga, *Chlamydomonas reinhardtii*. Although the hydrogenase enzyme is localized within the chloroplast stroma, both *hydA1* and *hydA2* are encoded within the cell's nucleus (Happe et al., 1994), as is true of over 90% of all chloroplast proteins which are translated in the cytoplasm and trafficked post-translationally into the chloroplast via a signal peptide (Smith, 2006). Hydrogen production is only able to occur through co-expression of ancillary maturation proteins, HydE, HydF, and HydG, which are involved in assembly of the functional [FeFe]-hydrogenase system (Posewitz et al., 2004). Accordingly, heterologous expression of *hydA1* in a host lacking hydrogenase maturation machinery, such as *Escherichia coli*, results in buildup of HydA1 polypeptide and the inability to produce hydrogen. Subsequent expression of the necessary maturation proteins triggers high-level hydrogen production.

Biophotolysis, the biological splitting of water to yield hydrogen and oxygen gas, refers to the coupling of photosynthesis and hydrogen evolution. However, oxygenic photosynthesis is intrinsically antagonistic to photohydrogen production due to the concomitant production of molecular oxygen upon oxidation of water. The evolved oxygen potently and irreversibly inhibits hydrogenase activity and cripples attempts to sustain hydrogen production. The extreme oxygen sensitivity exhibited by most [FeFe]-hydrogenases is often cited as the single greatest challenge impeding the feasibility of an industrial biophotolysis process for the production of biohydrogen. Other challenges, predominantly low photochemical efficiencies, have been shown to hinder the feasibility of direct biophotolysis using green algae. In order for biophotolysis to compete with current petrochemical methods as a sustainable platform for the production of biohydrogen, such challenges must be resolved through a combination of genetic and bioprocess engineering methodologies. This section discusses current limitations of hydrogen production via biophotolysis and reviews corresponding strategies to improve photobiological hydrogen production from green algae.

3.1. O_2 sensitivity of [FeFe]-hydrogenases

Oxygen sensitivity of [FeFe]-hydrogenases is a multifaceted challenge, as hydrogenase transcription, maturation, and photocatalytic activity are all highly susceptible to inhibition by molecular oxygen. Relative to the cyanobacterial [NiFe]-hydrogenases, [FeFe]-hydrogenases have been shown to be much more sensitive to oxygen, as significant activity is lost within only a few seconds exposure to oxygen (Cohen et al., 2005b). Oxygen is speculated to bind to an iron atom within the double Fe subcluster and in turn competitively inhibit proton binding required for generation of H_2. Furthermore, oxygen has been shown to be involved in hydrogenase gene expression, as HydA and HydEFG gene transcription is triggered, at least in part, by anoxia. For this reason, anoxic

conditions are desirable in order to trigger hydrogenase gene expression and prevent enzyme inhibition. However, such conditions are economically unfeasible, as sparging with an inert gas such as helium is costly for a large scale production process. Two distinct means of overcoming this inherent challenge have been successfully applied in recent years, namely sulfur deprivation and hydrogenase protein engineering.

3.1.1. Sulfur deprivation

As the result of culminating efforts set forth to separate, either spatially or temporally, the coordinated processes of oxygenic photosynthesis and hydrogen evolution, a major breakthrough occurred in 1998. Following the speculation that nutrient starvation may serve as a tool for modulating photosynthetic activity, it was found that sulfur limitation afforded an efficient means of drastically decreasing light-saturated rates of photosynthetic oxygen generation (Wykoff et al., 1998). Although electron transport was still carried out by the cell in a light-dependent manner, PSII activity, and therefore oxygen generation, was significantly impaired, attributed to a defective PSII repair cycle. Specifically, biosynthesis of D1, a pivotal protein located within the PSII reaction-center, was found to be significantly impaired as a result of the chloroplast's inability to synthesize appropriate amounts of the sulfurous amino acids, cysteine and methionine (Wykoff et al., 1998). This places drastic constraints on the PSII repair cycle, a fundamental process required to cope with photo-oxidative damage and sustain active photosynthesis (Melis, 1999).

In light of this initial observation, a novel two-stage process was developed allowing temporal separation of oxygenic photosynthesis and photobiological hydrogen production (Melis et al., 2000). In the first stage, cells are grown under typical, sulfur-replete conditions leading to vigorous cell growth and high rates of photosynthesis. Once a desired cell density is reached (approximately 3–6 $\times$ 10^6 cells/mL), cells are transferred to a medium deficient in sulfur. At this time, cultures are sealed to induce anoxia and hydrogenase expression, and hydrogen production is allowed to occur uninterrupted for up to 3–4 days (Melis and Happe, 2001). The sulfur-deprivation cycle can be broken down into three distinct time periods, i.e. 0–24, 24–72, and 72–120 h following initiation of sulfur limitation (Fig. 3) characterized by aerobic accumulation of energy reserves, onset of anoxia and hydrogen production, and termination of hydrogen generation, respectively (Matthew et al., 2009).

Following growth of algal cells to a sufficiently high density, sulfur limitation is typically achieved through suspending the cells in a fresh medium lacking sulfur. During the first 24 h following sulfur depletion, cells undergo a global metabolic shift in order to acclimatize to the nutrient-deprived conditions. This adaptive response is characterized by an exceptionally sharp decline in the amount of Rubisco and a relatively steady decrease in PSI and PSII activities (Zhang et al., 2002). Despite rapidly declining levels of Rubisco, cells are able to increase endogenous starch reserves by 8- to 10-fold during this phase in order to store carbon in a form that can be easily catabolized following the alleviation of sulfur deprivation (White and Melis, 2006). It has been speculated that the Rubisco catabolism and concurrent starch accumulation that occur during the initial aerobic phase of sulfur deprivation represent significant prerequisites for subsequent photoevolution of hydrogen to occur (White and Melis, 2006). Such events are light-dependent and do not occur in the dark, as

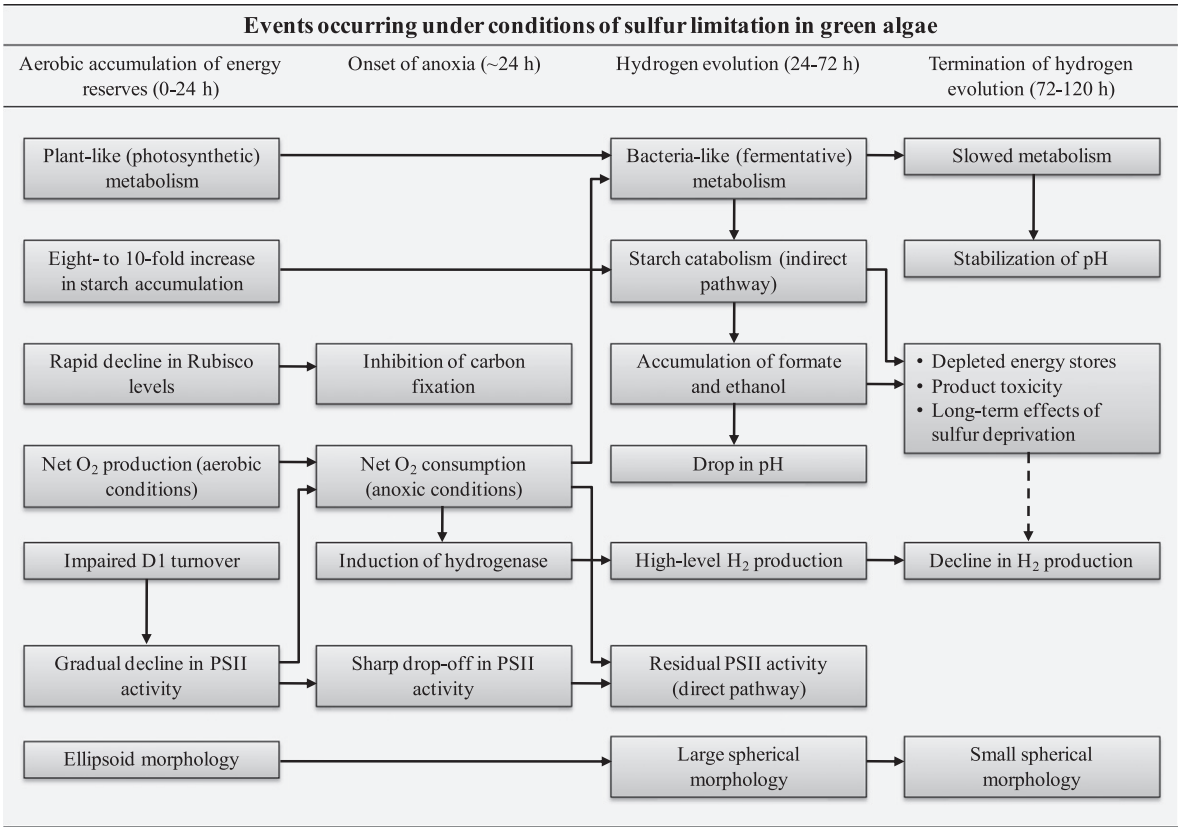

Fig. 3. Model showing the possible relationship of molecular and metabolic events which occur under distinct phases of sulfur deprivation in green algae. Four phases of sulfur deprivation can be observed based on the time at which they occur following the onset of sulfur limitation and the unique events occurring at each phase. They are characterized by (i) aerobic accumulation of energy reserves, (ii) onset of anoxia, (iii) hydrogen evolution, and (iv) termination of hydrogen evolution, occurring at approximately 0–24, 24, 24–72, and 72–120 h following the initiation of sulfur-deprived conditions, respectively.

electrons required for starch accumulation are derived from water through PSII activity.

As a result of steadily declining PSII activity, the absolute rate of photosynthetic oxygen evolution falls below that of respiratory oxygen uptake after approximately 20 h of sulfur deprivation (Ghirardi et al., 2000). Constant net oxygen consumption eventually leads to the establishment of anoxia at approximately 24 h of sulfur deprivation. This triggers a drastic global metabolic shift by which cells convert from a plant-like photosynthetic metabolism to a bacteria-like fermentation. At the exact point when the oxygen concentration reaches zero within the cell suspension, a rapid drop off in PSII photochemical activity has been observed (Antal et al., 2003). Downregulation of PSII at the outset of anoxia has been attributed to overreduction of the PQ pool as a result of a lack of electron sinks since hydrogenase expression has yet to be induced and carbon fixation has been almost completely inhibited due to undetectable levels of Rubisco (Antal et al., 2003; Zhang et al., 2002). Under these conditions, cells switch from linear to cyclic electron transport, in which electrons are shuttled around PSI as a means of generating ATP. The lack of available electron sinks, in addition to a state of anoxia, has been speculated to act as a trigger for the induction of hydrogenase gene expression and subsequent hydrogen evolution (Zhang et al., 2002). Hydrogen production has been detected as early as 10 min following the onset of anoxia in sulfur limited algal cultures (Antal et al., 2003). Sustained high level production of hydrogen typically occurs between 24 and 72 h following the initiation of sulfur deprivation at a rate of 2.0–2.5 mL H_2 L^{-1} culture h^{-1} (Melis and Happe, 2001). Due to a shift to predominantly fermentative metabolism, H_2-producing algal cultures accumulate fermentative end products, such as ethanol, acetate, and formic acid, in a pyruvate–formate–lyase-dependent pathway which is co-induced with *hydA* following anearobiosis (Hemschemeier et al., 2008). This causes a significant pH drop over the course of hydrogen photoproduction (Matthew et al., 2009).

The source of electrons associated with hydrogen production has been an area of active speculation and investigation. It is widely held that hydrogenase is fed with both electrons derived from a direct (i.e. PSII-dependent) and indirect (i.e. PSII-independent) pathway (Chochois et al., 2009; Fouchard et al., 2005). Whereas the direct pathway relies on residual water-splitting activity of PSII and subsequent oxygen consumption through respiratory activities, the indirect pathway utilizes electrons derived from the catabolism of endogenous starch to reduce the PQ pool in a non-photochemical process (Fouchard et al., 2005). The chief enzyme responsible for the non-photochemical reduction of PQ, NPQR, encoded by *nda2*, has recently been located within the genome of *C. reinhardtii* (Jans et al., 2008), implying that starch, rather than lipid or protein, serves as the main electron donor during PSII-independent hydrogen evolution. The precise contribution from each of these pathways has yet to be fully resolved. However, it is clear that low-level PSII activity is required to sustain hydrogen production, as sulfur-deprived cells do not produce hydrogen in the absence of light (Melis et al., 2000). It has also been shown that the direct pathway contributes approximately 80% of the reductant required to drive hydrogen photoevolution (Antal et al., 2003; Ghirardi et al., 2000), substantiated by the finding that mutants deficient in starch accumulation are capable of producing hydrogen at a level comparable to that of the wild type (Chochois et al., 2009). However, these findings occur in contrast to those obtained using Nda2-RNAi cell lines, in which hydrogen production was found to be substantially decreased due to severely downregulated non-photochemical PQ reduction (Jans et al., 2008). Such seemingly contradictory findings are often attributed to the differences in experimental conditions, such as the mode of induction of hydrogen photoproduction and the use of DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea), a potent inhibitor of the direct PSII-dependent hydrogen production pathway, which leads to a state of complete anaerobiosis, characterized by absent oxygen evolution, rather than more typical micro-aerobic conditions in which low-level oxygen generation is still present (Ghysels and Franck, 2010).

Following approximately 72 h of sulfur deprivation, the rate of fermentative starch catabolism begins to slow, pH partially stabilizes, and hydrogen evolution starts to decrease (Matthew et al., 2009). After 4 or 5 days of sulfur deprivation, hydrogen production is almost exhausted and the cells exhibit a spherical morphology and significant reduction in cell mass (Zhang et al., 2002). The exact events leading to the termination of hydrogen photoevolution are not entirely known, although diminished energy resources, product toxicity, and other effects associated with prolonged sulfur deprivation have been implicated as potential factors (Matthew et al., 2009). However, metabolomic analysis of algal cultures following termination of hydrogen production have revealed significant stores of endogenous starch, in addition to triacylglycerides, suggesting that depleted energy stores does do not lead to cessation of hydrogen evolution (Matthew et al., 2009). The effect of accumulated fermentation end products, predominantly ethanol and formate, may potentially lead to toxicity and the end of hydrogen evolution, as is the case in many classical fermentations. Although sulfur limitation imposes significant metabolic stress upon the cell, this process is reversible, as cultures can be cycled numerous times between media containing and lacking sufficient supplies of inorganic sulfur (Ghirardi et al., 2000).

3.1.2. Hydrogenase protein engineering

The sulfur limitation method discussed above depends largely on the development of a two-step process, i.e. oxygenic photosynthesis and hydrogen photoevolution, controlled by sulfur concentration in the culture medium. Another efficient means of sustaining hydrogen generation may involve decreasing the intrinsic oxygen sensitivity of algal [FeFe]-hydrogenases so as to allow the operation of a single stage hydrogen bioprocess, in which hydrogen evolution is carried out in the presence of oxygenic photosynthesis. This approach requires the construction of mutant strains with significantly decreased sensitivity to oxygen, as the algal [FeFe]-hydrogenases exhibit potent irreversible inactivation upon exposure to air.

Rational attempts to engineer algal hydrogenases for decreased oxygen sensitivity are presently rather limited due to the lack of the crystal structure for such enzymes. However, crystal structures of the homologous bacterial [FeFe]-hydrogenases from *Clostridium pasteurianum* (Peters et al., 1998) and *Desulfovibrio desulfuricans* (Nicolet et al., 1999) have been available. At present, these structures serve as the closest approximations to actual algal hydrogenase structures and form the basis of theoretical investigations into the mechanism of oxygen inhibition. However, [FeFe]-hydrogenases from green algae are substantially simpler in structure and much more sensitive to atmospheric oxygen than their bacterial counterparts (Cohen et al., 2005b). These drastic differences in oxygen sensitivities imply that algal hydrogenases likely possess distinctive structural pathways for oxygen diffusion, as oxygen appears to diffuse through the algal protein structure more easily (Cohen et al., 2005b).

Hydrogen and oxygen gas exhibit significantly different permeation through the hydrogenase protein structure. The crystal structure of the *D. desulfuricans* hydrogenase revealed a putative hydrophobic gas channel allowing diffusion of hydrogen, with a molecular size at 1.0 Å, from the protein surface to the active site (Nicolet et al., 1999). However, the much larger size of oxygen at 1.6 Å could potentially prevent its access to the catalytic site via this pathway (Nicolet et al., 1999). In depth computational simulation of the *C. pasteurianum* hydrogenase suggested a mechanism in

which hydrogen molecules are able to penetrate the protein's structure through an array of random and undefined pathways, whereas oxygen is restricted to a small number of pre-defined channels (Cohen et al., 2005a). Two oxygen diffusion pathways were identified based on nanosecond-scale simulation of oxygen diffusion (Cohen et al., 2005a), one of which corresponded to the aforementioned hydrophobic hydrogen-channel (Nicolet et al., 1999).

Based on the above findings, efforts were set forth in order to manipulate the oxygen sensitivity of the model *C. pasteurianum* hydrogenase by attempting to block diffusion of oxygen through the respective gas channels using rational mutagenesis (Ghirardi et al., 2006). Using "in silico" mutagenesis and volumetric oxygen accessibility maps, three hydrophobic amino acids in the central cavity were predicted to be located in environments favorable for oxygen to occupy (Ghirardi et al., 2006). In order to sterically hinder oxygen diffusion to these locations within the hydrogenase structure, targeted residues were mutated to hydrophobic amino acids with substantially larger side chains. Only one such mutation, L283W within channel A, afforded a modest improvement in oxygen tolerance, with the other mutations significantly increasing oxygen sensitivity. Nevertheless, all mutations adversely affected hydrogen production, as hydrogen evolution was decreased by more than 90% for all mutations except L283W which exhibited only a 29% reduction compared to the wild type. These findings suggest the profound sensitivity of mutations close to the active site of [FeFe]-hydrogenases and the inability to rationally engineer oxygen tolerance into inherently oxygen-sensitive enzymes.

Although hydrogenase exhibits irreversible inhibition by oxygen, the sensitivity of photobiological hydrogen production is a multifaceted problem since hydrogenase transcription, and likely maturation and assembly, might be also inhibited by exposure to atmospheric oxygen. Therefore, a comprehensive solution to this technical issue should focus on all aspects of inhibition. To this end, hydrogenase has been expressed heterologously under a promoter that is induced by factors other than the establishment of anoxic conditions (King et al., 2006). However, little work has been done in characterizing or manipulating the oxygen sensitivity of [FeFe]-hydrogenase maturation proteins (Posewitz et al., 2004), although it has been proposed that a hydrogenase with a half-life of several h would overcome this challenge, as the enzyme can be resynthesized and assembled during the night following activation of the hydrogenase maturation proteins (Ghirardi et al., 2007).

3.2. Sub-optimal photochemical efficiencies

The maximum sunlight-to-hydrogen conversion efficiency by green algae is roughly 12.5%. For photobiological hydrogen production to compete economically with current petrochemical methods, an ambitious photochemical efficiency of 10% is often cited as a future target (Mussgnug et al., 2007). However, current photochemical efficiencies of algal systems are often below 1% (Hallenbeck and Benemann, 2002), even under optimal conditions of sulfur deprivation. Under bright sunlight, the linear electron transfer between PSII and PSI is poor and occurs at approximately one tenth the rate of photon absorption by Chl (Hallenbeck and Benemann, 2002). Due to the absence of a chloroplastic system for storing excess absorbed photons, up to 90% of photons are dissipated, via a mechanism termed non-photochemical quenching, in the form of heat or fluorescence, rather than being siphoned into the photosynthetic electron transport chain (Melis, 1999). Particularly in high-density algal cultures, this phenomenon is largely associated with overabsorption by cells on the surface of a photobioreactor as light is unable to penetrate efficiently into the culture, causing an unequal and sub-optimal distribution of photon absorption. Although this does not directly hinder hydrogen production, sub-optimal photon utilization places significant constraints on photosynthetic productivity. Therefore, more efficient utilization of absorbed photons indirectly impacts photobiological hydrogen production by allowing a greater proportion of cells to contribute to highly active photosynthetic electron transfer. In order to overcome this inherent photochemical inadequacy, truncation of light-harvesting Chl antenna size is likely the most plausible strategy.

3.2.1. Engineering light-harvesting complexes

Sub-optimal photon utilization was first recognized as a salient limitation of photobiological hydrogen production in the 1950s. This led to speculation that decreasing photon absorption of growing algal cells could increase the transmittance of light through a high-density culture (Polle et al., 2002). In this respect, the rate of photon absorption should approach that of photon utilization via linear electron transport between PSII and PSI in order to minimize wasteful photon dissipation by over-illuminated cells at the surface of an algal culture. At the same time, light could penetrate deeper into a cell suspension, allowing a greater number of cells for efficient oxygenic photosynthesis and photobiological hydrogen production. An efficient molecular means of decreasing photon absorption involves the truncation of light-harvesting Chl antenna size, i.e. a decreased number of Chl molecules within the light-harvesting complexes of PSI and PSII, which has led to a 2- to 3-fold greater sunlight conversion efficiency compared to untruncated wild-type cells (Melis et al., 1998; Neidhardt et al., 1998).

Photosynthetic organisms possess the unique natural ability to alter the size of Chl antenna in order to cope with changing levels of illumination. Within a natural environment, this adaptive response imparts a competitive advantage to cells by allowing optimal light capture (Mussgnug et al., 2007). Under conditions of bright light, chloroplasts adapt by decreasing PSI and PSII Chl antenna size through downregulation of light-harvesting complex (LHC) transcription and post-translation in order to minimize the deleterious effects imposed by photoinhibition (Melis, 1999). Indeed, cells grown under high-light conditions exhibit significantly lower pigment content (yellow–green cells) and adversely truncated Chl antenna size, in addition to suffering from detrimental levels of photoinhibition (Neidhardt et al., 1998). It has been estimated that such inhibitory levels of light are prevalent for approximately 10 h during a spring day at mid-latitudes in the Northern Hemisphere (Mussgnug et al., 2007). Effects of high-level light are largely reversible, as PSII photodamage is repaired and Chl begins to accumulate (deep green cells) when cells acclimated to conditions of saturated illumination are placed under limiting light (Neidhardt et al., 1998). As a result of a delayed response between these molecular events, a brief period of time, typically 2–3 h following conversion from high-light to low-light conditions, triggers a small Chl antenna size phenotype characterized by a 6- to 9-fold higher light-saturated rate of photosynthesis (Neidhardt et al., 1998). Since this natural response can be produced only transiently under laboratory conditions and mutants possessing truncated Chl antenna sizes do not possess a selective advantage in nature, genetic methods are required in order to generate strains with permanently truncated Chl antenna size for use in algal biotechnology for the production of biohydrogen under high-light conditions.

Under low-light conditions, PSI and PSII contain approximately 250 and 500 Chl molecules (Chl *a* and *b*), respectively, in contrast to only 100 and 60 under conditions of high-light (Melis et al., 1998). It has been estimated that the minimum antenna size required to yield assembled and functional PSI and PSII complexes is 95 and 37 Chl *a* molecules, respectively (Glick and Melis, 1988). As a result, significant process enhancements stand to be gained through the genetic manipulation of Chl antenna size in algal chloroplasts. Genetic methods were first applied to generate a

Table 3
Mutant algal strains exhibiting improved solar energy to hydrogen conversion efficiencies.

Mutation	Deficiency	Truncation of Chl antenna size		Characteristics	References
		PSI	PSII		
cao insertion	Chl *b*	No	Yes	50–70% PSII truncation (95 Chl *a*) 10% less LHCII 2-fold higher P_{max}	Polle et al. (2000) Polle et al. (2002) Tanaka et al. (1998)
npg2 lor1 insertions	α-Car and β,β-epoxyCar derived from zeaxanthin	No	Yes	33–50% PSII truncation (90 Chl *a* + *b*) 1.5-fold higher P_{max} 50% greater photosynthetic capacity	Polle et al. (2001) Polle et al. (2002)
tla1 insertion	Chl *a* + *b*	Yes	Yes	20–50% PSI truncation (159 Chl *a* + *b*) 35–50% PSII truncation (114 Chl *a* + *b* 40% less LHCII 1.9-fold higher P_{max}	Mitra and Melis (2010) Polle et al. (2002) Polle et al. (2003)
RNAi of LHC gene family	Down-regulation of all 20 genes encoding LHCI, LHCII, CP26, and CP29	Yes	Yes	0.1–26% down-regulation 68% less total Chl 81% greater photosynthetic yield	Mussgnug et al. (2007)
NAB1* expression	Permanently repressed LHC translation	No	Yes	20% less Chl 15% PSII truncation 25-28% greater photosynthetic activity	Beckmann et al. (2009)

C. reinhardtii mutant deficient in Chl *b* biosynthesis (Polle et al., 2000) through DNA insertional mutagenesis of the gene encoding Chl *a* oxygenase (CAO), responsible for the formation of Chl *b* from Chl *a* (Tanaka et al., 1998) (Table 3). This mutant displayed a markedly truncated PSII antenna size, containing 96 Chl *a* molecules, compared to 320 Chl *a* and *b* molecules for the wild type, whereas PSI was essentially unaffected (Polle et al., 2000). Although a significant reduction in PSII antenna size relative to the wild type was observed, this Chl *b*-less mutant still possessed approximately 2.5-fold more Chl *a* molecules than the minimum supply required for assembly of the PSII-core antenna, as speculated by Glick and Melis (1988). Nevertheless, PSII Chl *a* antenna size truncation proved effective in decreasing wasteful photon dissipation, as the Chl *b*-less mutant exhibited a 2.5-fold greater light-saturated rate of oxygen evolution (Polle et al., 2000). A similar mutant (*npq2 lor1*) truncated in PSII antenna size but largely unaffected in PSI has been generated which lacks the most abundant carotenoids (Car) in higher plants and green algae, i.e. lutein and violaxanthin, in addition to other β,ε-carotenoid and β,β-epoxycarotenoid pigments (Polle et al., 2001). Levels of Chloroplast Protein 24 (CP24) and CP26, associated with PSII complexes, were decreased by 75 and 40%, respectively, in addition to a 40% decrease in LHCII. Although the growth rate and photon conversion efficiency were essentially identical to the wild type, the Car-deficient mutant exhibited a 50% greater photosynthetic capacity (Polle et al., 2001).

In a further effort to identify additional candidate genes responsible for dictating Chl antenna size, random insertional and chemical DNA mutagenesis methodology was applied to *C. reinhardtii* (Polle et al., 2002). This led to the generation of arguably the most efficient light-utilizing algal mutant strain to date. A single insertion was located within a previously unidentified coding sequence, termed *tla1* (*t*runcated *l*ight-harvesting Chl *a*ntenna size), and conferred a severely Chl-deficient phenotype (Polle et al., 2002, 2003). The *tla1* mutant was found to be truncated in both PSI and PSII antenna sizes by 50% and 35%, respectively, representing the first mutant possessing truncation of both photosystem antenna sizes. However, PSI and PSII antenna sizes were still larger than the minimum size (Glick and Melis, 1988) required to carry out functional oxygenic photosynthesis (by a factor of approximately 1.7 and 3.1, respectively), suggesting a potential increase in photochemical efficiency upon further truncation of Chl antenna size in *tla1* mutants (Polle et al., 2003). Nevertheless, the *tla1* mutant exhibits significantly increased light-saturated rates of oxygen evolution and substantially greater photosynthetic productivity compared to wild type untruncated *C. reinhardtii* (Polle et al., 2003). The precise physiological function of TLA1 in algal growth and development is presently unknown. However, its putative role in organelle biogenesis via signal transduction has been outlined recently due to the ubiquity of TLA1 homologs in diverse eukaryotic organisms (Mitra and Melis, 2010).

As a result of recent advancements in algal genetic systems, mutants exhibiting truncated Chl antenna sizes have been generated by means other than DNA insertional mutagenesis, including RNAi technology (Mussgnug et al., 2007) and nuclear transformation (Beckmann et al., 2009). Rather than targeting single genes implicated in regulating Chl antenna size, Mussgnug et al. (2007) used a comprehensive RNAi approach in order to simultaneously down-regulate all 20 LHC family genes, including the genes encoding LHCI, LHCII, CP26, and CP29. The resulting strain, exhibiting significantly lower levels of both LHC mRNA (0.1–26% of wild type) and therefore truncation of both PSI and PSII antenna size, possessed approximately 68% less Chl leading to an 81% increase in photosynthetic quantum yield, signifying decreased non-photochemical quenching due to dramatically improved photon utilization (Mussgnug et al., 2007). However, concerns regarding phenotypic instability for strains constructed using RNAi technology led to an alternative means of generating algal strains with permanently truncated Chl antenna size. The identification of NAB1 (nucleic acid binding protein), a potent cytosolic repressor of LHCII translation, and elucidation of its unique thiol-dependent, mRNA-sequestration mechanism led to its manipulation as a means of permanently truncating LHCII antenna size (Beckmann et al., 2009). It was found that mutation of two key cysteine residues, C181S and C226S, led to conversion of redox-inducible NAB1 into a variant, NAB1*, locked in a permanent repression state (Wobbe et al., 2009). The resulting strain exhibited a significantly truncated PSII antenna size, characterized by 20% lower levels of Chl, reduction in PSII antenna size by 20%, and 25–28% greater photosynthetic activity under high-light conditions (Beckmann et al., 2009). However, PSI antenna size was largely unaffected and PSII antenna size was truncated to a much lesser extent compared to other strains such as the *tla1*-deficient and NAB1* expression mutants, outlining the need for strains exhibiting a pronounced global truncation of antenna size, including all LHC family genes. Such mutants are central to the biotechnological production of renewable hydrogen, as they allow high photosynthetic productivity under conditions of bright sunlight and mass culture with significantly less wasteful dissipation of photons through non-photochemical quenching.

4. Biophotolysis by cyanobacteria

The faculty for hydrogen production in cyanobacteria (referred to as blue-green algae then) was first observed in an *Anabaena* sp.

at the end of the 19th century. It was discerned that this species, found in a Massachusetts reservoir, evolved hydrogen in an anaerobic state. Several decades later, the phenomenon of photobiological hydrogen production was deduced through the discovery of the bacterial hydrogenase. From this period and onward, photoconversion of water to hydrogen through the use of a photosynthetic system proved to be a feasible method. However, the algal photosystem for hydrogen production suffered several drawbacks, such as low productivity, unsustainable production, and, most importantly, oxygen sensitivity to hydrogenase. To tackle these technical issues that riddled algal biophotolysis, Benemann and Weare (1974) first explored the possibilities of using heterocyst-forming cyanobacterial strains in the 1970. From their initial study using *Anabaena cylindrica* B-629, it was reported that hydrogen and oxygen evolved concomitantly under nitrogen fixing conditions and, therefore, a nitrogenase-mediated hydrogen production was deemed as an alternative strategy. The apparent advantage is the spatial separation achieved through the use of a heterocyst, whereby hydrogen is evolved in an oxygen-free environment. The heterocyst maintains this anaerobic environment due to its oxygen impermeable membrane and lack of functional PSII. Moreover, unlike the algal photosystems which require a lengthened period of adaptation (generally between 2 and 30 h), hydrogen production in cyanobacteria is almost immediate at the onset of heterocyst formation. In addition to *A. cylindrica* B-629, other heterocystous strains with the capability to yield hydrogen, such as *Nostoc muscorum* IAM M-14 (Yoshino et al., 2007) and the filamentous *Anabaena varibilis* ATCC 29413 (Sveshnikov et al., 1997), have been discovered (Table 4a). Heterocystous hydrogen evolution is generally conducted in two stages with different atmospheric conditions. In the first stage, cell growth is promoted to attain a dense culture and the atmosphere is kept aerobic with varying light intensities. While some non-diazotrophic strains can produce hydrogen under dark anoxic conditions, most if not all heterocystous strains require light (preferentially far red light at 680 nm with an intensity ranging from 20 to 100 μmolE m^{-2} s^{-1}) for hydrogen production. In the second stage, a metabolic stress (i.e. nitrogen starvation) is brought upon the vegetative cells as a means to induce the formation of heterocysts and this is commonly conducted by using argon (occasionally supplemented with 2–5% CO_2). In addition to inducing heterocyst formation, the removal of nitrogen gas in this stage, also concomitantly enhances nitrogenase activity (Yu and Takahashi, 2007).

Although the spatial separation scheme associated with the heterocyst overcomes oxygen sensitivity issues, the nitrogenase-mediated hydrogen production is an energetically unfavorable reaction since 16 ATP molecules are siphoned into the nitrogen fixing cycle for the production of a single hydrogen molecule. Moreover, nitrogenases, in comparison to bidirectional [NiFe]-hydrogenases, proceed at a considerably slower turnover rate (6.4 s^{-1} vs. 98 s^{-1}) (Mathews and Wang, 2009). This is problematic as a large quantity of nitrogenase is required to sustain hydrogen production for a longer period of time; placing a potential physiological burden on cells. To circumvent these shortcomings, non-diazotrophic (and some diazotrophic) cyanobacterial strains have been explored as a potential platform for hydrogen production, a process mediated solely by bidirectional [NiFe]-hydrogenases. This is a two-stage operation conducted in a similar fashion to that of green microalgae, namely photosynthesis (for starch accumulation) and hydrogen evolution (via dark fermentation) are temporally separated. In the first stage, a nutrient-enriched medium sealed by a mixed atmosphere of air and CO_2 is used to grow cells. When the growth ceases, the cells are removed from the enriched medium, washed, and then placed into a medium deficient in macronutrients, such

Table 4
Hydrogen production via a (a) spatial or (b) temporal separation scheme for selected cyanobacterial strains.

Strain Name	Relevant phenotype	Atmosphere; light intensity		Maximum H_2 evolution	References
		Growth stage	H_2 assay stage		
(a) Spatial					
Anabaena cynlindria B-629	Filamentous, diazotrophic marine cyanobacteria	Air enriched with 0.3% CO_2; 92 μmolE m^{-2} s^{-1}	97% Ar, 3% CO_2 276 μmolE m^{-2} s^{-1}	0.74 μmol mL^{-1} h^{-1}[a]	Benemann and Weare (1974)
Nostoc muscorum IAM M-14	Filamentous, diazotrophic fresh and salt water cyanobacteria	Air enriched; 20 μmolE m^{-2} s^{-1}	95% Ar, 5% CO_2; 100 μmolE m^{-2} s^{-1}	7.7 μmol mg Chl α^{-1} h^{-1}	Yoshino et al. (2007)
Anabaena variabilis ATCC 29413	Filamentous, diazotrophic marine cyanobacteria	Argon enriched, with 25% N_2, 2% CO_2; 90 μmolE m^{-2} s^{-1}	95% Ar, 5% N_2; 90 μmolE m^{-2} s^{-1}	3.07 μmol mg Chl α^{-1} h^{-1}	Sveshnikov et al. (1997)
Anabaena variabilis IAM M-58	Filamentous, diazotrophic marine cyanobacteria	Air enriched; 20 μmolE m^{-2} s^{-1}	95% Ar, 5% CO_2; 100 μmolE m^{-2} s^{-1}	3.4 μmol mg Chl α^{-1} h^{-1}	Yoshino et al. (2007)
Oscillatoria sp. Miami BG7	Filamentous, diazotophic (non heterocystous) marine cyanobacteria	Air enriched; 100 μmolE m^{-2} s^{-1}	Ar; 89 μmolE m^{-2} s^{-1}	0.15 μmol mg (dry weight)$^{-1}$ h^{-1}	Kumazawa and Mitsui (1985)
(b) Temporal					
Synechococcus PCC 602	Unicellular, non-diazotropnic marine cyanobacteria	Air enriched; 20 μmolE m^{-2} s^{-1}	Ar with CO (13.4 μmol) and C_2H_2 (1.34 mmol); dark	0.66 μmol mg Chl α^{-1} h^{-1}	Howarth and Codd (1985)
Synechococcus PCC 6803	Unicellular, non-diazotropnic marine cyanobacteria	Air enriched; 20 μmolE m^{-2} s^{-1}	Ar with CO (13.4 μmol) and C_2H_2 (1.34 mmol); dark	0.26 μmol mg Chl α^{-1} h^{-1}	Howarth and Codd (1985)
Gloeobacter violaceus PCC 142	Unicellular, non-diazotropnic marine cyanobacteria	Air enriched; 20 μmolE m^{-2} s^{-1}	Ar with CO (13.4 μmol) and C_2H_2 (1.34 mmol); dark	0.59 μmol mg Chl α^{-1} h^{-1}	Howarth and Codd (1985)
Gloeocapsa alpicola CALU 743	Unicellular, non-diazotrophic salt water cyanobacteria	Air enriched with 2% CO_2[a], 165 μmolE m^{-2} s^{-1}	Ar; dark	0.22 μmol mg protein^{-1} h^{-1}	Troshina et al. (2002)
Gloeocapsa alpicola CALU 743	Unicellular, non-diazotrophic salt water cyanobacteria	Air enriched with 4% CO_2[b], 165 μmol photons m^{-2} s^{-1}	Ar; dark	0.14 μmol mg protein^{-1} h^{-1}	Antal et al. (2005)
Synechocystis PCC 6803	Unicellular, non-diazotropnic marine cyanobacteria	Air enriched with 4% CO_2[b]; 165 μmol photons m^{-2} s^{-1}	Ar; dark	0.09 μmol mg protein^{-1} h^{-1}	Antal et al. (2005)

[a] NO_3^- limited media for growth.
[b] SO_4^{2-} limited media for growth.

as nitrogen and sulfur, to further accumulate the intracellular glycogen. The second stage of anaerobic and dark incubation is often induced through sparging with argon and hydrogen production ensues immediately at the onset of anaerobiosis when glycogen is catabolized. Unlike green algae, it is postulated that enzymes involved in dark fermentation in cyanobacteria are constitutively expressed. As a result, the organism has the ability to switch immediately from photoautotrophic metabolism to dark fermentation without the need of an adaptation phase. Cyanobacteria are proficient photoautotrophs capable of accumulating large amounts of glycogen granules (with some strains accruing as much as 50% of the dry cell mass) (Yu and Takahashi, 2007). Species frequently employed for hydrogen evolution via temporal separation include *Gloeocapsa alpicola*, a strain which holds a great promise (Troshina et al., 2002), and the genetically amenable *Synechocystis* PCC 6803 (Antal et al., 2005) (Table 4b).

Given the existence of numerous spatial and temporal separation strategies described above, cyanobacterial hydrogen production is still in its infancy due to several inherent obstacles. For example, the cyanobacterial hydrogenases and nitrogenases are highly sensitive to oxygen, produced through oxygenic photosynthesis. Additionally, hydrogen productivity in cyanobacteria is inherently low due to various limitations, such as the competition for electrons from non-hydrogenase pathways, the low quantum solar efficiency of the photosynthetic antennae, the presence of the uptake [NiFe]-hydrogenase within the heterocyst, and the low frequency of the heterocyst formation. As a result, there has not been a major advancement in enhancing hydrogen photoproduction in cyanobacteria since its initial inception in the 1970s. Nevertheless, research and development over the last 40 years have led to fundamental understanding of hydrogen-producing metabolism and an array of methodologies for hydrogen production in appreciable quantities, paving an avenue for sustainable hydrogen production by cyanobacteria in the future. Herein, we survey recent genetic and bioprocessing stratagems employed to enhance hydrogen production in cyanobacteria; whilst providing insights that can be beneficial and applicable to future advances in this area.

4.1. O_2 sensitivity of [NiFe]-hydrogenases

As previously mentioned, the majority of redox metalloenzymes involved in hydrogen evolution are subject to inactivation in the presence of oxygen. While [FeFe]-hydrogenases, such as algal hydrogenases, are irreversibly crippled when exposed to oxygen, [NiFe]-hydrogenases, such as cyanobacterial hydrogenases, are more tolerant to oxygen and only temporarily inactivated upon the exposure to oxygen. Crystallographic and various spectroscopic analyses of the [NiFe]-hydrogenase from the bacterium *Desulfovibrio fructosovorans* reveal a putative oxygen binding site between nickel and iron. The bound oxygen molecule forms a hydroxyl group between the two metals, preventing protons from accessing the binding site (Volbeda et al., 2002). Under anoxic conditions, the oxygen can be removed from the binding site using a strong reducing agent (Ghirardi et al., 2007). Another hypothetical strategy would be to modify the amino acid composition of the gas diffusion channel to make it narrower so that the diffusion of protons can be permitted whilst molecular oxygen is restricted. With the recent establishment of genetic engineering tools for cyanobacterial manipulations (Koksharova and Wolk, 2002), the oxygen sensitivity of [NiFe]-hydrogenases can be alleviated. For example, novel cyanobacterial strains harboring hydrogenases either less sensitive or completely tolerant to oxygen can be derived. Another genetic engineering approach yet to be examined is the incorporation of an 'enzymatic deoxygenation system' that can sequester oxygen and decrease its concentration within the cell. This would include the incorporation of genes encoding oxygen-scavenging enzymes, such as oxidases and superoxide dismutases (Shestakov and Mikheeva, 2006).

4.1.1. Hydrogenase engineering

With the recent discovery of oxygen-tolerant [NiFe]-hydrogenases from certain photosynthetic microorganisms, heterologous expression of these hydrogenases in cyanobacteria has been explored. The characterization of the soluble oxygen-tolerant [NiFe]-hydrogenase from the soil bacterium *Rastania eutrophia* H16 has revealed that the oxygen tolerance is associated with the distinct shape of the intramolecular gas cavity that is hard to be accessed by molecular oxygen (Casalot and Rousset, 2001). Although genetically tractable cyanobacterial strains are available (e.g. *Synechococcus* PCC 6301 and PCC 7942 and *Synechocystis* PCC 6803), heterologously expressing a hydrogenase remains challenging primarily due to complex posttranslational processings. In order to yield a fully functional hydrogenase, co-expression of a set of essential maturation and assembly machineries is required.

Another exploration was initiated to derive novel recombinant cyanobacterial strains harboring oxygen-tolerant hydrogenases and to probe metagenomic data for identification of additional oxygen-tolerant hydrogenases (Xu et al., 2009). To date, all the necessary structural and ancillary genes of the *Thiocaspa roseopersicina* hydrogenase have been successfully integrated into the chromosome of *Synechococcus* PCC 7942. On the other hand, significant progress has been made in deriving a recombinant *Synechocystis* PCC 6803 expressing functional *Rubrivivax gelatinosus* hydrogenase by transforming the two structural genes of the *Rubrivivax* hydrogenase and most of the associated maturation genes into the cyanobacterial host. However, the functionality of these recombinant hydrogenases requires further characterization to achieve sustained hydrogen evolution under illumination (Xu et al., 2009).

Recently, a light-driven recombinant system for hydrogen production was derived by constructing an artificial fusion comprised of the oxygen-tolerant [NiFe]-hydrogenase from *Rastania eutrophia* H16 and PSI subunit (PsaE) from cyanobacterium *Thermosynechococcus elongatus* using innovative genetic engineering techniques (Ihara et al., 2006). The hybrid fusion instinctively associates with a PsaE-free PSI of an engineered *Synechocystis* PCC 6803 strain and, as a result, has the capacity to shuttle electrons for hydrogen evolution at a reported rate of 0.58 μmol H_2 mg Chl^{-1} h^{-1}.

Coupling [Fe]-hydrogenases from anaerobic fermentative bacteria with the photosynthetic machinery from cyanobacteria is another noteworthy approach to genetically tailor cyanobacteria for hydrogen production. Compared to algal and cyanobacterial hydrogenases, bacterial [Fe]-hydrogenases are far more superior in terms of possessing a greater tolerance to oxygen and being unidirectional without consuming molecular hydrogen (Cohen et al., 2005b). Accordingly, heterologous expression of the structural genes of *Clostridium pasteurianum* [Fe]-hydrogenase in *Synechococcus* PCC 7942 was explored (Asada et al., 2000). However, the recombinant [Fe]-hydrogenase was not functional due to the lack of knowledge in the accessory genes associated with the maturation process.

Given the above technical advances in hydrogen production associated with the hydrogenase engineering in cyanobacteria, more efforts are needed particularly to increase the availability of molecular biological tools and genetically amenable strains for enhancing functional expression of recombinant hydrogeneases. Moreover, only four crystal structures of [NiFe]-hydrogenaes are available to date, all from *Desulfovibrio* species (Ghirardi et al., 2007). The availability of more crystallographic data will help the understanding of catalytic activities, functionalities, maturation mechanics, and structure of cyanobacterial hydrogenases as well

as the genetic engineering of endogenous cyanobacterial hydrogenases that are insensitive to oxygen.

4.1.2. Macronutrient deprivation

Photosynthetic hydrogen evolution increases drastically when essential macronutrients, such as sulfur, nitrogen, and phosphorous used for the biosynthesis of essential biomolecules, are removed from the culture media because, upon these stresses, photoautotrophic organisms can exhibit a wide range of abrupt metabolic changes such as re-routing cellular energy for the accumulation of glycogen reservoirs. In addition, by depriving sulfur which is a key element of methionine and cysteine, certain proteins such as the D1 subunit of the PSII reaction center cannot be properly synthesized and an increased hydrogen evolution is observed concomitantly with this stressing strategy.

Although sulfur stress strategies are commonly used to enhance hydrogen production in algae, its use for cyanobacterial systems has not been extensively examined. Cyanobacterial sulfur deprivation is different from its algal counterpart in that hydrogen evolution does not require illumination as cyanobacteria are capable of evolving hydrogen in appreciable quantities under dark anaerobic conditions. This feature suggests that hydrogenase expression is likely constitutive and independent of the circadian clock. The effects of depriving micronutrients on the hydrogen production have been examined using the non-diazotrophic strains of *G. alpicola* CALU 734 and *Synechocystis* sp. PCC 6803 (Antal et al., 2005). Upon nitrogen deprivation, *G. alpicola* CALU 734 increases its glycogen reservoirs by almost 40%. Similarly, there is a pronounced increase in glycogen content for both *G. alpicola* CALU 734 and *Synechocystis* sp. PCC 6803 upon sulfur deprivation. As a result, hydrogen production can last for at least 8 h with a peak rate of 0.1–0.15 μmol H_2 mg Chl α^{-1} h^{-1} with a significant increase in hydrogenase activity, leading to a speculation that the cellular concentration of oxygen is diminished due to the impairment of the photosynthesis associated with sulfur deprivation.

Further studies into cyanobacterial sulfur stress could aim at utilizing strains, such as *Sirulina platensis*, which can effectively accumulate glycogen upon deprivation of micronutrients (Yu and Takahashi, 2007). A combinatorial study through stressing phosphorous, nitrogen, and sulfur is another possible avenue to explore (Antal et al., 2005; Berchtold and Bachofen, 1979; Zhang et al., 2008). Finally, little is known about the regulation of hydrogenases and other enzymes involved in glycogen fermentation upon the sulfur stress as well as whether sulfur deprivation is a reversible metabolic stress. Impetus for novel approaches in establishing cyanobacterial sulfur deprivation for sustained hydrogen production lies in the understanding of these biochemical mechanisms.

4.2. Overall low hydrogen productivity

A fundamental tenet for hydrogen production in biological systems, including cyanobacteria, is that hydrogen is released as a secondary metabolite to dispose of the excess reductants associated with cellular metabolism, resulting in unsustainable hydrogen production with a diminutive yield. In order to address this innate technical issue, genetic manipulation of cyanobacteria has been employed to overcome at least the following challenges: (1) the short turnover rate of the heterocysts; (2) the presence of an uptake [NiFe]-hydrogenase; (3) the competition for reductants by other assimilatory pathways; (4) the low photochemical efficiency of the light-harvesting antennae. In conjunction with various biochemical engineering approaches, such as innovative cultivation techniques and efficient photobioreactor systems, these genetic strategies have contributed major advances to enhance hydrogen production in cyanobacteria.

4.2.1. Increasing heterocyst frequency

As previously stated, heterocyst is a unique feature associated with diazotrophic cyanobacteria in which nitrogen fixation takes place. Technically, it is favorable to utilize diazotrophic cyanobacteria that are heterocystous for hydrogen production due to the anoxic environment inside the heterocyst that allows the nitrogenase to function (Lee, 2008). However, nitrogenase-mediated hydrogen evolution is energetically expensive as it requires 16 ATP molecules for every hydrogen molecule being produced. Although heterocyst formation can be activated through nitrogen deprivation, the frequency (number of heterocysts per 100 vegetative cells) is fairly low. For a heterocystous strain *Anabaena* sp. PCC 7120, the frequency was approximately 10% under nitrogen starvation (Buikema and Haselkorn, 2001). When vegetative cells differentiate into heterocysts, a wide variety of physiological changes take place and these changes are orchestrated through a number of regulatory pathways. Based on a transcriptomic study, nearly 600–1000 genes are specifically activated during the heterocyst formation (Callahan et al., 2001). In light of this observation, it is of utter importance to deduce the molecular machinery and identify the key genes involved in heterocyst formation and nitrogen fixation.

Currently, only a few genes are known to be associated with the regulation of heterocyst formation. Cyanobacteria monitor and sense the overall level of nitrogen indirectly through the TCA cycle intermediate 2-oxoglutarate. When cellular nitrogen levels (e.g. ammonia) are plentiful, the genes encoding for the enzymes involved in nitrogen fixation (namely nitrogenase and uptake [NiFe]-hydrogenase) and the genes involved in heterocyst formation are repressed. Conversely, when cyanobacteria are starved in nitrogen, the presence of 2-oxoglutarate signals the binding of NtcA (a transcriptional activator) to the promoter of its gene *ntcA* (as well as other genes such as *glnA* in *Synechococcus* PCC 7942). This in turn causes the activation of a cascade of genes involved in heterocyst formation and maturation. It is also important to note the expression of the uptake [NiFe]-hydrogenase genes (collectively known as *hup* genes) is also coupled with heterocyst formation (Golden and Yoon, 2003). Through mutagenesis and complementation studies (Buikema and Haselkorn, 1991), it was shown that the key regulatory gene responsible for the formation of the heterocyst is *hetR*. Although the biological role of HetR has not yet been determined, strains that lack *hetR* are unable to form a heterocyst. Interestingly, plasmid-based overexpression of *hetR* causes an overall increase in heterocyst frequency, but the heterocyst size is often irregular and the formation is not synchronized. The *hetR* gene belongs the *het* gene family which also includes *hetF*, *hetC*, and *hetL*. These genes are critical for the formation and maintenance of a heterocystous state. The formation of heterocyst is also suppressed by a number of genes, most importantly *hetN* and *patS* (Golden and Yoon, 2003). It is been speculated that the initial patterning of approximately one heterocyst per every 10 vegetative cells is influenced by *patS*, whereas *hetN* is then responsible to upkeep this pattern. In addition, it is of vital importance that the cells surrounding a heterocyst (conducting nitrogen fixation) remain vegetative, as heterocysts lack the ability to fix carbon. Thus, carbohydrates which are required for supplying the reducing power during nitrogen fixation in heterocysts are solely provided by the vegetative cells. While the above characterized genes can be rational targets for manipulation (e.g. upregulating *hetR* expression and downregulating *patS* and *hetN* expressions), other candidate genes remain to be identified. For example, upregulating *hglK* expression potentially fortifies the already present glycolipid layer, thus strengthening the heterocyst and minimizing its breakage (Black et al., 1995). A thorough understanding of these candidate genes associated with the formation, maintenance, and regulation of heterocyst will be required for effective genetic

Table 5
Hydrogen production via a spatial separation scheme for selected uptake [NiFe]-hydrogenase-deficient cyanobacterial strains.

Strain Name	Mutation Description	Atmosphere; light intensity		Maximum H_2 Evolution	References
		Growth stage	H_2 assay stage		
Anabaena sp. strain CA N9C	Nitroglycerin mediated chemical mutagenesis	Air enriched with 2% CO_2; 250 μmolE m^{-2} s^{-1}	Argon environment; 1200 μmolE m^{-2} s^{-1}	24 μmol mg Chl α^{-1} h^{-1}	Kumar and Kumar (1991)
Anabaena variabilis AVM13	Transposon mediated disruption of *hup*LS operon	Air enriched with1% CO_2; 100 μmolE m^{-2} s^{-1}	Same as growth condition	68 μmol mg Chl α^{-1} h^{-1}	Happe et al. (2000)
Nostoc punciforme NHM5	Transposon mediated disruption of the *hupL* gene	Air enriched with1% CO_2; 40 μmolE m^{-2} s^{-1}	99% Ar, 1% CO_2; 40 μmolE m^{-2} s^{-1}	14.5 ± 3.5 μmol mg Chl α^{-1} h^{-1}	Lindberg et al. (2002)
Anabaena sp. PCC 7120: *hupL*$^-$	Insertional inactivation of *hup*L gene through recombination	Air enriched with1% CO_2; 70 μmolE m^{-2} s^{-1}	99% Ar, 1% CO_2; 70 μmolE m^{-2} s^{-1}	~52 μmol mg Chl α^{-1} h^{-1}[b]	Masukawa et al. (2002)
Anabaena sp. PCC 7120: *hupL*$^-$/*hoxH*[a]	Insertional inactivation of *hup*L and *hoxH* genes through recombination	Air enriched with1% CO_2; 70 μmolE m^{-2} s^{-1}	99% Ar, 1% CO_2; 46 μmolE m^{-2} s^{-1}	~50 μmol mg Chl α^{-1} h^{-1}[b]	Masukawa et al. (2002)
Anabaena sp. PCC 7422 Δ*hupL*	Insertional inactivation of *hup*L gene through recombination	Air enriched; 20 μmolE m^{-2} s^{-1}	95% Ar, 5% CO_2; 100 μmolE m^{-2} s^{-1}	100 μmol mg Chl α^{-1} h^{-1}	Yoshino et al. (2007)

[a] Bidirectional [NiFe]-hydrogenase gene.
[b] Numerical values estimated from graphical readings.

engineering of cyanobacterial strains to sustain the heterocystous state for hydrogen production.

4.2.2. Inactivating uptake [NiFe]-hydrogenase

Hydrogen consumption is an undesirable process during hydrogen production, particularly for cyanobacteria. Generally, hydrogen consumption is mediated by the [NiFe]-uptake hydrogenase, which is the smallest hydrogenase found in cyanobacteria probably because it only catalyzes the oxidation of hydrogen. Structurally, uptake [NiFe]-hydrogenase contains two subunits, HupL and HupS, respectively encoded by the *hup*L and *hup*S genes (Shestakov and Mikheeva, 2006). An apparent strategy to improve hydrogen production is to prevent hydrogen consumption by inactivating uptake [NiFe]-hydrogenase. Mutants deficient in uptake [NiFe]-hydrogenase have been derived through various library screening or targeted gene knockout techniques (Table 5). While the approach can increase hydrogen yield noticeably, hydrogen consumption is rerouted entirely to an alternative pathway via bidirectional [NiFe]-hydrogenase (Tamagnini et al., 2007). Since hydrogen consumption serves as a physiological means to balance cell's redox potential by refilling the reducing power lost during nitrogen fixation, lack of this process would perturb nitrogen assimilation and consequently more carbohydrates would be dissimilated to maintain the electron supply. It appears that hydrogen consumption is physiologically inevitable, particularly for nitrogen-fixing cyanobacteria, since further inactivation of bidirectional [NiFe]-hydrogenase drastically decreases hydrogen productivity (Masukawa et al., 2002).

4.2.3. Eliminating competition for electrons

Fd is the central hub in the electron transfer chain for supplying electrons to various assimilatory pathways with their key enzymes (such as nitrogenase, hydrogenase, nitrite reductase, sulfite reductase, and FNR) as electron acceptors. The chief competitor of nitrogenase, and hydrogenase, which are involved in hydrogen evolution, is FNR. FNR primarily shuttles electrons to reduce $NAD(P)^+$ to NAD(P)H, most of which is subsequently fed into the Calvin-Benson cycle to fix CO_2 (Seibert et al., 2008). Thus, an apparent strategy would be to re-route the electron flow to effectively reach nitrogenase and hydrogenase for hydrogen evolution. The fusion of hydrogenase with PSI (discussed in Section 4.1.1) was a typical study to demonstrate this strategy (Ihara et al., 2006). When utilizing this hybrid protein complex, the electron flow forms a 'shortcut' towards hydrogen production by elimination of the reliance on Fd for supplying electrons. Although this unique hydrogen production system had a considerably higher yield in hydrogen production, the demonstration was conducted "in vitro" and it would be valuable to incorporate this strategy "in vivo" by creating novel strains with an altered electron transport chain for effective shuttling of electrons to hydrogenase. Conceptually, the aforementioned sulfur deprivation strategy can reduce the competition for electrons by bringing photoautotrophy to a halt so that glycogen is used as the source of electrons to reduce $NAD(P)^+$. Unlike the algal [FeFe]-hydrogenases, cyanobacterial [NiFe]-hydrogenases can utilize NAD(P)H as a substrate to evolve hydrogen (Yu and Takahashi, 2007). Furthermore, genetic engineering strategies can focus on knocking out nonessential assimilatory pathways (e.g. xanthine oxidation mediated by xanthine oxidase) to reduce the competition for electrons.

4.2.4. Increasing quantum solar efficiencies

Cyanobacterial Chl *a* molecules are housed in a specialized proteinous structure called a phycobilisome. In total, there are four types of phycobilisomes, each absorbing light at a different wavelength. Two of these phycobilisomes, i.e. phycocyanin and allophycocyanin absorbing light at wavelengths of 620 and 650 nm, respectively, are universal to cyanobacteria. Similar to green algae, a low photochemical efficiency can riddle biophotolysis for hydrogen production in cyanobacteria, in which only a small fraction of the photons from sunlight are used for photosynthesis. An idealistic strategy (effectively adopted for green algae) to circumvent this issue is the truncation of the light harvesting complexes (i.e. antennae). Although several genes associated with the regulation of the light harvesting complexes in green algae have been extensively identified and studied (see Table 3), such work for cyanobacteria is still in its infancy. To date, only one phycocyanin-deficient mutant (PD1) has been derived from the non-nitrogen fixing strain *Synechocystis* PCC 6714 (Nakajima et al., 2001). Characterization of this mutant suggests that the genes involved in the regulation of phycobilisomes in *Synechococcus* PCC 7942 may reside in the *cpc* operon. Moreover, the phycocyanin-deficiency phenotype was brought upon through a single nucleotide mutation to the *cpcC* gene. Although hydrogen production studies have yet to be conducted utilizing PD1, this strain is promising by showing 50% higher photosynthetic activities at high light intensities, in comparison to its wild-type counterpart.

5. Conclusions

Typically, using the cell factories of photosynthetic algae and cyanobacteria with the feedstock of carbon dioxide represents both an environmentally-friendly and economic solution to hydrogen production. However, large-scale photosynthetic hydrogen production is often limited by various genetic, metabolic, and

physiological defects associated with these organisms, in particular the inherent incompatibility between oxygenic photosynthesis and hydrogen evolution and poor photochemical efficiencies due to non-photochemical quenching. Significant progress has been made to overcome these challenges through the development of novel bioprocesses and genetic engineering strategies, including temporal or spatial separation of aerobic photosynthesis and anoxic hydrogen production, protein engineering to reduce oxygen sensitivity of hydrogenases, increasing photosynthetic efficiency by truncating the antenna size, and developing more effective photobioreactors. Given these technological advances, this biotransformation platform will require further biochemical and genetic revamping in major areas to be able demonstrate its economic feasibility.

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Glossary

Biophotolysis: Decomposition of water carried out by biological systems through the absorption of light and, as a result, molecular hydrogen is released.

Chemoheterotrophy: A light-independent biosynthesis using organic compounds to provide carbon skeletons, and organic and inorganic compounds as electron donors and energy sources.

Dark fermentation: A light-independent catabolism of organic compounds (e.g. carbohydrates) acting as the electron donors.

Nitrogen fixing: A process in which organisms (known as diazotrophs) convert atmospheric nitrogen into a more useable form of ammonia by nitrogenase.

Photoautotrophy: A light-dependent biosynthesis using water as an electron donor and simple inorganic molecules (e.g. CO_2) as a carbon source to form complex organic compounds.

Photofermentation: A light-dependent complete catabolism of organic compounds (e.g. organic acids) to yield CO_2 and molecular hydrogen. Also known as pseudofermentation.

Photoheterotrophy: A light-dependent biosynthesis using organic compounds to provide carbon skeletons and electrons.