6 Characterization and Screening of Algal Strains for Sustainable Biohydrogen Production: Primary Constraints

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Abstract

Algae have emerged as one of the most promising sources for biofuel production. In particular, microalgae can provide several different types of renewable biofuels like biodiesel, ethanol, and biohydrogen. Oxygenic photosynthesis splits water to release oxygen gas and uses the hydrogen atoms to drive the reduction of carbon dioxide to sugars. Under some circumstances, cyanobacteria are able to release the reductant as hydrogen gas. Hydrogen is an excellent source for fuel cells and has some attractive features such as three times more potentiality than ethanol. Algal communities including cyanobacteria can produce H_2 through three main routes: (1) H_2 production directly from native bidirectional hydrogenase, (2) H_2 production from a native nitrogenase, and (3) H_2 production from an introduced hydrogenase. Over the last decade or so, several new algal hydrogenases have been reported in literature, and efforts have been undertaken by manipulation of genetic pathways and metabolic engineering approaches. However, such approaches have shown constraints in terms of scale-up at the industrial level. This chapter highlights the aspect of metabolic engineering approaches and underlying constraints for biohydrogen production from algae. This chapter mainly discusses biohydrogen production potential of algae with a focus on understanding of biomass production, optimization of H_2 production in response to strength of selected solution, and pH of the culture medium.

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6.1 Introduction

The progress of human civilization over centuries has been always dependent on energy. Supply of clean and sustainable energy is arguably the most important scientific and technical challenge facing humanity in this century (Lewis and Nocera 2006). Global energy consumption is projected to increase by at least twofold in the middle of this century due to population outburst and rapid economic growth. Presently, global energy consumption is approximately 15 TW, while it is estimated to increase to 27 TW in the year 2050 and may surpass 40 TW by 2100 (Lewis and Nocera 2006). The high energy demand could be met, in principle, from fossil energy resources, primarily derived from plant biomass. However, resulting carbon dioxide (CO_2) emissions due to burning of fossil fuels will severely affect global climate along with rise in air temperature (IPCC 2014). Therefore, it is important to develop technologies for carbon-neutral energy production which can meet global energy demand and at the same time safeguard environment. Except nuclear energy, other promising alternative renewable energy sources are mainly ethanol and hydrogen gas, which are derived from biological organisms. Organisms that produce hydrogen photobiologically can be broadly divided into two groups: photosynthetic bacteria and oxygenic photosynthetic organisms. Photosynthetic bacteria require organic compounds or reduced sulfur compounds as electron donors which are generally limited. On the other hand, oxygenic photosynthetic organisms use water as an electron donor, which is available in plentiful. Among oxygenic photosynthetic organisms, prokaryotic and eukaryotic microalgae have emerged as one of the most promising sources for biofuel production. In particular, microalga can be excellent source for various renewable biofuels such as biodiesel, ethanol, and biohydrogen (Darzin et al. 2010; Lakaniemi et al. 2011; Nayak et al. 2014). During oxygenic photosynthesis, water is split to oxygen gas (O_2), and hydrogen atoms are used to reduce CO_2 to sugars. Under certain conditions, microalgae are able to release the reductant as hydrogen gas (Melis 2002). Hydrogen (H_2) is an excellent fuel due to its highest energy efficiency (143GJ $tonne^{-1}$) (Nayak et al. 2014).

Algal communities including cyanobacteria can produce H_2 through three main routes: (i) H_2 production mediated by native bidirectional hydrogenase enzyme, (ii) H_2 production by native nitrogenase enzyme, and (iii) H_2 production from an introduced hydrogenase enzyme. Hydrogen production in prokaryotic microalgae (cyanobacteria) was first reported more than a century ago (Jackson and Ellms 1896). In cyanobacteria, an alternate nitrogenase-based H_2 production pathway is present, thereby catalyzing unidirectional production of hydrogen. In 1942, Hans Gaffron and co-workers demonstrated hydrogenase activity in the eukaryotic green microalga *Scenedesmus obliquus* (Gaffron and Rubin 1942; Homann 2003; Melis and Happe 2004). Hydrogenase activity is not observed in all eukaryotic microalgal groups which also include green algae (Brand et al. 1989; Boichenko et al. 2004; Melis and Happe 2004). It has been reported that some eukaryotic algae can produce H_2 using low-potential electrons derived either from light-driven photosynthetic pathways or during organic substrate fermentation (Brand et al. 1989; Happe and Kaminski 2002; Boichenko et al. 2004; Ghirardi et al. 2007). Over the last decade or so, several new algal hydrogenases have been reported in literature (e.g., Leino et al. 2014), and efforts involving manipulation of genetic pathways coupled with metabolic engineering approaches have been attempted for large-scale production of biohydrogen. However, such approaches have shown considerable constraints and challenges in terms of scale-up at the industrial level (Levin et al. 2004). Hydrogen production in microalgae is currently limited by technological constraints (Dubini et al. 2014). Specific limitations include:

(i) Extreme sensitivity of hydrogenases to molecular oxygen
(ii) Low reductant availability for hydrogenase activity due to the existence of competing metabolic pathways that converge at the level of ferredoxin (FD)

(iii) Downregulation of photosynthetic electron transport and establishment of cyclic electron transfer around photosystem I (PSI) under anaerobic H_2-producing conditions
(iv) Low level at which light saturation occurs during photosynthesis
(v) Reversible nature of hydrogenases that results in consumption of H_2 under high H_2 partial pressure
(vi) Low level of hydrogenase expression

The main emphasis of this chapter has been focused on elucidating biohydrogen production potential in microalgae such as prokaryotic cyanobacteria and eukaryotic green algae. Pertinent issues such as types of enzyme that catalyze biohydrogen production, microalgal biomass optimization, and the role of culture conditions from the context of pH and carbon source, in addition to selection of strains for H_2 production, have been thoroughly discussed. In the last part of this chapter, application of metabolic engineering approaches toward large-scale production of biohydrogen from microalgae and associated underlying constraints have been also highlighted.

6.2 Types of Biofuels from Algae

Algal biomass can be converted into biofuel by several processes including thermochemical and biological processes (Bridgwater 2003). Thermochemical processes such as gasification, pyrolysis, liquefaction, or even direct combustion can convert stored energy into gases like hydrogen (Bridgwater 2003; Murphy et al. 2013). Biological processes such as fermentation of biomass produce energy carriers, namely, bioethanol, biomethane, and biohydrogen (Hu and Gao 2003; Hu et al. 2008; Mata et al. 2010; Lakaniemi et al. 2011; Nayak et al. 2014) (Fig. 6.1). Optimization of microalgal biomass production is dependent on several biotic and abiotic factors. The selection of algal strain with most efficient biohydrogen potential is of utmost importance during scaling up of biomass.

6.3 Fundamentals of Biohydrogen Gas Production by Algae

6.3.1 Types of Algal Group

Algae represent a heterogeneous group of chlorophyll a containing oxygenic photosynthetic organisms (Falkowski et al. 2004). They are capable of fixing large amounts of carbon dioxide (approximately 50 Gt carbon fixed per year) while contributing approximately 40–50 % oxygen in the atmospheric pool (Field et al. 1998; Uitz et al. 2010; Giering et al. 2014). Algal communities can be found in terrestrial and aquatic habitats and adapt to varying temperature, pH

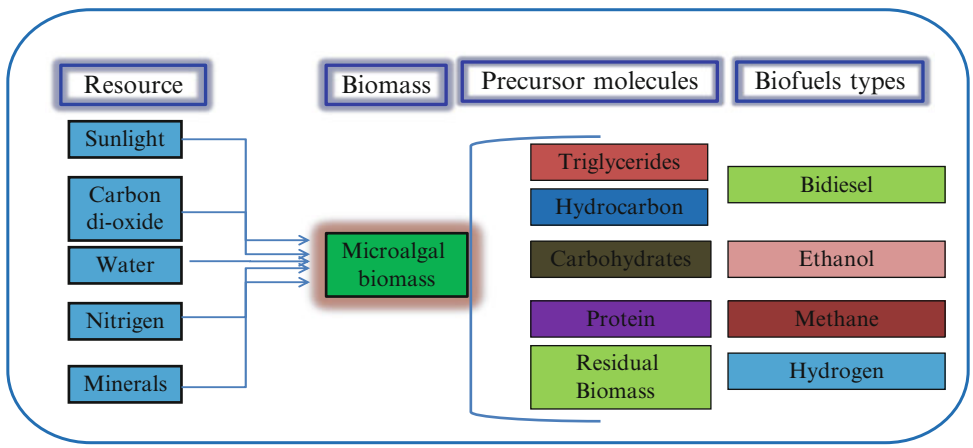

Fig. 6.1 Types of biofuel derived from microalgae grown in the presence of natural resources and inorganic minerals (Modified from Darzins et al. 2010)

and salinity conditions (Uitz et al. 2010). Microalgae are highly productive, represent 0.2 % of the world's total photosynthetic biomass inventory on a global scale, with rapid turnover of biomass (cell doublings of 1–4 per day) (Gallagher 2011; Slade and Bauen 2013). In microalgae, photosynthetic efficiency is higher by almost 20 % under available photosynthetically active radiation (PAR). In terrestrial crops, generally lower photosynthetic conversion efficiencies have been observed (Dismukes et al. 2008). For example, sugarcane, one of the most productive of all terrestrial crops, has a photosynthetic efficiency of 3.5–4 % (Odum 1971; Dismukes et al. 2008). The sugars formed during photosynthesis are converted to cellular molecules such as carbohydrates, proteins, and lipids that ultimately make up the biomass. Therefore, sustainable and carbon-free renewable energy sources such as biohydrogen can be effectively obtained from microalgal biomass while safeguarding the environment. In this context, microalgae have become a target for bioenergy production on an industrial scale.

Over 40,000 species of algae have been identified, and that number almost certainly represents a small fraction of the true unexplored diversity (Hu et al. 2008; Falkowski and Raven 2013). Algal classification is primarily dependent upon morphological and physiological attributes such as whole organismal morphology, cellular anatomy and ultrastructure, photosynthetic pigments, and metabolism (Lee 2009). The biological divisions that encompass various groups of algae are Cyanophyta (cyanobacteria); Prochlorphyta, Glaucophyta, and Rhodophyta (red algae); Cryptophyta (cryptomonads); Chlorophyta (green algae); Euglenophyta, Chloroarachniophyta, and Pyrrophyta (dinoflagellates); and Chromophyta (heterokonts) (Graham et al. 2000; Lee 2009). Generally macroalgae are not considered as potential candidates for commercial scale biohydrogen production due to slower growth rate and specific nutritional requirements (Pedersen and Borum 1996). According to Lee (2009), algae can be divided into four major evolutionary groups consisting of ten division including cyanobacteria and green microalgae (Chlorophyta) which are of global interest as major biohydrogen feedstock. There are other systems of algal classification where groupings are different under Chlorophyta and Chromophyta (Van den Hoek et al. 1995; Graham et al. 2008).

6.3.2 Microalgal Biohydrogen Gas Production

Microalgae use solar energy to convert water into hydrogen gas. Inside a microalgal cell, water is split into proton (H^+) and oxygen (O_2) in the presence of light. The H^+ gets converted into molecular hydrogen in the presence of hydrogenase enzyme through a process known as direct photolysis (Ayhan 2009). Hydrogen production in this process is low because (i) immediately after formation, H_2 and O_2 are converted into water and (ii) sensitivity of the enzyme hydrogenase to oxygen (Nath and Das 2004). Advantages and disadvantages of different light-dependent hydrogen production process have been summarized in Table 6.1.

The inhibitory effect can be overcome by adopting indirect biophotolysis during large-scale biohydrogen production. Indirect biophotolysis consists of two stages: in stage I, the cells synthesize organic compounds (mostly glucose) by photosynthesis, and during the process, oxygen is released. In stage II, algal cells degrade stored organic compounds under anaerobic condition (Melis and Melnicki 2006). During stage II, oxygen and hydrogen are evolved separately. Stage II can happen in the presence or absence of light, also known as photofermentation and dark fermentation, respectively (Guan et al. 2004). The concept of two-staged hydrogen production by microalgae and factors that affect hydrogen yield in stage I and stage II has been illustrated in Fig. 6.2. It has been also shown that growing algal cells in stage I produce optimum level of hydrogen (Rashid et al. 2013). Photosynthetic cyanobacteria and green algae provide a more promising pathway for generation of hydrogen on a large scale compared to hydrogen produced during non-photosynthetic fermentation (Roy

Table 6.1 Summary of advantages and disadvantages of light-dependent hydrogen production processes in cyanobacteria and eukaryotic microalgal biomass

Microalgal groups	Preferable light-dependent metabolic pathway for H_2 production	Advantages	Disadvantages
Cyanobacteria	Indirect biophotolysis through nitrogenase	H_2 evolution is separated from O_2 evolution	High-energy-dependent process
		Spatial separation in heterocystous N_2-fixing cyanobacteria	Biosynthesis and maintenance of heterocysts
		Temporal separation (light/dark) in nonheterocystous cyanobacteria	Significant ATP requirement for nitrogenase. The presence of uptake hydrogenase
			Reoxidize produced molecular hydrogen
Green microalgae	Direct and indirect biophotolysis through bidirectional hydrogenase	Hydrogen-economy strategy based on a virtually limitless and renewable source	Production of O_2 and H_2 simultaneous
		Energy cycle is carbon-free	Inhibition of hydrogenase by O_2
		Theoretical energy efficiency is much higher for hydrogen production from biophotolysis (40 %) compared to hydrogen production from biomass (1 %)	
		One of the most promising processes due to separate production of O_2 and H_2	

Modified from Dasgupta et al. 2010a, b

et al. 2014). Cyanobacteria and green microalgae absorb light through pigments that are associated with two photosystems, photosystem I (PSI) and photosystem II (PSII) (Fig. 6.3).

The absorbed light energy is transferred from antenna pigments to chlorophyll reaction center molecules where charge separation occurs, yielding oxidants and reductants (Hallenbeck 2012). The strong oxidant generated by PSII extracts electrons from water while releasing oxygen and protons as by-products (Fig. 6.2). The generated electrons reduce a series of membrane-bound and membrane-soluble carriers, ultimately reducing oxidant generated by PSI (Falkowski and Raven 2013). Photosystem I generates a reductant that eventually reduces the iron-sulfur protein ferredoxin, which plays a vital role in several metabolic processes such as cyclic and noncyclic photophosphorylation and nitrogen fixation (Bothe et al. 2010). The main function of PSI is to provide electrons to generate NADPH via ferredoxin-NADP oxidoreductase (FNR). The NADPH molecule, along with ATP, is needed for fixing carbon dioxide via Calvin-Benson-Bassham cycle ultimately resulting in the production of carbohydrate molecules. However, under anaerobic conditions in the absence of carbon dioxide, NADPH or reduced ferredoxin reduces protons to yield hydrogen gas, a reaction catalyzed by hydrogenase. In cyanobacteria, NADPH is the likely electron donor to hydrogenase, whereas ferredoxin links photosynthetic electron transport directly to hydrogen production in case of green microalgae (Hallenbeck 2012).

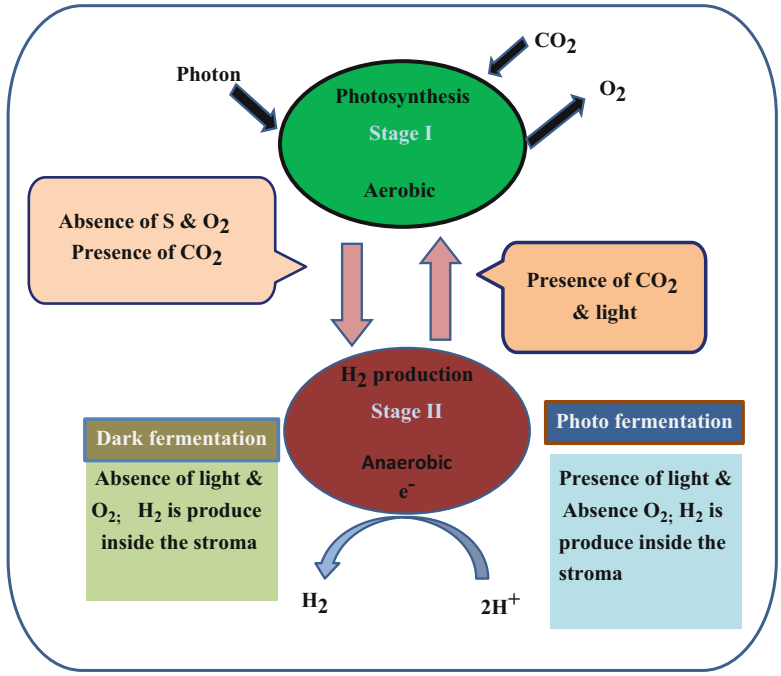

Fig. 6.2 Schematic representation of two-staged hydrogen production within the chloroplast or thylakoid of an oxygenic photosynthetic eukaryotic/prokaryotic cell (Modified from Rashid et al. 2013)

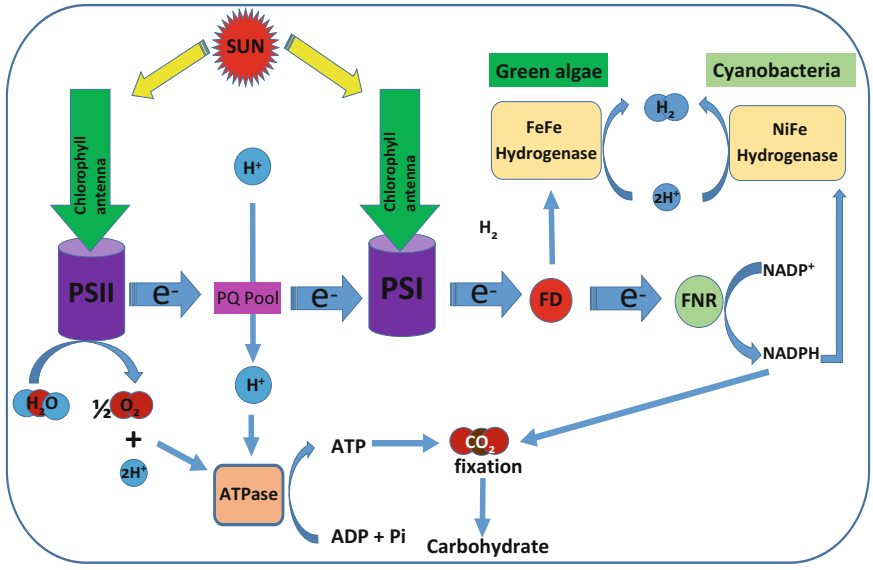

Fig. 6.3 Hydrogen production in cyanobacteria and green algae through photosynthesis-based pathways using different hydrogenases. *PS* photosystem, *PQ* plastoquinone, *FD* ferredoxin, *FNR* ferredoxin-NADP oxidoreductase (Modified from Hallenbeck 2012)

Immobilization and sulfur (S) starvation are the key intermediate steps of stage I and stage II, respectively, during indirect photobiolysis. For immobilization, microalgal cells are suspended in a solidifying material and cut into small pieces. Immobilized cells have higher stability and produce more hydrogen than free cells. Sulfur-deprived cells yield more hydrogen than sulfur-provided cells. In the presence of sulfur, the cell synthesizes proteins which suppress hydrogen production. In cyanobacteria, key enzymes involved in hydrogen production are hydrogenase and nitrogenase (Hallenbeck 2012; Nayak et al. 2014). Under in vivo condition, the enzyme nitrogenase is activated in the presence of light and absence of nitrogen (Bothe et al. 2010), whereas hydrogenase is activated at high light intensity and pH (Tamburic et al. 2011; Rashid et al. 2013). The mechanism during stage II as part of photobiological H_2 production is relatively well understood compared to stage I (Kosourov et al. 2002; Das and Veziroglu 2008; Rashid et al. 2011). An in-depth study of both stages is essential for optimization of hydrogen production in algal cells. Characteristics of photobiological hydrogen production processes along with mediating enzymes in cyanobacteria and green microalgae have been summarized in Table 6.2.

6.3.3 Types of Enzymes for Biohydrogen Production

Three enzymes, namely, hydrogenase (Hox), uptake hydrogenase (Hup), and nitrogenase (nif), are known to be involved in hydrogen generation in cyanobacteria and green microalgae (Tamagnini et al. 2007; Bothe et al. 2010; Berggren et al. 2013) (Fig. 6.4). In this subsection, characteristic features, mode of action, and H_2 production of these three enzymes have been detailed.

6.3.3.1 Hydrogenase

Hydrogenase is a key enzyme for biological hydrogen production which is present across all domains of life including bacteria and plant kingdom (Tamagnini et al. 2007).

Cyanobacteria and green algae also contain this particular class of enzyme. In a cyanobacterial cell, hydrogenase is present in the cytoplasm (Mathews and Wang 2009), whereas in a green algal cell, it is found in the chloroplast (Dubini et al. 2014). In cyanobacteria and green algae, protons and oxygen are produced by splitting of water, and generated protons are converted into hydrogen in both photosystems (PSI and PSII) and mediated by enzymes such as ferredoxin and hydrogenase (Fig. 6.3). The enzyme hydrogenase is sensitive to oxygen and gets deactivated at 2 % O_2 partial pressure (Ghirardi 1997). Hydrogenase oxidizes the low redox electron carrier ferredoxin during reversible reaction. In direct biophotolysis, light drives simultaneous O_2 evolution on the oxidizing side of PSII and H_2 production on the reducing side of PSI, with a maximum H_2:O_2 (mol/mol) ratio of 2:1 (Melis et al. 2000). Under anaerobic conditions, the activity of this enzyme is known to increase significantly (Melis et al. 2000; Bothe et al. 2010). Three phylogenetically distinct classes of this enzyme are known based on the composition of its metal center: [NiFe], [FeFe], or [Fe] (Berggren et al. 2013). The first two classes contain binuclear metal cores with unusual ligand spheres as catalytic centers, whereas the third class contains a mononuclear iron next to a special organic cofactor. Two types of hydrogenases, [FeFe] hydrogenase and [NiFe] hydrogenase, are known to be present in microalgae. The [FeFe] hydrogenase is 10–100 times more efficient than [NiFe] hydrogenase (Madden et al. 2011). The [FeFe] hydrogenase has protein containing [FeFe] catalytic core, while [NiFe] hydrogenase has selenium also in the form of selenocysteine (Volgusheva et al. 2013). The [FeFe] hydrogenases thermodynamically favor hydrogen production relative to [NiFe] hydrogenases, which are frequently regarded as predominantly H_2 uptake enzymes (Ducat et al. 2011). This has led to intense research focusing on the application of [FeFe] hydrogenase for sustainable production of H2 based on metabolic engineering approaches.

In all hydrogenases, including those found in microalgae, the active site has been found to be buried within the proteins. The hydrogenases contain three types of channels or pathways for

Table 6.2 Characteristic features of photobiological hydrogen production strategy and types of enzyme involved in hydrogen production across different microalgae

H_2 production strategies	H_2-evolving enzymes	Characteristic features	Requirements/comments	Microalgal groups
Photobiological hydrogen production utilizing oxygen-evolving photosynthesis				
1. Single stage: direct production of hydrogen				
A. Truly single stage	Hydrogenase	Simultaneous production of H_2 and O_2 in a single cell	Quick separation of H_2 from O_2	Cyanobacteria and green microalgae
B. Operationally single stage		Apparently continuous production of H_2	Operationally simple	
a. Spatial separation	Nitrogenase	Different H_2-producing cells and O_2-producing cells	Subsequent separation of H_2 from O_2	Heterocystous cyanobacteria
b. Temporal separation	Nitrogenase	Cyanobacteria and O_2-producing stages in the same cells	Subsequent separation of H_2 from O_2	Nonheterocystous cyanobacteria
2. Two stage				
Stage I: Accumulation of organic compounds by oxygenic photosynthesis				
Stage II				
A. Anaerobic light-driven H_2 production				
a. Linked to PSI	Hydrogenase	The presence of air	Change of gas phase	Cyanobacteria and green microalgae
b. Linked to PSI	Hydrogenase	PSII inactivation by sulfate depletion	Change of gas phase	Green microalgae
B. Dark fermentation	A variety of pathways			Bacteria

Modified from Bothe et al. (2010), Rashid et al. (2013)

gas access, proton as well as electron transfer. Such channels facilitate the movement of substrate and educts to move between active site and molecular surface (Fontecilla-Camps et al. 2007).

A soluble or loosely membrane associated [NiFe] hydrogenase present in some cyanobacteria. This [NiFe] hydrogenase can produce and take up hydrogen, known as bidirectional hydrogenase (Schmitz et al. 2002) (Fig. 6.4c). The bidirectional hydrogenase, purified from the cyanobacterium *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 6301, is a complex dimer of proteins (HoxF, HoxU, HoxY, HoxH, HoxE) with a molecular weight of 375 kDa (Schmitz et al. 2002; Schwarz et al. 2010; Hallenbeck 2012). It has been also shown that some of the [NiFe] hydrogenase shows tolerance to oxygen (e.g., Ghirardi et al. 2007). Green microalgal cells are known to encode two distinct hydrogenases, namely, HYDA1 and HYDA2, with recent RNA interference-based approach showing that HYDA1 is the predominant H_2-producing enzyme (Godman et al. 2010). It has been shown that HYDA homologs are similar across members of green algae including *Scenedesmus obliquus* and *Chlamydomonas reinhardtii*, and they encode only H-cluster-active site domain and lack additional N-terminal iron-sulfur (FeS) cluster-binding domains (Fe-cluster) (Florin et al. 2001; Happe and Kaminski 2002). Fe-clusters are thought to mediate electron transfer between physiological donors/acceptors

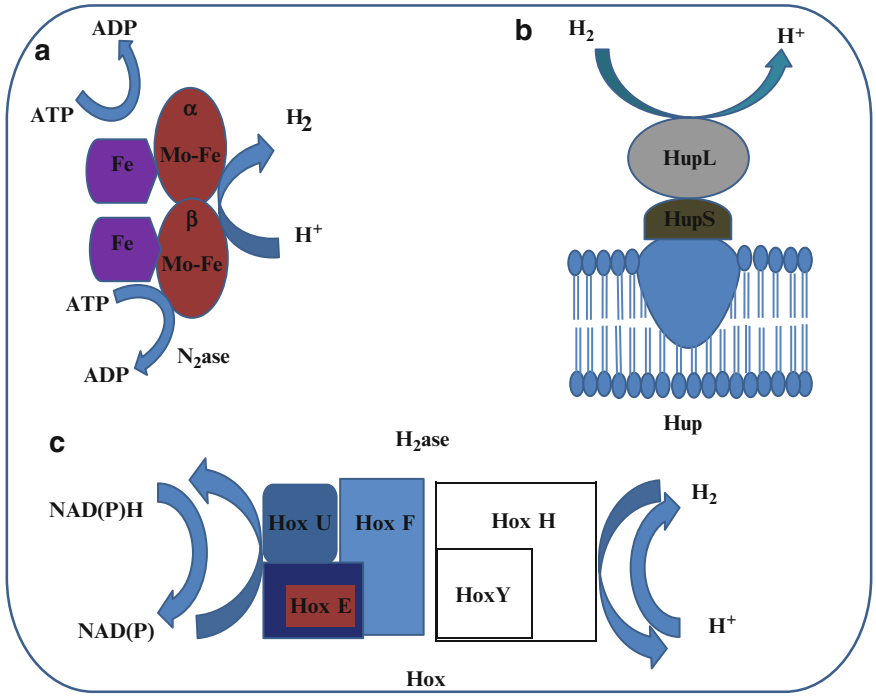

Fig. 6.4 Enzymes involved in hydrogen metabolism inside a cyanobacterial cell (Modified from Bothe et al. 2010; Hallenbeck 2012)

(Vignais and Billoud 2007). It has been also shown that *Chlorella variabilis* NC64A, a green algae, contain *HYDA* genes which code for accessory FeS cluster-binding domains (F-cluster), in contrast to other members of this group. In general, the two hydrogenases coded by *HYDA1* and *HYDA2* genes have different promoter regions and get transcribed and regulated differently in response to varying environmental conditions (Forestier et al. 2003). The Fe hydrogenases from green algae are monomeric proteins of about 45–50 kD in size (Roessler and Lien 1984; Happe and Naber 1993). The nucleus-encoded polypeptides are synthesized in the cytosol as precursor proteins, but the mature protein is localized in the chloroplast stroma of green alga *Chlamydomonas reinhardtii* (Happe et al. 1994).

6.3.3.2 Uptake Hydrogenase

Heterocystous cyanobacterial cells that fix nitrogen appear to have an uptake hydrogenase, whose function is to recover electrons lost during hydrogen production by nitrogenase (Tamagnini et al. 2000). The uptake hydrogenase comprises of two subunits encoded by *hupS* and *hupL* (Schmitz and Bothe 1996). In cyanobacteria, the *hupSL* genes appear to be transcribed predominantly or exclusively in heterocysts such as in *Anabaena* and *Nostoc*, consistent with their role in nitrogen fixation. However, there is one report that indicates that *hupL* is expressed in vegetative cells of *Anabaena variabilis* (Boison et al. 2000). The uptake hydrogenase is known to be resistant to oxygen. Two different mechanisms of oxygen tolerance have been noted in microalgal uptake hydrogenases. First, an additional CN^- ligand bound to nickel of [NiFe] site contributes to oxygen tolerance in this enzyme. Mutant proteins devoid of the nickel-bound CN^- ligand turned out to be oxygen sensitive (Burgdorf et al. 2005). Second, X-ray absorption spectroscopy revealed that the active site of this hydrogenase is coordinated by more oxygen ligands and less sulfur ligands. The different coordination of [NiFe]-active site may also contribute to oxygen tolerance in this enzyme (Burgdorf et al. 2005). For

increased yield of hydrogen, uptake hydrogenase has been modified making them more oxygen tolerant based on protein engineering approaches in cyanobacteria and green algae (Das and Veziroglu 2001; Dasgupta et al. 2010a); however, this enzyme require high redox potential. Because of the limitation of this enzyme, it is not useful for maintaining a stable hydrogen production in microalgae (Lindblad et al. 2001; Rashid et al. 2013).

6.3.3.3 Nitrogenase

Nitrogenase converts N_2 into ammonia and produces hydrogen. Among oxygenic photosynthetic microalgae, only cyanobacteria contain this enzyme. The enzyme nitrogenase can be categorized based on metal, molybdenum (Mo), vanadium (V), and iron (Fe) present in the prosthetic group, namely, Mo-nitrogenase, V-nitrogenase, and Fe-nitrogenase (Tamagnini et al. 2002; Bothe et al. 2010). The reaction of nitrogenase enzyme is energetically inefficient due to irreversible reaction coupled by hydrolysis. Cyanobacteria can be broadly divided into nitrogen-fixing and nonnitrogen-fixing groups. Nitrogen fixation requires ATP (10–16 molecules for per molecule of dinitrogen) and reductants such as NADP or NADPH in cyanobacterial cell (Bothe et al. 2010). Majority of cyanobacterial cells show nitrogenase activity under anaerobic or microaerobic conditions (Bothe et al. 2010).

6.3.4 Biohydrogen Production in Cyanobacteria

Hydrogen production in cyanobacteria is potentially feasible using either reversible hydrogenase or nitrogenase (Bothe et al. 2010). The uptake hydrogenase recycles hydrogen produced by nitrogenase; thus, high levels of hydrogen production require inactivation of *Hup SL* (Fig. 6.5). In a study conducted by Weissman and Benemann (1977), *Anabaena cylindrica* was shown to produce hydrogen continuously for 7–19 days under an argon-CO_2 atmosphere in the presence of nitrogenase; however, the highest rates of production declined to half of the maximum within 5–7 days (in 30 ml per liter culture per hour). In *Anabaena* sp. CA and *Anabaena* sp. IF, hydrogen was produced at a rate of about 20–40 µl mg dry weight^{-1} hr^{-1} in air with higher yields at higher

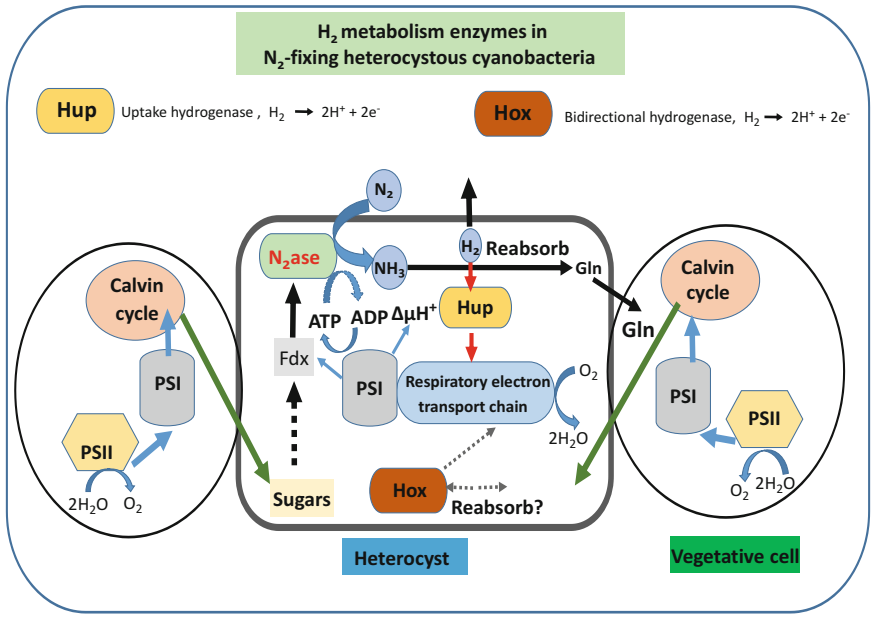

Fig. 6.5 Hydrogen metabolism in heterocystous cyanobacterial filament under active nitrogen-fixing condition (Modified from Schutz et al. 2004)

light intensities (≤180 μEm^{-2}) (Zhang et al. 1983). In *Anabaena* sp. CA, hydrogen production by uptake hydrogenase is dependent on nickel concentration present in the growth medium. Uptake hydrogenase in this train becomes inactivated in the presence of 100 nM Ni^{2+} (Smith et al. 1985). In most nitrogen-fixing cyanobacteria, net hydrogen production by nitrogenase is virtually nonexistent unless the uptake hydrogenase is eliminated (Happe et al. 2000; Masukawa et al. 2002; Tsygankov et al. 2002; Lindberg et al. 2004). A cluster of accessory proteins for [NiFe] hydrogenases, including *hypAB*, *hypCD*, and *hypEF*, is located about 4 kb upstream from *hupSL* in *Nostoc punctiforme*, *Anabaena variabilis*, and *Anabaena* sp. PCC 7120 (Table 6.3).

The functions of Hyp protein and *hyp* gene have not been thoroughly studied in cyanobacteria. In *E. coli*, Fe binds to *HypCD*, which then binds CO and CN^- ligands with the help of *HypEF* in an ATP-dependent reaction (Vignais and Colbeau 2004). Different types of hydrogenase and nitrogenase genes present in members representing cyanobacteria have been summarized in Tables 6.3 and 6.4.

In a comparison of hydrogen production among several heterocystous strains, higher levels of H_2 production have been noted in *Anabaena* sp. strain PCC 7120 compared to *Nostoc* sp. strains ATCC 73102, ATCC 38901, and ATCC 91911 (Schutz et al. 2004). Experiments with *A. variabilis* PK84 were also conducted outdoor in a 4.35 L bioreactor in summer months (United Kingdom) with the longest experiment lasting for 40 days (Tsygankov et al. 2002). The authors found that hydrogen production was highest at outdoor temperature of 30 °C, and they concluded that higher temperature can provide even higher yields of hydrogen (Tsygankov et al. 2002). The amount of hydrogen produced by various cyanobacteria (*Nostoc* spp., *Anabaena* spp., and *Anabaena variabilis* PK84) lacking the uptake hydrogenase in an aerobic environment is in the range of 50–150 nmol H_2 μg Chl*a*$^{-1}h^{-1}$ (Borodin et al. 2000; Happe et al. 2000; Masukawa et al. 2002; Tsygankov et al. 2002; Lindblad et al. 2002; Schutz et al. 2004). Major finding in terms of comparative biohydrogen production has been summarized in Table 6.5.

The uptake hydrogenase may perform differently in different strains of cyanobacteria. A strain of *Cyanothece* sp. PCC 51142 when grown aerobically and incubated under argon produced higher amount of H_2. The H_2 production rate reached 373 mol mg chlorophyll *a* (Chl a)$^{-1}h^{-1}$ under aerobic conditions and over 400 mol mg Chl a$^{-1}h^{-1}$ when grown in the presence of 50 mM glycerol (Bandyopadhyay et al. 2010). The H_2 production rate in a *hupL* mutant strain of *Nostoc* sp. PCC 7422 increased by threefold compared to wild type (80 mol mg Chl a$^{-1}h^{-1}$) (Yoshino et al. 2007). In another *hupL* mutant strain of *Nostoc punctiforme* 29133, hydrogen production increased to 9 mol mg Chl a$^{-1}h^{-1}$ in contrast to trace amounts produced in wild type (Ekman et al. 2011). Increased hydrogen production has been also reported in several other mutant strains of *Anabaena* sp. The uptake hydrogenase-deficient mutant of *Anabaena variabilis* PK84 strain produces more hydrogen (1670.6 mol/mg Chl a/h) compared to wild-type *A. variabilis* (39.4 mol mg Chl a$^{-1}h^{-1}$) when grown anaerobically with 25 % N_2, 2 % CO_2, and 73 % Ar (Sveshnikov et al. 1997; Dutta et al. 2005). Transcription regulation of uptake hydrogenase plays an important role in hydrogen production under nitrogen-fixing condition in cyanobacterial cells (Happe et al. 2000). In N_2-fixing cyanobacteria, *hupSL* transcription is coordinately regulated with nitrogenase along with the differentiation of heterocyst (Happe et al. 2000). In *Anabaena variabilis* ATCC 29413, *Anabaena* sp. PCC 7120, *Nostoc punctiforme* ATCC73102, and *Gloeothece* sp. strain ATCC 27152, the transcription of *hupSL* increases with the decrease in nitrogen fixation (Happe et al. 2000; Lindberg et al. 2000; Oliveira et al. 2004).

6.3.5 Biohydrogen Production in Eukaryotic Microalgae

In eukaryotic microalgal domains, *Chlamydomonas reinhardtii* has emerged as a model organism for studying H_2 metabolism,

Table 6.3 Different type of hydrogenase genes present in cyanobacteria

Strain	Hup	Hox	Hyp	References
Lyngbya majuscula	+	+	+	Tamagnini et al. (2000) and Schutz et al. (2004)
Nostoc sp. Mitsui 38901	+	–	+	Tamagnini et al. (2000) and Schutz et al. (2004)
Nostoc sp. Mitsui 56111	+	+	+	Tamagnini et al. (2000) and Schutz et al. (2004)
Anabaena sp. PCC 7120	+	+	+	Kaneko and Tabata (1997)
Unpublished data	Web site			
Synechococcus sp. PCC 7942	–	+	+	http://genome.jgipsf.org
Prochlorococcus marinus MIT9312	–	–	–	http://genome.jgipsf.org
Prochlorococcus marinus NATL 2A	–	–	–	http://genome.jgipsf.org
Crocosphaera watsonii WH8501	+	–	+	http://genome.jgipsf.org
Trichodesmium erythraeum IMS101	+	–	+	http://genome.jgipsf.org
Nostoc punctiforme ATCC 29133	+	–	+	http://genome.jgipsf.org
Anabaena variabilis ATCC 29413	+	+	+	http://genome.jgipsf.org
Unpublished data	GenBank accession number			
Synechococcus sp. PCC 6301	–	+	+	AP008231GR (Genome)
Synechococcus sp. PCC 7002	–	+	+	AAN03569.;AAN03573
Prochlorococcus marinus MIT9313	–	–	–	BX548175GR (Genome)
Prochlorococcus marinus CCMP1378	–	–	–	BX548174GR (Genome)
Prochlorococcus marinus CCMP1375	–	–	–	AE017126GR (Genome)
Prochlorothrix hollandica	NA	+	NA	AAB53705
Gloeobacter violaceus PCC7421	–	–	–	BA000045GR (Genome)
Arthrospira (spirulina)	NA	+	NA	AAQ63961
Spirulina subsalsa	NA	+	NA	AAO962
Anabaena siamensis TISTR8012	+	+	NA	AAN65267, AAN65266

and substantial progress has been made in recent years to understand mechanisms of H_2 production (Melis et al. 2000; Kruse et al. 2005; Kosourov and Seibert 2009). Detailed genetic and physiological studies describing several aspects of H_2 production in *C. reinhardtii* are available, and several of its mutants related to H_2 metabolism have been made and studied. It has been shown that *C. reinhardtii* has an unprecedented repertoire of metabolic capabilities that allow it to adapt to rapidly changing environmental conditions, including anoxia, which is important for H_2 production. The metabolic flexibility of *C. reinhardtii* and other phototrophic microorganisms (*Chlorella* sp., *Scenedesmus* sp., and *Nannochloropsis* sp.) likely facilitates acclimation to natural energetic fluxes arising from environmental conditions and diurnal light/dark cycles, allowing these organisms to readily adjust their metabolic flux in response to diverse

Table 6.4 Uptake and bidirectional hydrogenases as well as conventional and alternative nitrogenases in top 10 H_2-producing cyanobacterial strains

	Nitrogenase	Alternative nitrogenases	Uptake hydrogenase	Bidirectional hydrogenase	
Cyanobacterial strain	nifHDK1	nifHDK2	vnfDGK	hupLS	hoxY
Synechocystis sp. PCC 6803	–	–	–	–	+
Nostoc sp. Becid 19	+	–	–	+	+
Nostoc sp. XHIID A6	+	–	–	+	+
Anabaena sp. XSPORK 7B	NA	NA	NA	+	+
A. variabilis ATCC 29413	+	+	+	+	+
Anabaena sp. PCC 7120	+	–	–	NA	NA
Nodularia sp. AV33	+	–	–	+	+
Nodularia sp. TRO31	+	–	–	+	+
Calothrix sp. 336/3	+	–	–	+	+
Calothrix sp. XPORK 5E	+	–	–	+	+
Calothrix sp. XSPORK 11A	NA	NA	NA	–	–
Calothrix sp. XSPORK 11A	+	–	–	–	–
Calothrix sp. Becid 33	+	–	–	+	+

Strains source: University of Helsinki Cyanobacteria Collection, UHCC) (Modified from Leino et al. 2014

Table 6.5 Hydrogen production in heterocystous cyanobacteria (wild type/mutant strain) grown under varying growth conditions

Strain	Characteristic features	Growth conditions	H_2production nmol.µg $Chla^{-1}h^{-1}$(maximum)	References
Nostoc punctiforme	$hupSL^-$	Air	6	Lindberg et al. (2004)
N. muscorum	Wild type	Air	4	Scherer et al. (1980)
A. variabilis AVM13	$hupSL^-$	Air	135	Happe et al. (2000)
A. variabilis PK84	$hupSL^-$ – Mo + V grown cell	Air	106	Borodin et al. (2000)
Anabaena sp. PCC7120	Wild type	Air	10	Masukawa et al. (2002)
Anabaena sp. PCC7120	$hupSL^-$	Air	52	Masukawa et al. (2002)
Anabaena sp. PCC7120	$hupSL^-$ $hoxH^-$	Air	50	Masukawa et al. (2002)
Anabaena cylindrica	Wild type	Air + 0.2 % CO + 10 % C_2H_2 + 3 % CO_2	66	Lambert et al. (1979)
Anabaena sp. CA	$hupSL^-$+ Ni and Ni-free	Air	0.4	Smith et al. (1985)
A. variabilis	Wild type	Air	10	Happe et al. (2000)
Anabaena sp. TU37-1	Wild type	Air	3	Kumazawa (2003)

Table 6.6 Hydrogen production by green microalgae in laboratory photobioreactor through direct biophotolysis process

Organism	Maximum evolution rate ($mmolg^{-1}$ hr^{-1})	Maximum productivity ($mmolL^{-1}hr^{-1}$)	Gas for growth; light intensity (wm^{-2})	Gas for H evolution; light intensity (wm^{-2})	References
Chlamydomonas reinhardtii CC124	5.94	0.094	97 % air 3 % CO_2; acetate (17 mM)	Argon; S-free acetate (17 mM)	Kosourov et al. (2002)
Chlamydomonas reinhardtii CC1036	5.91	0.48	Air; acetate (17 mM)	Argon; S-free acetate (17 mM)	Laurinavichene et al. (2006)
Platymonas subcordiformis	0.001	0.002	Air; seawater nutrients; (L/D)	N_2; S-free seawater	Guan et al. (2004)

Table 6.7 Hydrogen production by green microalgae grown in different culture medium

Green microalgae	Growth medium	Maximum rate of H_2 production (ml L^{-1} h^{-1})	References
Chlamydomonas reinhardtii	Acetate(TAP-S medium)	4.3	Laurinavichene et al. (2006)
Chlamydomonas reinhardtii	TAP-S medium	4.5	Winkler et al. (2002a)
Chlamydomonas reinhardtii	TAP(acetate) medium	80–140	Skjånes et al. (2010)
Chlamydomonas moewusii	TAP-S medium	10.0	Winkler et al. (2002a, b)
Chlamydomonas noctigama	TAP(acetate) medium	30–80	Skjånes et al. (2010)
Chlamydomonas euryale	TAP (acetate) medium	22	Skjånes et al. (2010)
Chlorella sorokiniana	TAP (acetate) medium	148	Kumar et al. (2013)
Chlorella pyrenoidosa	TAP (acetate) medium	10	Skjånes et al. (2010)
Scenedesmus obliquus	TAP-S medium	3.6	Winkler et al. (2002a, b)

challenges (Tables 6.6 and 6.7). Several proteins, including hydrogenases, are typically found in strictly anaerobic organisms and are used as part of anoxic metabolism. Although the presence of fermentation pathways in an oxygenic phototroph was initially considered somewhat paradoxical since these pathways are found in anaerobic chemotrophs, however it is now apparent that photosynthetic microbes frequently experience extended periods of limited O_2 availability (Quinn et al. 2002; Steunou et al. 2006; Mus et al. 2007). In eukaryotic microalgae, hydrogenases are solely responsible for H_2 production. The [FeFe] hydrogenase gene sequences derived from green algae indicate that majority of microalgal [FeFe]-hydrogenase genes encode small, monomeric proteins (approximately 45–50 kDa) containing only H-cluster-binding domain (Florin et al. 2001; Wunschiers et al. 2001; Happe and Kaminski 2002; Winkler et al. 2002a, b, 2004; Forestier et al. 2003). However, it was observed that a strain of *Chlorella* sp. NC64A possess an [FeFe] hydrogenase with F-cluster domains and exhibit both fermentative and H_2 photoproduction activities (Das and Veziroglu 2008). These additional FeS F-clusters are found in most bacterial [FeFe] hydrogenase enzymes and are putatively required for electron

Table 6.8 Genes coding for hydrogenase in green microalgae belonging to the division Chlorophyta

Green microalgae	Genes				References
	HYDA	HYDA1	HYDA2	HYDA3	
Chlamydomonas noctigama	–	+	–	+	Skjånes et al. (2010)
Chlamydomonas moewusii	–	–	+	–	Skjånes et al. (2010)
Chlamydomonas reinhardtii	–	+	+	–	Happe et al. (2002)
Chlorella vulgaris	–	+	–	–	Hwang et al. (2014)
Chlorella fusca	+				Hwang et al. (2014)
Scenedesmus obliquus	+	–	–	–	Florin et al. (2001)

+ and – denote the presence and absence

transport from/to electron mediators. Majority of algal [FeFe] hydrogenases lack additional F-clusters. This type of truncated [FeFe] hydrogenases are found only in green microalgae (Dubini et al. 2014). Eukaryotic microalgal hydrogenase genes have been detailed in Table 6.8. A third protein with [FeFe] hydrogenase homology is also present in the genome of *C. reinhardtii* (Accession number EDP03395). However, this protein has similarity with Narf-like protein family that are thought to play a role in FeS cluster assembly in some eukaryotes and possibly do not exhibit activities typical to that of hydrogenase (Balk et al. 2004). The microalgal [FeFe] hydrogenase contains transit peptides of variable length in N-terminus, which are required for translocation to the chloroplast. Majority of microalgal hydrogenase enzymes sequenced to date also contain an insertion of 15–54 amino acids in C-terminus and a smaller insertion, approximately nine amino acids to the N-terminal side of L1 motif. The physiological significance of these insertions is currently unknown; however, they may have roles in regulating enzyme activity, protein interactions, and cellular localization. Anaerobiosis is required to induce hydrogenase activity in *C. reinhardtii*, which is achieved in the laboratory in a variety of ways including (i) purging with inert gas, (ii) providing exogenous reductant, and (iii) allowing cellular respiration to metabolize dissolved O_2. Following the establishment of anaerobiosis, cultures are sealed to prevent introduction of O_2 from the atmosphere. Cultures grown in nutrient-replete media must be maintained under dark condition or at very low light levels to prevent O_2 accumulation from endogenous photosynthetic activity. Hydrogen photoproduction is observed at high initial rates immediately after illumination of dark in anaerobically adapted cells. However, in cultures grown in a nutrient-replete medium, these initial rates of H_2 photoproduction rapidly diminish as O_2 levels from photosynthesis increase and cells adapt to an aerobic metabolism along with fixation of CO_2. In Tables 6.6, 6.7, and 6.8, the rate of hydrogen production by green microalgae under direct biophotolysis using different growth medium in the presence of a variety of hydrogenase genes as reported in the literature has been summarized.

6.4 Primary Constraints of Algal Hydrogen Gas Production

6.4.1 Limiting Factors for Growth and Hydrogen Gas Production

In microalgae, hydrogen production efficiency is determined by physical and chemical factors such as light intensity, temperature, pH, carbon source, and composition of growth medium. Among these factors, we will discuss about the role pH and carbon sources for hydrogen production in microalgae. Some of the above factors have been summarized in Table 6.9.

Table 6.9 Rate of hydrogen production in cyanobacteria and green microalgae under optimal physicochemical condition

Microalgal groups	Strains	Optimum physicochemical conditions				Hydrogen production rate			References
		pH	T °C	Light intensity	Carbon source	(ml L^{-1} culture h^{-1})	µmol mg dw^{-1} h^{-1}	µmol mg chl a^{-1} (protein) h^{-1}	
Heterocystous									
Cyanobacteria	*Aphanocapsa montana*	NA	NA	4–6 Wm^{-2}	Air	NA	0.4	NA	Howarth and Codd (1985)
	Gloeobacter PCC 7421	NA	NA	20 $\mu Em^{-2}s^{-1}$	CO	NA	NA	1.38	Moezelaar et al. (1996)
	Anabaena cylindrica B 629	NA	NA	7000 lux	5 % CO_2	NA	0.103	NA	Lambert and Smith (1977)
	Anabaena variabilis	NA	30	150 $\mu Em^{-2}s^{-1}$	1 % CO_2	NA	0.25	NA	Berberoglu et al. (2008)
	Anabaena azollae	NA	NA	140 $\mu Em^{-2}s^{-1}$	2 % CO_2	13	NA	38	Tsygankov et al. (1998)
Nonheterocystous									
Cyanobacteria	*Synechococcus* PCC 602	NA	NA	20 $\mu Em^{-2}s^{-1}$	CO	NA	0.66	NA	Howarth and Codd (1985)
	Synechocystis sp. PCC 6803	NA	NA	50 $\mu Em^{-2}s^{-1}$	$NaHCO_3$	NA	NA	0.081	Burrows et al. (2008)
	Chroococcidiopsis thermalis CALU 758	NA	NA	70 $\mu Em^{-2}s^{-1}$	1 % CO_2	NA	NA	0.7	Serebryakova et al. (2000)
	Microcystis sp. PCC 7820	NA	NA	20 $\mu Em^{-2}s^{-1}$	CO	NA	NA	0.16	Moezelaar et al. (1996)
	Spirulina platensis	NA	NA	8 Wm^{-2}	NA	4.032	NA	NA	Aoyama et al. (1997)
	Oscillatoria limosa	NA	20	1200 lux	CO_2	NA	NA	0.83	Heyer et al. (1989)
	Oscillatoria miami BG7	7.75	35	100 $\mu Em^{-2}s^{-1}$	CO_2	NA	NA	0.25	Phlips and Mitsui (1983)
Green microalgae	*Chlamydomonas reinhardtii*	7	NA	100 $\mu Em^{-2}s^{-1}$	Acetate (17 mM); S deprived	2.1	NA	NA	Laurinavichene et al. (2006)
	Chlamydomonas sp. MGA 161	8	30	25 Wm^{-2}	5 % CO_2	4.48	NA	NA	Ohta et al. (1987)
	Platymonas subcordiformisa	NA	NA	22 Wm^{-2}	NA	0.048	NA	NA	Guan et al. (2004)
	Chlorella sorokiniana Ce	NA	NA	120 $\mu Em^{-2}s^{-1}$	Acetate;S deprived	1.35	NA	NA	Chader et al. (2009)

6.4.1.1 pH

In microalgae, cellular processes are dependent on intracellular pH (close to neutral), and most algae have limited abilities to tolerate variable pH conditions (Andersen 2005). In growth medium, hydrogen production process significantly depends upon pH. A subtle change in pH can change the end products (CO_2, acetate) of anaerobic process (Khanal et al. 2004; Dsagupta et al. 2010). During photosynthesis, initial pH decreases due to formation of carbonic acid as a result of a chemical reaction between CO_2 and water. After certain period, pH increases due to evolution of oxygen via photosynthesis. Microalgae usually grow at a pH ranging from 5.0 to 9.0 (Song et al. 2011). High pH in culture medium shortens lag time of hydrogen production and increases its rate of production (Khanal et al. 2004). Any changes in pH alter metabolic pathways that mediate hydrogen production. In mixed microbial flora, sucrose degradation increased with pH and maximum efficiency (95 %) was found at pH 9.0 (Lee et al. 2002). This fact can be explained in terms of enzyme activity; hydrogen-producing enzymes (hydrogenase and nitrogenase) are sensitive to pH. Initially, protons, generated by the degradation of endogenic or exogenic carbon source and by the splitting of water, are converted into hydrogen. Later, proton concentration increases; a few of them are entrapped by hydrogenase or nitrogenase (depending upon the light condition applied) and get converted into hydrogen; the rest of them remain unutilized. At low pH value (5.0), hydrogen-producing enzyme inactivates, thereby reducing hydrogen production rate. During sulfur (S) deprivation, pH of the culture medium fluctuates which may cause metabolic disturbance resulting in production of low amount of hydrogen in green microalgae (Khanal et al. 2004). According to Kosourov et al. (Kosourov et al. 2003), hydrogen production rate in S-deprived *Chlamydomonas reinhardtii* was high at pH 7.7 but decreased at pH 6.5. In cyanobacteria and green microalgae, pH requirement for photosynthesis and fermentation vary from species to species. Marine microalgae require different pH compared to freshwater microalgae due to low nitrate requirement in former case (Andersen 2005).

Some algal groups have adapted themselves in highly acidic environments by pumping protons out of the cell using efficient ATP-driven H^+ pumps, one example being the acidophilic *Chlamydomonas acidophila* (Gerloff-Elias et al. 2006). Under very low pH, as much as 50 % of the synthesized ATP inside an algal cell has been observed to be consumed by proton pumps (Bethmann and Schönknecht 2009). Cellular metabolic processes such as increase fatty acid saturation, production of acid-tolerant cell wall proteins, reduction of cell volume, reduction of starch reserves, and production of antioxidants in microalgal cell depend on low pH. Nitrate uptake along with carbon fixation can influence alteration of pH (Bothe et al. 2010, Rashid et al. 2013). It has been shown that in *Gloeocapsa alpicola*, optimal pH for H_2 production has been found to be ranging from 6.0 to 7.0 (Antal and Lindblad 2005). In *C. reinhardtii*, hydrogen production depends on pH of the medium (Antal et al. 2003). Optimization of pH is essential in aerobic and anaerobic phases of photobiological hydrogen production. The production of undesirable intermediate metabolic products can be controlled by developing a correlation between photosynthetic by-products in stage I and intermediate by-products during stage II with pH. Extensive research is available on pH optimization in stage I, but only few studies deal with the effect of pH on stage II (Das and Veziroglu 2008; Rashid et al. 2011, 2013; Dubini et al. 2014). Table 6.9 shows the pH tolerance for different microalgae species under anaerobic (stage II) conditions. Therefore, maintenance of pH is extremely difficult as well as costly when microalgal biomass are grown on a large scale with the objective to produce hydrogen at the industrial scale.

6.4.1.2 Carbon Sources

Carbon sources are also known to influence hydrogen production significantly while having an effect on nitrogenase activity (Mata et al. 2009). The flow of electron from carbon source to the nitrogenase may vary and thus influence hydrogen production in microalgae. Some strains

of green microalgae are able to grow under both very high CO_2 concentrations (20–100 % bubbling of cultures) as well as high temperatures (49–56 °C) (Wang et al. 2008). One example is a strain of *Chlorella sorokiniana* isolated from a hot spring (Sakai et al. 1995); other known examples are strains of *Scenedesmus* sp. (de Morais and Costa 2007; Hanagata et al. 1992) and *Chlorococcum littorale* (Satoh et al. 2002). It is hypothesized that tolerance toward high CO_2 is connected to state transition in favor of PSI (Miyachi et al. 2003; Satoh et al. 2002). Microalgae store carbon in the form of starch or glycogen during photosynthesis and use them under anaerobic condition. These cells can accumulate limited amount of glycogen and starch, and ultimately there is a low yield of hydrogen during anaerobic phases. A significant increase in hydrogen yield is possible by introducing exogenic carbon source in early phase of anaerobiosis (Nayak et al. 2014). A wide variety of exogenic carbon sources are known to be used by algal cells for hydrogen production, namely, glucose, fructose, sucrose, malt extract, malic acid, acetate, and organic wastewater (Rashid et al. 2011). The yield of hydrogen varies according to the source of carbon as well as the cultured microalgal strains. Therefore, selection of carbon source is a prerequisite for establishment of large-scale cultures of microalgae with the objective to produce biohydrogen. Microalgae can use inorganic carbon (CO_2) as well as organic carbon sources (glucose, mannitol, acetate, sucrose) (Hu et al. 2003). Microalgae grown under heterotrophic condition (using organic carbon) could have more potential to produce hydrogen compared to autotrophic condition (inorganic carbon). In heterotrophic condition, high biomass is achieved. Unlike autotrophic condition, light is also not a prerequisite in heterotrophic cultivation. Therefore, heterotrophic cultivation can be cheaper than autotrophic cultivation (Nayak et al. 2014). However, bacterial contamination can be a serious concern in heterotrophic microalgal growth system (Das and Veziroglu 2008; Rashid et al. 2013). As soon as organic carbon is introduced in the medium, bacterial growth starts which outperforms the microalgae and consumes all the nutrients unless necessary steps are taken. Antibiotics are therefore needed to control the contamination at this stage (Das and Veziroglu 2008; Rashid et al. 2013). In biohydrogen production process, the effect of carbon source on microalgae cultivation is not fully explored. Wei et al. used glucose, sucrose, fructose, and malt extract as substrates for growing cells of *Microcystis aeruginosa* (cyanobacterium) and *Chlorella vulgaris* (green microalgae). Malt extract turned the maximum hydrogen yield of 1300 ml L^{-1} (of microalgae medium) in *Chlorella vulgaris*. Chen et al. (2008) used glucose, fructose, galactose, and sucrose as substrates with a concentration of 200 mg L^{-1} for growing cells of *Anabaena* sp. CH3 during hydrogen production. The authors found that preferred substrate in case of *Anabaena* sp. CH3 was fructose and glucose, producing 0.0016 and 0.004 mol of hydrogen, respectively. In *C. reinhardtii*, acetate was the most effective substrate producing 1.7 mol of hydrogen (Rashid et al. 2013). Table 6.9 shows the carbon sources used for growing different microalgae species during hydrogen production.

6.4.2 Photobioreactor and Its Utility for Hydrogen Gas Production

Bioreactors facilitated with illumination are essential for production of hydrogen and hence are called photobioreactors. In microalgae, H_2 production through a photobioreactor depends on two important steps: (i) culture specification for biomass (aerobic, stage I) production and (ii) culture conditions for H_2 production (Cuaresma et al. 2011). All photobioreactors require entry of light, which usually is sunlight, but in some photobioreactors, other artificial sources are also used for providing controlled light. Inside a photobioreactor, there should be a photic zone, close to the illuminated surface and a dark zone, further away from this surface. The dark zone is due to light absorption by algal cells and mutual shading. Hydrogen production inside a photobioreactor is light limited and tends to decrease at higher light intensities (photosynthesis diverts hydrogen

production pathway); hence, light regime is determined by light gradient (must be diluted and distributed as much as possible; absolute dark condition responsible for highest production) (Oncel and Sabankay 2012). Liquid circulation time or aeration (enzymes for H_2 production are oxygen labile; anaerobic condition or inert gas environment is required) rate has a limiting effect on hydrogen production by microalgal biomass. The position of light source as well as gas liquid hydrodynamics also affects microalgal growth and resulting hydrogen production. To achieve maximum biomass from microalgae in photobioreactor, light panels are constructed along with speed controlled agitator (Menetrez 2012; Oncel and Kose 2014). As a result of agitation, microalgal cells will circulate between light and dark zone of the photobioreactor at a certain frequency and regular intervals, based on photobioreactor design and gas input (Oncel and Kose 2014). Photobioreactors used for hydrogen production can be broadly divided into three categories: vertical column photobioreactor, tubular-type photobioreactor, and flat panel photobioreactor (Table 6.10). Comparison of performance of reactor with respect to hydrogen production is given in Table 6.11. A photobioreactor for microalgal hydrogen production should meet following conditions:

1. Photobioreactor should be an enclosed system so that the produced hydrogen may be collected without any loss.
2. The photobioreactor design must allow sterilization with convenience and ease.
3. To maximize the area of incident light (thus allowing high growth and hydrogen production), photobioreactor design should provide high surface to volume ratio.

6.5 Progress in Metabolic Engineering for Hydrogen Gas Production

In case of eukaryotic microalgae, *Chlamydomonas reinhardtii* is an attractive candidate for hydrogen production due to its relatively high hydrogenase activity, through the [FeFe] hydrogenase HYDA1 (Meuser et al. 2012). Although hydrogen is naturally produced by *C. reinhardtii* under sulfur starvation and hydrogenase activity can be externally induced by adding 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU, a PSII electron chain uncoupler), the production cannot be sustained while photosynthesis is actively occurring since oxygen inactivates hydrogenase (Esquivel et al. 2011). In 2000, Melis and co-worker used sulfur deprivation strategy for lowering partial pressure of oxygen in *C. reinhardtii*. Oxygen sensitivity is a proton gradient-related problem which can be avoided by genetic insertion of a hydrogenase promoter programmed polypeptide proton channel into the algal thylakoid membranes. This necessitates a biphasic production strategy in which cells grow photosynthetically to accumulate biomass, which is then exploited for H_2 production under anoxic conditions. Several approaches to overcome this limitation have recently shown promising results (Oncel and Kose 2014; Dubini et al. 2014). Incorporation of leghemoglobin proteins (oxygen sequester in the nitrogen-fixing root nodules of legumes) within *Chlamydomonas* sp. cell can facilitate a fourfold increase of H_2 production (Wu et al. 2011). It is well known that in green microalgae about 90 % of the photons being captured by antenna systems are not being utilized (Hallenbeck and Benemann 2002). In a truncated antenna mutant of *C. reinhardtii* CC-4169, initially engineered for increased photosynthetic performance, an eightfold increase in H_2 production was observed under sulfur deprivation in presence of high light (350 $\mu Em^{-2}s^{-1}$) (Kosourov et al. 2011). In *C. reinhardtii*, photosynthetic efficiency improved by truncating chlorophyll antenna size of PSII (Perrine et al. 2012). A PSII protein D1 mutant of *C. reinhardtii* exhibit increased carbohydrate storage and H_2 production (Scoma et al. 2012); however, the highest reported yields for this alga (*C. reinhardtii* D1 mutant) are still three to five times below light to H_2 conversion than through direct photolysis (Esquivel et al. 2011). The H_2 production pathways predicted from in silico reconstruction suggest that increased production can occur under conditions of inhibited cyclic

Table 6.10 Type of photobioreactor with their optimal features

Type of photo bioreactor	S/V ratio	Agitation system	Temperature control	Gas exchange	Advantages	Disadvantages	References
Tubular reactors							
Vertical tubular	Small	Airlift, bubble column	NA	Open gas exchange at headspace	Good mixing, efficient CO_2 supply and O_2 removal	Scale-up is limited, major light is reflected due to angle	Martnez-Jeronimo and Espinosa-Chavez (1994) and Tamagnini et al. (2002)
Horizontal tubular	Large	Recirculation with diaphragm/mechanical pumps	Shading, overlapping, water spraying	Injection into feed, and dedicated degassing units	Adequate angle toward sunlight,	High shear due to pumps, risks of O_2 buildup, biofouling, separate gas exchange unit required	Iqbal et al. (1993) and Tredici et al. (1998)
Helical tubular	Large	Centrifugal pumps	Heat exchanger	-do-	High S/V, easy scale-up by increasing the number of units	O_2 buildup, separate gas exchange, pumps exert more shear, cell debris accumulate inside	Tsygankov et al. (1998) and Morita et al. (2000)
Table 10 (continued)							
Alpha shape reactor	Large	Airlift	-do-	Injection in the vertical units and degassed at top	High unidirectional flow rate with low air flow rate, high S/V	Foam formation due to high cell density	Lee et al. (1995)
Flat plate reactors							
Flat panel bubbled at bottom	Medium	Bubbling at bottom or from sides, recirculation	Heat exchange coils	Bubbling	Open gas transfer avoids O_2 buildup	Shear due to entrainment of cells till bubbles burst	Hu and Richmond (1996) and Tredici and Zittelli (1998)
Flat panel pivoted at center	Medium	Pulsating motion	Heat exchange coils	Degasser	Good mixing, low shear	Scale-up is difficult	Dasgupta et al. (2010a, b)

Panel with V-shape	Medium	Bubbling	-do-	-do-	Very high mixing rate, low shear	Agitation system can dilute H_2 formed	Iqbal et al. (1993)
Alveolar panel	Large	Bubbling	water circulation in lower row	-do-	High S/V due to alveolar panels, uniform distribution of light	O_2 buildup, high air flow rates required to move across the channels	Tredici et al. (1998) , Tredici et al. (1991), and Tredici et al. (1993)
Floating type bioreactor	Medium	Sea saw motion	No cooling required	-do-	Low energy for operation, good agitation, can be installed on lakes and sea floor	NA	Otsuki et al. (1998)
Fermentor type with internal/external lighting	Small	Impellers	Heat exchange coils	By sparger	High degree of control of various parameters	Light conversion efficiency is less	Pohl et al. (1988)
Torus shaped reactor	Medium	Marine impeller	Cooling fans	CO_2 inlet after impeller, outlet at top	Good mixing conditions owing to shape avoiding dead zones	NA	Pottier et al. (2005) and Fouchard et al. (2008)
Annular triple jacketed with lighting from innermost chamber	Medium	Magnetic stirrer	Outer water jacket	Open gas exchange	Good S/V and temperature control, open gas exchange	Scaling up is difficult, biofouling	Basak and Das (2009)

Adapted from Dasgupta et al. 2010a, b
NA not available

Table 6.11 Performance of different photobioreactor in terms of hydrogen production in microalgae

Reactor type	Organism	Surface to volume ratio	Volumetric productivity ($g\ L^{-1}\ d^{-1}$)	Areal productivity ($g\ m^{-2}\ d^{-1}$)	Percentage of photosynthetic efficiencies	References
Flat plate reactor	*Arthrospira platensis*	40	1.09	NA	4.84	Tredici and Zittelli (1998)
Helical tubular	*Arthrospira platensis*	53	0.9	NA	0.66	Tredici and Zittelli (1998)
Near horizontal tubular	*Arthrospira platensis*	70	1.4	28	5.6	Tredici and Zittelli (1998)
Panel type	*Chlamydomonas reinhardtii* CC124	NA	1.3 ± 0.05 ml $L^{-1}\ h^{-1}$	NA	NA	Oncel and Kose (2014)
Conical tubular	*Chlorella* sp.	NA	1.01	28.3	6.84	Morita et al. (2000)
Airlift	*Phaeodactylum tricornutum* UTEX 640	NA	1.2	20	NA	Fernandez et al. (2001)
Alveolar	*Nannochloropsis* sp.	80	1.45	NA	0.48	Tredici et al. (1991)

NA not available

electron flow (Dal'Molin et al. 2011), which is indeed observed in high H_2-producing *C. reinhardtii* mutant *Stm6Glc4* (Kruse et al. 2005). RNA interference (RNAi) has also been recently used to downregulate the entire family of light-harvesting complexes (LHC) in *C. reinhardtii*. The simultaneous knockdown of three LHC proteins (LHCMB 1, 2, and 3) was undertaken in the high H_2-producing *C. reinhardtii* mutant *Stm6Glc4* using an RNAi triple knockdown strategy (Oey et al. 2013), and this tool may prove to be extremely useful as part of metabolic engineering approaches.

In order to use cyanobacteria for biological production of hydrogen, it is important to thoroughly understand the regulation of hydrogen production machine and identification of bottlenecks that limit H2 production. The bidirectional hydrogenase from cyanobacteria does not require ATP to function and can suffer from a buildup of ATP which then inhibits electron flow (Lubitz et al. 2008). A variety of genetic tools also exist to express the bidirectional, oxygen-tolerant [NiFe] hydrogenase genes (such as *hydS* and *hydL*) in cyanobacterial species (Lukey et al. 2011). Recent studies have shown that incorporation of a heterologous [FeFe] hydrogenase (from *Shewanella oneidensis* MR-1) into the heterocysts of *Anabaena* sp. PCC 7210 could potentially provide a way to increase hydrogen production in this organism (Gartner et al. 2012). Major strategies developed for higher rate of H_2 production in cyanobacteria are detailed in Table 6.12:

- *Inactivation of uptake hydrogenase:* Preparation of single and double mutant of *Hup* and *Hox*, respectively, showed enhanced production of hydrogen in several *Hup* disrupted mutant strains of *Anabaena* sp., especially in strain PCC7120 (Masukawa et al. 2012). Thus, parental strain with high nitrogenase activity can perform high hydrogen production after *Hup* inactivation.
- *Modification of the catalytic activity center of nitrogenase*: In the presence of N_2, *Anabaena* wild strain PCC7120 effectively produces H_2 when *nif*V1 was inactivated along with high heterocyst frequency (Kufryk 2013).

Table 6.12 Major limitation and remedies for biohydrogen production in cyanobacteria and green microalgae

Microalgal groups	Challenges	Strategies	References
Cyanobacteria	Low H_2 production rate of [NiFe] hydrogenases	Requires constant sparging of inert gas	Silbert et al. (2001)
		Expressing the bidirectional, oxygen-tolerant [NiFe] hydrogenase genes, hudS and hydL in cyanobacteria	Lukey et al. (2011)
		Incorporation of [FeFe] hydrogenase into heterocysts	Gartner et al. (2012)
Green microalgae	Light conversion efficiency to H_2 (theoretically about 10%)	Truncating the chlorophyll antenna size of PSII using RNAi method	Oey et al. (2013)
	Inhibition of [FeFe] hydrogenases by oxygen production through PSII	Sulfur deprivation	He et al. (2012)
		Genetic insertion of a hydrogenase promoter proton channel into the thylakoid membranes	

- *Overexpression of bidirectional hydrogenase*: In *Synechocystis* sp. PCC6803, increase in H_2 production was noted after overexpression of bidirectional hydrogenase and deletion of NDH-1 respiratory complex (Kufryk 2013).
- *Expression of heterologous hydrogenase*: Low specific activity of [NiFe] hydrogenase was 500 times more active in *Synechococcus elongatus* 7942, after expression of *Clostridium acetobutylicum* [FeFe] hydrogenase (*Hyd A*) (Weyman et al. 2011).
- *Inactivation of competitive biochemical pathways*: In *Synechococcus* sp. PCC 7002, genetic manipulation led to increased hydrogen production by increase in the ratio of $NADPH/NADP^+$ through bidirectional hydrogenase (Kufryk 2013).

Major limitation and strategies including metabolic and genetic engineering processes for overcoming such bottlenecks during biohydrogen production by microalgae are summarized in Tables 6.12 and 6.13.

6.6 Economic Viability

The research attention on biological hydrogen production has substantially increased over the last 10 years. However, only a limited number of studies have looked into the economic viability of biohydrogen production on a commercial scale. Reported analysis suggests that the cost of photobiologically produced hydrogen is much lower ($25 m^{-3}) compared to that produced by photovoltaic process ($170 m^{-3}) (Dutta et al. 2005). The experimental studies have shown that dark fermentation is a cheap method for generation of biohydrogen; however, yields are usually lower. On the contrary, photofermentation is a more efficient method, but it is relatively more expensive. The application of indirect photolysis methods of hydrogen production is predicted to cost around 1220$ per GJ/year, while the capital cost is predicted to be 2.4$/gigajoule/year (Resnick 2004; Menetrez 2012). For this reason, a hybrid production system has been proposed which integrates both light and dark fermentation

Table 6.13 Metabolic and genetic engineering strategies for overcoming bottlenecks during hydrogen production in cyanobacteria and green microalgae

Strategies	Advantages	Microalgae	References
Pigment reduction	Increasing the photosynthetic efficiency	*Chlamydomonas reinhardtii*, *Dunaliella salina*	Polle et al. (2003) and Mussgnug et al. (2007)
		Synechocystis sp. PCC 6803	Bernat et al. (2009)
Generating anaerobic environment	Activating hydrogen-producing enzyme	*C. reinhardtii* (arp mutant)	Melis (2007) and Ruhle et al. (2008)
		C. reinhardtii (D1 protein mutant)	Torzillo et al. (2009)
Oxygen-tolerant enzyme	Producing hydrogen in the presence of oxygen	*Chlamydomonas* sp.	Chen et al. (2003)
		Synechococcus sp. PCC7942	Chen et al. (2005)
Eliminating competitive inhibition by other e^- acceptor	Redirecting the e^- flux toward the hydrogen-producing enzyme	*C. reinhardtii* 137c	Lee and Greenbaum (2003)
Introducing foreign efficient hydrogen-producing enzyme	Enhancing hydrogen production in that particular microorganism, that may be efficient in other criteria	*Synechococcus elongatus*	Miyake and Asada (1997) and Asada et al. (2000)
Enhancing the capacity of deriving e^- from carbohydrates	Contributing in hydrogen production in anoxic dark condition	*C. reinhardtii* (Stm6 strain)	Kruse et al. (2005) and Doebbe et al. (2007)
Inhibiting or overexpressing the crucial metabolic enzymes	Redirecting the e^- flux toward hydrogen-producing enzyme	*Synechocystis* sp. (mutant M55)	Vignais et al. (2006)

processes for maximum biohydrogen yield from algal systems (Nayak et al. 2014). The use of external enzymes (e.g., amylase) for the breakdown of microalgal cell wall under dark fermentation is effective and can ultimately maximize H_2 production (Nayak et al. 2014). Hydrogen production using photobiological systems has the potential to become the most effective method for large-scale requirements (Dasgupta et al. 2010a, b). Hence, high setup cost is one of the major constraints that limit commercial scaling up of biohydrogen to meet large-scale energy demand in the present time. Summary of benefits of biohydrogen over fossil fuels through various routes of production and possibilities is represented in Fig. 6.6.

6.7 Conclusions

In the beginning of this chapter, we argued about sustainable and environmentally friendly carbon-free green energy for mankind and thus discussed mainly on the metabolism and mechanism of biological hydrogen production including scale-up and associated constraints. Biological hydrogen production has several advantages over conventional hydrogen production processes. Microalgae are sustainable and low-cost renewable source for biohydrogen production. However, biohydrogen production faces two major problems: (i) low hydrogen yield in dark fermentation (in microalgae, stored carbohydrates are converted into H_2 via pyruvate in dark) and (ii) high energy cost in photofermentation (in photosynthetic bacteria, stored organic compound produce H_2 under light). Therefore, hybrid production system is proposed to maximum biohydrogen yield from algal systems. Hydrogen production using photobiological systems has the potential to become the most effective method for large-scale requirements. However, innovative research approaches need to be formulated so as to improve the efficiency of microalgae for H_2 production including discovery and identification of new strains, species consortium, novel enzymes, and

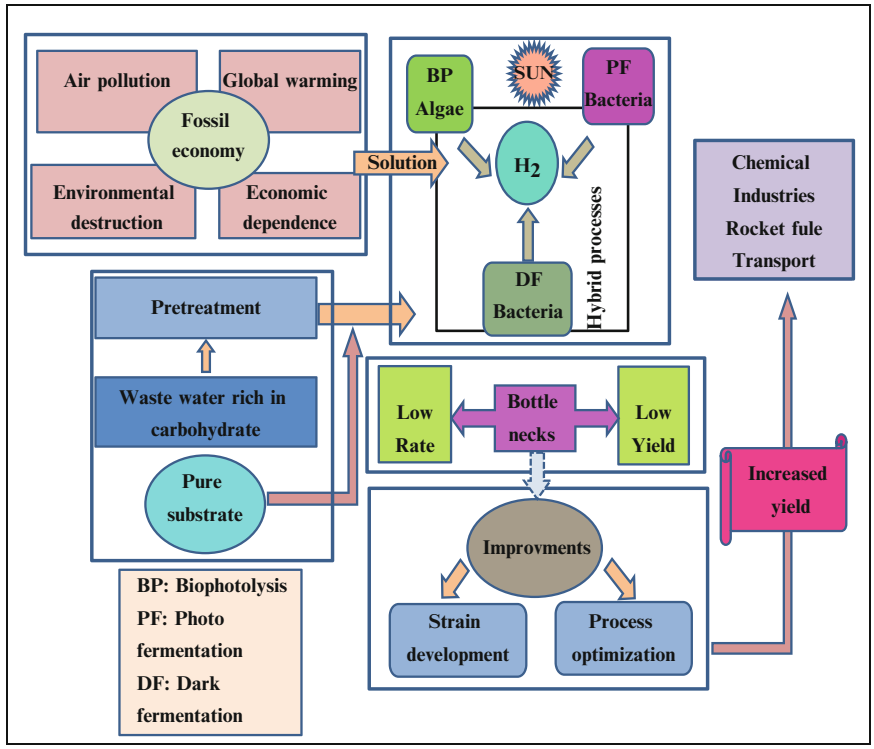

Fig. 6.6 Schematic summary of benefit of biohydrogen over fossil-based fuels and constraints in terms of production along with underlying challenges

manipulation of culture conditions. Development of low-cost photobioreactors (with maximum utilization of PAR) and fermentors is another challenge; when addressed, it can lead to commercial scale production of H_2. Detailed understanding of system biology including supply of carbon, reducing power, oxygen inactivation, metabolic engineering, and genetic manipulation can ultimately help toward cost-effective production of biohydrogen from microalgal biomass.

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