Chapter 10
Biofuels from Microalgae: Biohydrogen

Harshita Singh and Debabrata Das

Abstract Rapid industrialization and urbanization are mainly responsible for the energy crisis, environmental pollution and climate change. In addition, depletion of the fossil fuels is a major concern now. To confront these problems, it is essential to produce energy from sustainable and renewable energy sources. Hydrogen is widely considered as a clean and efficient energy carrier for the future because it does not produce carbon-based emission and has the highest energy density among any other known fuels. Due to the environmental and socioeconomic limitation associated with conventional processes for the hydrogen production, new approaches of producing hydrogen from biological sources have been greatly encouraged. From the perspective of sustainability, microalgae offer a promising source and have several advantages for the biohydrogen production. Microalgae are characterized as high rate of cell growth with superior photosynthetic efficiency and can be grown in brackish or wastewater on non-arable land. In recent years, biohydrogen production from microalgae via photolysis or being used as substrate in dark fermentation is gaining considerable interest. The present chapter describes the different methods involved in hydrogen production from microalgae. Suitability of the microalgae as a feedstock for the dark fermentation is discussed. This review also includes the challenges faced in hydrogen production from microalgae as well as the genetic and metabolic engineering approaches for the enhancement of biohydrogen production.

Keywords Microalgae · Biohydrogen · Photolysis · Dark fermentation
Genetic engineering

H. Singh · D. Das (✉)
Advance Technology Development Center, Indian Institute
of Technology, Kharagpur 721302, India
e-mail: ddas.iitkgp@gmail.com

D. Das
Department of Biotechnology, Indian Institute of Technology,
Kharagpur 721302, India

E. Jacob-Lopes et al. (eds.), *Energy from Microalgae*, Green Energy
and Technology, https://doi.org/10.1007/978-3-319-69093-3_10

1 Introduction

The exponential increase in the world population and rapid industrialization has resulted in continuous rise of the global energy demand. This has led to the depletion of fossil energy reserves, climate change and environmental pollution. To address these problems, we are compelled to find sustainable, renewable and carbon-neutral energy sources. In this regard, hydrogen is considered as a clean and efficient energy carrier. It has the highest energy density (142 kJ/g) among any other known fuels (Kumar et al. 2013), and on combustion it only produces water vapour. In addition, hydrogen can easily get converted into electricity in a fuel cell without any pollution (Batista et al. 2014) and may be used directly as a transportation fuel in an internal combustion engine (Das et al. 2014).

Different conversion technologies can be used for hydrogen production, but till today, it is produced through conventional technologies which include reforming processes, gasification and water splitting. Among the conventional processes, steam reforming of methane is widely used thermo-chemical technology and contributes 48% of the global hydrogen demand. About 30% of world hydrogen is produced by the reforming of oil/naphtha and 18% from the coal gasification (Das et al. 2014). Water electrolysis is another efficient method which produces hydrogen of very high purity and accounts for 3.9% of hydrogen production (Das et al. 2014), but this technology is challenged by the high cost of electricity. Biomass (crop residues, animal wastes, waste paper, municipal solid wastes, etc) gasification is also employed for hydrogen production, but it has the drawback of low thermal efficiency (Holladay et al. 2009). To overcome the various socioeconomic and environmental limitations associated with the currently existing industrial processes of hydrogen production, research focusing the biological hydrogen production technology has received substantial importance. This technology is not only environmentally benign but also requires less energy input as it can be carried out under ambient operating conditions (Das and Veziroglu 2001). Production of hydrogen through biological pathways is primarily controlled by the domain of bacteria and algae. In recent days, microalgae are considered valuable and tremendously potential source for the sustainable generation of biohydrogen. Interest in microalgae for hydrogen production has been ensued due to the fact that they can carry out the production of hydrogen through the process of photosynthesis utilizing most abundant natural resources, sunlight and water. Evidence of microalgal hydrogen production through biophotolysis of water was firstly put on record by Gaffron and Rubin in 1942. They studied the hydrogen metabolism in a unicellular green microalga, *Scenedesmus obliquus,* and reported the hydrogen production by this microorganism in the presence of light energy under anaerobic condition after an adaptive dark phase (Gaffron and Rubin 1942). Although photobiological hydrogen production by algae has been studied for several years, in recent decades considerable advances in this field have been made (Torzillo et al. 2015; Marquez-Reyes et al. 2015). Apart from this, over the past few years, dark fermentation utilizing microalgal substrate for biohydrogen production has also

gained attention (Roy et al. 2014; Batista et al. 2014; Ortigueira et al. 2015). In comparison with other biomass, microalgae present several advantages to be used as feedstock for biohydrogen production: (1) they have higher growth rate with superior photosynthetic efficiency; (2) they can grow on non-arable land; (3) they can grow in wide variety of water sources (fresh, salt, brackish and wastewater); (4) they do not contain lignin, so no rigorous pretreatment is required (Sambusiti et al. 2015).

This chapter is aimed to describe about biohydrogen production from microalgae both by biophotolysis as well as dark fermentation. The barriers in biohydrogen production from microalgae and the molecular approaches to enhance the hydrogen production are taken into consideration.

2 Microalgal Hydrogen Production Processes

Microalgae can participate for hydrogen production mainly by two processes: (1) photolysis of water, which requires light and is closely related to the process of photosynthesis; (2) light-independent process in which microalgal biomass, rich in carbohydrate and protein, is used up as a feedstock for dark fermentation.

2.1 Biophotolysis

Biophotolysis is the action of light energy on the biological systems that results in the dissociation of substrate, usually water molecule, into hydrogen and oxygen. Unicellular green algae and cyanobacteria are organisms known to perform both oxygenic photosynthesis and biohydrogen production (Happe et al. 2000). In microalgae, the process of photolysis is closely related to the process of photosynthesis. Unlike photosynthesis, where the reductants released by the dissociation of water are consumed in the Calvin cycle or pentose phosphate pathway to reduce CO_2 for cell growth, in biophotolysis the reductants are directed for hydrogen evolution. The photosynthetic machinery of eukaryotic green algae and prokaryotic blue-green algae is similar to higher terrestrial plants. In eukaryotic microalgae, the photosynthetic machinery is embedded in the thylakoid membranes present inside an intracellular organelle, the chloroplast. In contrast, the photosynthetic apparatus of cyanobacteria lacks compartmentalization and the thylakoid membranes are present in the cytoplasm, adjacent to the plasma membrane. The thylakoid membranes contain several light-absorbing pigments such as chlorophyll a, antenna chlorophylls, carotenoid and phycobiliproteins which are arranged in two different kinds of functional arrays called photosystems (PSI and PSII). Photosystem I and photosystem II consist of distinct photochemical reaction centre, P700 and P680 respectively. Absorption of photons by the chlorophyll molecules of P700 and P680 causes their excitations and drives the electrons through thylakoid membrane to

reduce ferredoxin (Fd). Under aerobic and light condition ferredoxin: $NADP^+$ oxidoreductase transfers the electrons from reduced ferredoxin to $NADP^+$ which generates NADPH. This reducing power (NADPH) is used to fix carbon for cell growth and for carbohydrates and/or lipids production. However, under some conditions, the reduced ferredoxin generated by the water splitting can be directed to reduce hydrogenase or nitrogenase enzymes for the hydrogen production. There are two types of biophotolysis for H_2 production from microalgae: direct and indirect biophotolysis.

2.1.1 Direct Biophotolysis

In this biological process, the reductive equivalents required for the hydrogen production are generated directly by the photolysis of water. Photosynthetic machinery of green algae utilizes the solar energy to split the H_2O molecule into O_2 and H_2. The electrons generated by the oxidation of H_2O molecule flows to the ferredoxin which under the optimal conditions donates the electrons directly to hydrogenase enzyme for H_2 production. In direct biophotolysis, production of H_2 at the reducing side of the PSI is associated with the simultaneous evolution of O_2 at the oxidizing side of the PSII (Melis et al. 2000). Presence of O_2 limits the hydrogen production as the hydrogenase gets deactivated at O_2 partial pressure of <2% (Ghirardi et al. 1997). Thus, in this process H_2 evolution occurs for transient period upon illumination, before the hydrogenase gets inactivated by the accumulated O_2 (Eroglu and Melis 2011). Several green microalgae such as *Chlamydomonas reinhardtii*, *Chlorella fusca*, *S. obliquus*, *Chlorococcum littorale* and *Platymonas subcordiformis* possess genomically encoded [FeFe]-hydrogenase enzyme for hydrogen generation (Eroglu and Melis 2011). Among the green algae, *C. reinhardtii* has been mostly investigated by many researchers for biohydrogen production (Melis et al. 2000; Torzillo and Seibert 2013; Tsygankov et al. 2006). In order to prevent the inactivation of H_2-evolving enzyme by O_2 and for the sustained production of H_2, different methods have been investigated such as purging the reaction mixture with inert gases (Greenbaum 1982), addition of oxygen scavenger (Randt and Senger 1985) and depletion of sulphur in the cultivation media (Melis et al. 2000).

The method of sulphur deprivation is a two-stage approach for the sustained hydrogen production by green microalgae. First stage is the growth phase in which generation of biomass takes place under suitable conditions. Second stage is the non-growth phase in which the carbohydrate-rich algal biomass is transferred to sulphur-deprived cultivation medium for H_2 production (Melis et al. 2000). Sulphur is essential for the biosynthesis of PSII protein, which is made up of sulphur-containing amino acids (cysteine or methionine). Due to the partial suppression of PSII activity, evolution of O_2 reduces and the mitochondrial respiration further helps in the depletion of oxygen, developing the essential anaerobiosis inside the cells. Anaerobic condition induces the [FeFe]-hydrogenase activity (Forestier et al. 2003) which produces H_2 by utilizing 60–90% electrons contributed

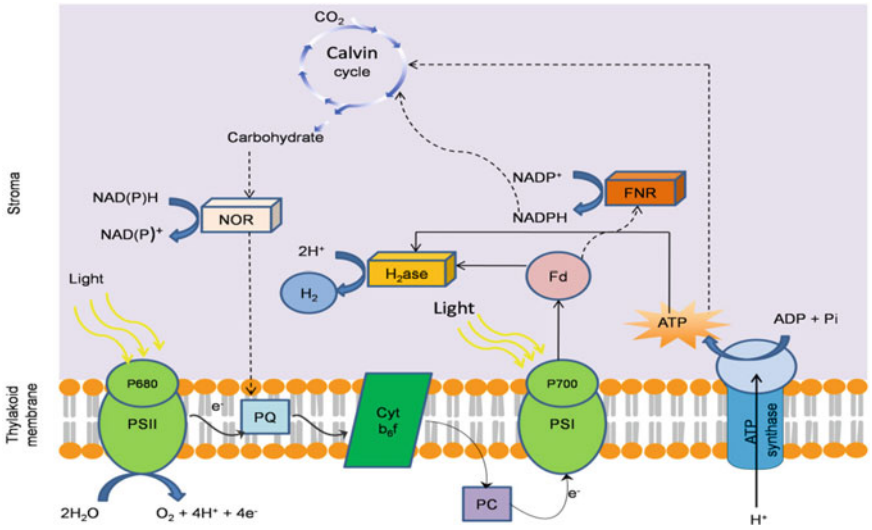

Fig. 1 Biohydrogen production via direct and indirect biophotolysis carried out by green microalgae

by the water splitting and remaining 20–30% comes from the catabolism of carbohydrate through fermentation pathway. In this way, sulphur deprivation mechanism employs both direct as well as indirect biophotolysis for H_2 generation (Fig. 1). Re-addition of sulphur in limiting amounts during the H_2 production phase helps in regenerating the depleted algal cells for another round of H_2 generation without re-establishment of aerobic condition (Kosourov et al. 2005). However, the cycling of algal suspension cultures between the sulphur deplete and sulphur replete conditions is challenging and might become simpler by using the immobilized, sulphur-deprived algal cells for sustained H_2 evolution (Laurinavichene et al. 2006).

Direct photolysis is an interesting process due to the fact that this process utilizes the most abundant natural resources, solar energy and water for the production of efficient fuel "hydrogen". However, this process suffers from the limitation of low yield and hydrogen production rate. The energy productivity via this process ranges from 0.02 to 0.12 kJ/L/h (Yu and Takahashi 2007).

2.1.2 Indirect Biophotolysis

In this process, the reductive equivalents or electrons are directly derived by the endogenously stored carbohydrates such as starch in green algae and glycogen in cyanobacteria (Fig. 2). In this method firstly, during the photosynthesis, CO_2 fixation and accumulation of carbohydrate take place. Secondly, fermentation of the carbon reserves occurs which leads to hydrogen production by the following reaction:

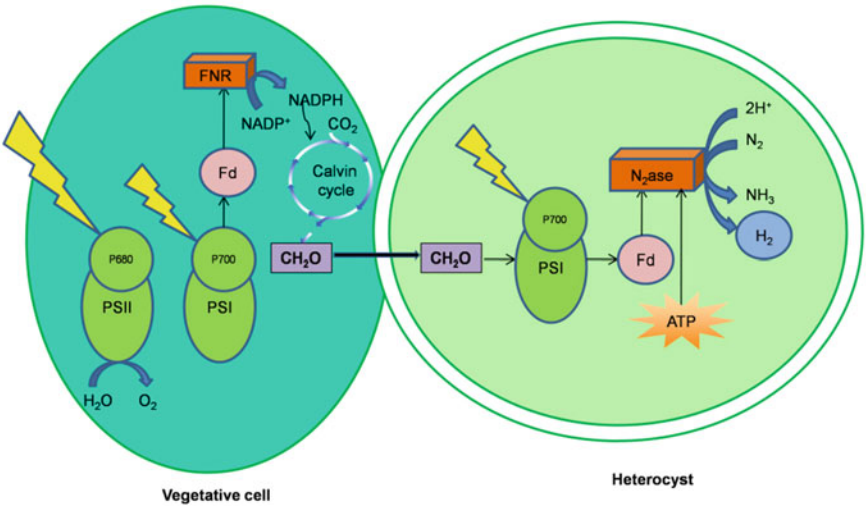

Fig. 2 Biohydrogen production via indirect biophotolysis carried out by cyanobacteria

$$12H_2O + 6CO_2 \rightarrow C_6H_{12}O_6 + 6O_2 \quad (1)$$

$$C_6H_{12}O_6 + 12H_2O \rightarrow 12H_2 + 6CO_2 \quad (2)$$

In comparison with green microalgae, H_2 production via indirect photolysis from cyanobacteria is more attractive (Yu and Takahashi 2007). In this process, the problem of H_2-producing enzyme sensitivity to O_2 is solved by the temporal or spatial separation of H_2 and O_2 evolving reactions. In spatial separation, the apparatus for photosynthesis and H_2 production is present at different locations. Temporal separation involves the reactions of O_2 and H_2 evolution to occur at different time by using light/dark cycles. In this process, during the daytime carbohydrate accumulation takes place via photosynthesis and during the night-time H_2 production occurs via fermentation of stored sugar (Miura et al. 1997).

Cyanobacteria are capable of carrying out the both, CO_2 as well as nitrogen fixation. In these organisms, nitrogen fixation occurs under anoxic conditions inside the specialized cells known as heterocysts whereas oxygenic photosynthesis and CO_2 fixation take place in the vegetative cells. Inside the heterocysts, anaerobic environment is maintained due to the absence of the O_2-evolving PSII. In addition, the O_2 impermeable cell walls of heterocysts do not allow the oxygen diffusion from the nearby vegetative cells thus helping further in creating anaerobiosis, required for the nitrogen fixation and H_2 generation by the O_2-sensitive nitrogenases (Das et al. 2014).

Nitrogen-fixing cyanobacteria known for H_2 production mostly include the genus *Anabaena, Nostoc, Calothrix* and *Oscillatoria.* In these organisms, nitrogen fixation and hydrogen evolution catalysed by the nitrogenase enzyme are described according to Eq. 3 (Eroglu and Melis 2011):

$$N_2 + 8e^- + 8H^+ \rightarrow 2NH_3 + H_2 + 16ADP + 16Pi \quad (3)$$

In the absence of N_2, nitrogenase acts as an ATP-powered hydrogenase and produces H_2 exclusively without any feedback inhibition by the following reaction (Eq. 4) (McKinlay and Harwood 2010):

$$8e^- + 8H^+ \rightarrow +16ATP \rightarrow 2H_2 + 16ADP + 16Pi \quad (4)$$

The process of heterocyst-based H_2 production by cyanobacteria suffers the problem of low photon conversion efficiency (Benemann 2000). This is due to the high metabolic energy requirement of the nitrogenase catalysis (2 ATP per e^- transfer). Moreover, half of the energy metabolism of the cyanobacteria also accounts for the biosynthesis and maintenance of heterocysts (Benemann 2000). This energy requirement is met by the solar energy via PSI-mediated cyclic phosphorylation occurring inside the thylakoid membrane of the heterocysts. During the N_2 fixation, nitrogenase is usually accompanied by uptake hydrogenase to reutilize and retrieve the H_2/e^- for minimizing the loss of energy (Tamagnini et al. 2002). Disruption of this uptake hydrogenase activity in cyanobacteria helps in H_2 accumulation (Masukawa et al. 2002). Non-nitrogen-fixing cyanobacteria such as *Gloeobacter, Synechococcus and Synechocystis* can also generate H_2 via indirect biophotolysis. These organisms possess bidirectional [NiFe] hydrogenase which has the capability of catalysing both the synthesis and oxidation of H_2 (Tamagnini et al. 2002). The physiological function of this enzyme is still unclear but the suggested role includes: removal of excess reducing power during anaerobic fermentation and allocation of electrons to the respiratory chain by H_2 oxidation (Baebprasert et al. 2010). H_2 production by this enzyme is energetically efficient as it does not require ATP like nitrogenases. However, the rate of H_2 production by non-nitrogen-fixing cyanobacteria is comparatively lower (0.02–0.40 μmol H_2/mg chl a/h) than heterocystous cyanobacteria (0.17–0.42 μmol H_2/mg chl a/h) (Levin et al. 2004). Biohydrogen production from microalgae via photolysis is summarized in Table 1.

2.1.3 Enzymes Involved in PhotoBiological Hydrogen Production from Microalgae

The key enzymes that can catalyse the reaction of hydrogen production in microalgae are hydrogenase and nitrogenase.

Hydrogenases

Hydrogenases are the metalloenzymes and on the basis of the metal composition at their catalytic sites, they can be classified as: [FeFe]-hydrogenase, [NiFe]-hydrogenase and [Fe]-hydrogenase (Kim and Kim 2011). Among these, [Fe]-hydrogenase is found in archaea so this will not be discussed here.

Table 1 Biohydrogen production from microalgae via photolysis

Microalgae	Process condition	Hydrogen production	Reference
Anabaena variabilis PK84	Indoor helical tubular photobioreactor, Allen and Arnon medium, air +2% CO_2, 12 h light and 12 h dark cycles, 332 μE/m^2/s	19.2 mL/h/PhBR	Borodin et al. (2000)
Anabaena variabilis PK84	Outdoor tubular photobioreactor, Allen and Arnon medium, air +2% CO_2, sunlight	45.8 mL/h/PhBR	Tsygankov et al. (2002)
Chlamydomonas reinhardtii cc-124	Flat glass photobioreactor, re-addition of sulphur (25 μM) in TAP-S medium, argon sparged, 300 μE/m^2/s	5.94 μmol mg/chl/h	Kosourov et al. (2002)
Gloeocapsa alpicola	Glass bottles, cells suspended in Tris-HCl buffer, argon sparged, 24 h dark	25 μL/h/mg d. w	Troshina et al. (2002)
Anabaena PCC 7120	Indoor tubular photobioreactor, BG 11_0 medium, argon sparged, 456 μE/m^2/s	1.4 mL/h/PhBR	Lindblad et al. (2002)
Anabaena AMC 414	Indoor tubular photobioreactor, BG 11_0 medium, argon sparged, 456 μE/m^2/s	13.8 mL/h/PhBR	Lindblad et al. (2002)
Immobilized *Chlamydomonas reinhardtii* CC-1036 pf18 mt+	Flat plate photobioreactor, TAP-S medium, argon sparged, 120 μE/m^2/s	6.4 μmol/mg chl/h	Laurinavichene et al. (2006)
Synechocystis sp. PCC 6803	BG 11 medium with optimized nutrients, nitrogen atmosphere, dark condition	0.81 μmol/mgchl/h	Burrows et al. (2008)
Chlamydomonas reinhardtii strain L159I-N230Y	Flat plate photobioreactor, TAP-S medium, 70 μmol/m^2/s	5.77 mL/L/h	Torzillo et al. (2009)
Synechocystis sp. PCC 6803	Glass vial, $BG11_0$-S medium, 750 mM β-mercaptoethanol, argon sparged, 24 h dark	14.32 μmol/mg chla/min	Baebprasert et al. (2010)
Aphanothece halophytica	Erlenmeyer flask, nitrogen-deprived BG 11 medium, 30 μmol/m^2/s for 18 h	13.8 μmol/mgchl/h	Taikhao et al. (2013)
Nostoc PCC 7120 Δ*hupW*	Flat panel photobioreactor, BG 11 medium, alternate argon/N_2 (20/80) and 100% argon sparged, 44 μmol/m^2/s	0.71 mmol/mgchla/h	Nyberg et al. (2015)
Lyngbya sp.	Glass reactors, medium containing benzoate (600 mg/L), argon sparged, 4000 lx	17.05 μmol/gchl 1/h	Shi and Yu (2016)

[FeFe]-hydrogenases are often involved in the reduction of protons to produce H_2. These are the only type of hydrogenases found in the eukaryotic microorganisms (Vignais and Colbeau 2004). In green microalgae they are located exclusively in the stroma of the chloroplast (Eroglu and Melis 2011). These hydrogenases are monomeric or dimeric with an average molecular weight of 50 kDa. The active site cluster of the enzyme also known as H-cluster consists of six Fe atoms arranged as [4Fe-4S] sub-cluster to which [2Fe-2S] extension is covalently bridged via cysteine residue. The Fe atoms of the active site are bound to non-protein ligands, CN^- and CO groups (Peters et al. 1998, 2015). The H-cluster of the [FeFe]-hydrogenases makes them different from the other H_2-producing enzymes and results in 100-fold higher enzyme activity (Happe et al. 2002). However, in spite of high specific activity these enzymes get easily inactivated by O_2 or CO_2. The green microalgae, *C. reinhardtii,* encodes two [FeFe]-hydrogenases (HydA1 and HydA2) which are 74% similar and are expressed under anaerobic condition (Forestier et al. 2003).

[NiFe]-hydrogenases are the most numerous hydrogenases found only in prokaryotes: cyanobacteria, bacteria and archaea. The core enzyme consists of the α–β heterodimer, where the larger α-subunit possesses the NiFe bimetallic centre and the smaller β-subunit consists of the Fe–S clusters which transfer the electrons from the active site to the e^- acceptor molecule (Kim and Kim 2011). In the active site, presence of non-protein ligands (CN^- and CO groups) bound to the Fe atom is the common structural characteristic of the [FeFe]- and [NiFe]-hydrogenases (Peters et al. 2015). In cyanobacterial species, these enzymes occur in two different types: hup-encoded [NiFe]-uptake hydrogenases and hox-encoded [NiFe]-bidirectional hydrogenases. Uptake hydrogenase catalyses the oxidation of H_2 to recover the energy lost during N_2 fixation. These are found in all nitrogen-fixing cyanobacteria, but their presence in non-nitrogen-fixing cyanobacteria is still under question (Tamagnini et al. 2002). The small subunit of the enzyme does not contain the signal peptide at N-terminal; therefore, the enzyme is localized on the cytoplasmic side of either the cytoplasmic or thylakoid membrane (Tamagnini et al. 2002). In the filamentous cyanobacteria, these enzymes are found in the thylakoid membrane of the heterocysts (Tiwari and Pandey 2012). Inactivation of the gene (*hupS*) encoding the small subunit of uptake hydrogenase led to the enhanced and sustained H_2 production in *Anabaena siamensis* TISTR 8012 under high light intensity (Khetkorn et al. 2012). Bidirectional hydrogenase is the reversible enzyme that can either evolve or consume H_2 according to the existing redox state of the cell's photosynthetic membrane (Eroglu and Melis 2011). This enzyme is present in both nitrogen-fixing and non-nitrogen-fixing cyanobacteria. The enzyme is multimeric because the dimeric module of the enzyme is associated with other subunits that can bind cofactors. In cyanobacteria, during the period of adaptation to higher light intensities the reversible hydrogenases may act as an electron valve (Vignais and Colbeau 2004).

Nitrogenases

Nitrogenase is present in cyanobacteria which catalyses the nitrogen fixation by reducing the molecular nitrogen into ammonium ions that can be easily utilized by the organisms. Nitrogen fixation is ATP-requiring irreversible reaction and is essential for the maintenance of the nitrogen cycle in the atmosphere. The reduction of nitrogen to ammonia by nitrogenase is accompanied by the reduction of protons (H^+) leading to H_2 production. Nitrogenases are the metalloenzyme, and depending upon the type of metal cofactor present at the catalytic site, they can be of three types: molybdenum, iron or vanadium nitrogenases. All these three variants of nitrogenases are capable to carry out the H_2 production during the nitrogen fixation but with variable stoichiometries (Eqs. 5–7). However, in the absence of nitrogen, nitrogenases can exclusively produce the H_2 as described in Eq. 4.

$$\text{Mo-nitrogenase} : N_2 + 8e^- + 8H^+ \rightarrow 2NH_3 + H_2 \tag{5}$$

$$\text{Fe-nitrogenase} : N_2 + 21e^- + 21H^+ \rightarrow 2NH_3 + 7.5H_2 \tag{6}$$

$$\text{Fe-nitrogenase} : N_2 + 12e^- + 12H^+ \rightarrow 2NH_3 + 3H_2 \tag{7}$$

Among all the nitrogenases, the most studied one is molybdenum nitrogenase. It consists of two proteins: the larger dinitrogenase (Mo–Fe–S protein or protein I) and the smaller dinitrogenase reductase (Fe–S protein or protein II). The dinitrogenase complex has an average molecular weight of 230 kDa and is a $\alpha_2\beta_2$heterotetramer encoded by the *nifK* and *nifD* genes. The dinitrogenase reductase subunit is a homodimer of around 65 kDa and is encoded by *nifH* gene. Reductase protein receives the electron either from flavodoxin or ferredoxin (external e^- donor) and transfers it to dinitrogenase protein with concomitant hydrolysis of ATP. Hydrogen produced by the nitrogenase activity is generally consumed by the uptake hydrogenases due to which the net H_2 evolution by cyanobacteria is barely observed, at least in aerobic condition (Almon and Böger 1988).

2.2 *Dark Fermentation Using Microalgal Biomass as Feedstock*

2.2.1 Anaerobic Fermentation Process

Dark fermentation for bioH_2 production is considered as a promising technology mainly due to following reasons: process simplicity, no requirement of light energy, higher rate of H_2 evolution and potentiality to utilize wide variety of substrates (different biomass and wastewater) for H_2 production. This process involves the anaerobic breakdown of the high molecular weight organic substrates

(carbohydrate, protein and lipid) into soluble metabolite products (volatile fatty acids and alcohols), H_2 and CO_2 by the facultative and obligate anaerobic bacteria. The genus of bacteria typically associated with dark fermentation includes *Clostridium*, *Klebsiella*, *Enterobacter*, *Citrobacter*, *Bacillus*, *Lactobacillus*, *Thermotoga*, *Anaerobiospirillum* and *Caldicellulosiruptor* (Xia et al. 2015). The hydrogen-producing bacteria utilizes the protons (H^+) as the electron acceptor and thus disposes the excess electrons generated by the oxidation of organic substrates in the form of H_2 (Das and Veziroglu 2001). There are two pathways for the formation of molecular H_2: NADH re-oxidation pathway and formate decomposition pathway. The H_2 production via NADH re-oxidation pathway is mediated by some specific bacteria such as *Clostridium* sp. by the following reaction (Eq. 8)

$$\mathrm{NADH} + \mathrm{H}^+ \rightarrow \mathrm{H}_2 + \mathrm{NAD}^+ \tag{8}$$

This NADH is generated due to the conversion of glucose into pyruvate during the glycolysis pathway, which could be represented as follows (Eq. 9):

$$\mathrm{C_6H_{12}O_6} + 2\mathrm{NAD}^+ \rightarrow 2\mathrm{CH_3COCOOH} + 2\mathrm{NADH} + 2\mathrm{H}^+ \tag{9}$$

Pyruvate-ferredoxin oxidoreductase catalyses the breakdown of pyruvate into acetyl CoA which could be further metabolized either into acetate or butyrate (Fig. 3). In both the cases, oxidation of one mole of ferredoxin by [Fe–Fe]-hydrogenase yields one mole of H_2. Maximum H_2 yield of 4 mol/mol glucose is achieved when acetic acid is the sole metabolic end product. However, H_2 yield of only 2 mol/mol glucose is achieved when butyrate is the final product Eqs. (10 and 11):

$$\mathrm{C_6H_{12}O_6} + 2\mathrm{H_2O} \rightarrow 4\mathrm{H_2} + 2\mathrm{CO_2} + 2\mathrm{CH_3COOH} \tag{10}$$

$$\mathrm{C_6H_{12}O_6} \rightarrow 2\mathrm{H_2} + 2\mathrm{CO_2} + \mathrm{CH_3CH_2CH_2COOH} \tag{11}$$

In contrast, few facultative anaerobes such as *Escherichia coli* can carry out the H_2 evolution via formate decomposition pathway. In this case, pyruvate is converted into formate and acetyl CoA by pyruvate formate lyase. Subsequently, under the anaerobic condition the formate is cleaved into H_2 and CO_2 catalysed by formate hydrogen lyase Eqs. (12 and 13):

$$\mathrm{CH_3COCOOH} + \mathrm{HCoA} \rightarrow \mathrm{CH_3COCoA} + \mathrm{HCOO} \tag{12}$$

$$\mathrm{HCOOH} \rightarrow \mathrm{H_2} + \mathrm{CO_2} \tag{13}$$

However, when fermentation is carried out by mixed microbial consortia, glucose can undergo some other biochemical pathways which generates undesired by-products such as lactate, propionate, succinate, 2,3 butanediol, ethanol, butanol and isopropanol. Generation of these metabolites hampers the H_2 production and

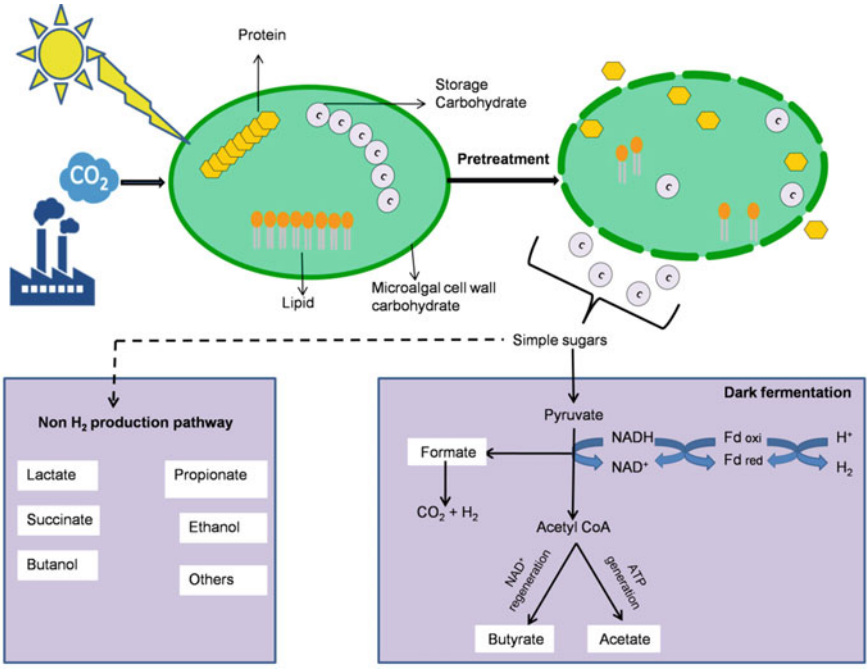

Fig. 3 Biohydrogen production via dark fermentation using algal biomass as substrate

lowers the overall yield of H_2. In such cases, the H_2 yield can be improved by inoculum pretreatment methods (enrichment of H_2-producing microorganisms) as well as by maintaining the proper operating conditions (Ghimire et al. 2015).

2.2.2 Suitability of Microalgal Biomass as a Substrate

Second-generation biofuels produced from the lignocellulosic biomass (agricultural residues and energy crops cultivated on non-arable lands) have no doubt provided the solution of the raised criticism regarding the sustainability of first-generation biofuels (biofuels produced from agricultural substrates). Nevertheless, the native recalcitrant structure of lignocellulosic biomass limits their hydrolysis by the fermentative bacteria. Indeed, to disrupt the rigid structure and to decrease the crystallinity of lignocellulosic biomass, required pretreatment methods are difficult and energy intensive. In this respect, third-generation biofuel production utilizing microalgal biomass as substrate has gained tremendous attention in recent years (Kumar et al. 2013; Nayak et al. 2014; Roy et al. 2014; Ortigueira et al. 2015; Khan et al. 2017).

Microalgal biomass offers several potential advantages to be used as an alternative to lignocellulosic feedstock for biofuel (biohydrogen) production, such as:

(1) relatively simple cell walls with no lignin therefore requires milder pretreatment, (2) high ability of CO_2 fixation, (3) higher productivity, (4) no need of arable land for mass cultivation and (5) can grow in brackish, saline and wastewaters thus reducing the freshwater footprint (Monlau et al. 2014; Sambusiti et al. 2015; Xia et al. 2015). During their growth, microalgae can synthesize and accumulate lipid, carbohydrate and protein (Monlau et al. 2014). The percentage of different components of microalgae varies according to algal species, environmental and cultivation conditions (Sambusiti et al. 2015).The first and most important task during the utilization of microalgal biomass as feedstock for dark fermentation is the selection of appropriate microalgal species having high biomass productivity and carbohydrate content (Wang et al. 2017). Microalgae store the polysaccharides either in the form of starch or glycogen.

Indeed, in microalgae the carbohydrates are also found entrapped within the cell wall mainly in the form of cellulose, hemicellulose, pectin and sulphated polysaccharide (Chen et al. 2013). Some microalgae can accumulate carbohydrate higher than 50% of their dry weight (Markou et al. 2012b). The microalgal storage and the cell wall polysaccharides upon efficient pretreatment can be released in the form of simple sugars (glucose) and contribute as a potential feedstock for dark fermentation. Appropriate cultivation condition and nutritional strategy can maximize the carbohydrate content and its productivity by altering the metabolic pathway of microalgae (Markou et al. 2012b). For instance, threefold higher carbohydrate content (39.19%) was obtained in *Scenedesmus* sp. CCNM 1077 under mixotrophic condition (glucose-supplemented medium) (Pancha et al. 2015). Ho et al. (2012) reported an increase in the carbohydrate content of *S. obliquus* CNW-N from 38.25 to 51.8%, when it was cultivated under high light intensity with nitrogen deficient condition. Moreover, Vitova et al. (2015) suggested sulphur deprivation as the most effective method of maximizing the carbohydrate content and productivity. Microalgae are also rich in macro- and microelements which are required for the growth of H_2-producing bacteria (HPB) (Sambusiti et al. 2015). The H_2 yield in dark fermentation mainly depends upon the monosaccharide content of the microalgal biomass because fermentation of lipid and protein by the HPB is thermodynamically unfavourable (Xia et al. 2015). Despite the lower potentiality of proteins for biohydrogen production, they are essential for balancing the C/N ratio of algal feedstock (Sambusiti et al. 2015). In dark fermentation, optimal C/N ratio of the substrate is an important factor for the growth and biological activity of HPB (Lay et al. 2013). The high protein content of the algal biomass results in low C/N ratio, which decreases the rate of H_2 production and limits the use of algal biomass as sole substrate. Excessive protein content leads to release and accumulation of nitrogen in the form of ammonium ion. High concentration of ammonium ion decreases the pH of the fermentation media, which may inhibit the growth of HPB or activity of enzymes participating in fermentative H_2 production. The C/N ratio of the algae can be increased by applying selected growth conditions (Montingelli et al. 2015). Moreover, an appropriate C/N ratio can be achieved by the addition of carbon-rich biomass with the microalgal biomass having high protein content. Xia et al. (2014) observed an increase in hydrogen

yield, when mixed biomass of *Chlorella pyrenoidosa* and cassava starch was used as feedstock in dark fermentation.

Finally, the economic viability of the hydrogen production from microalgal feedstock is an important aspect that ought to be contemplated. Therefore, to increase the economic feasibility of the process, a biorefinery approach where microalgal biomass residues after lipid and value-added product extraction that are still rich in sugars can be utilized as feedstock under dark fermentation.

2.2.3 Pretreatment of Microalgal Biomass for Hydrogen Production

Carbohydrates in algae are entrapped within the cell wall in form of complex polymer or stored intracellularly as starch or glycogen. Therefore, when algal biomass is used as feedstock, it is necessary to carry out algal cell wall disruption followed by conversion of polymeric carbohydrates into simple fermentable sugars (glucose, arabinose, galactose, xylose and mannose), which are readily accessible for hydrogen-producing bacteria (Ho et al. 2013; Kumar et al. 2013). Efficient pretreatment of algal biomass is required to enhance the saccharification and thereafter biohydrogen yield (Xia et al. 2013). For instance, Roy et al. (2014) reported very low hydrogen production (0.03 m^3/m^3) from untreated algal biomass compared to the pretreated biomass (1.33 m^3/m^3). The effectiveness of pretreatment process depends upon the cell wall characteristics of the microalgal species. Microalgae having carbohydrate (cellulose and hemicelluloses)-based cell wall (*Chlorella kessleri* and *S. obliquus*) are difficult to be pretreated. In contrast, microalgal species having protein-based cell wall (*C. reinhardtii*, *Arthrospira platensis, Euglena gracilis*) are easily degraded (Mussgnug et al. 2010). The *Scenedesmus* sp. has one of the most resistant cell walls consisting of trilaminar structure where inner layer is composed of cellulose covered by hemicellulose. The outer layer contains sporopollenin-like biopolymer which confers high resistant to pretreatment (Miranda et al. 2012).

Nevertheless, pretreatment is an indispensable step for efficient production of hydrogen from microalgal feedstock; unoptimized pretreatment and saccharification conditions can generate sugar degradation products (furfural, hydroxymethylfurfural (HMF), formic acid, acetic acid, propionic acid and lactic acid) (Harun et al. 2014; Hernández et al. 2015; Xia et al. 2015). The accumulation of such by-products is inhibitory to the microbial growth and fermentation process thereby decreasing the overall hydrogen production (Miranda et al. 2012; Xia et al. 2015). The biomass pretreatment step is associated with high price and significantly contributes to the overall cost of biohydrogen production process (Roy et al. 2014). In order to increase the feasibility of biohydrogen production process, the selected pretreatment procedure must be simple, energy efficient, cost-effective and must enhance the polymeric carbohydrate conversion into fermentable sugars without the formation of inhibitory by-products.

To date, the pretreatment methods used for the microalgal biomass hydrolysis are mechanical, thermal, chemical, biological and combination of any two

pretreatments. Mechanical pretreatment disrupts the algal cell wall by applying shear forces. Pretreatment method such as bead beating or milling is less dependent on microalgal species and can break the cell wall due to the collision of microalgal biomass with minute glass, ceramic or steel beads, under high agitation. Ultrasonication is another pretreatment method for cell wall disruption and solubilization of the organic matter. In this method, the repetitive compression and rarefaction of the sonic waves cause the formation of microbubbles which grow and then collapse, generating high pressure and heat, shear forces and free radicals thereby damaging the cell wall. Cheng et al. (2012) found that bead milling can disrupt some of the cyanobacterial cells releasing carbohydrate and protein to be utilized by hydrogenogens. However, through milling lower yield of H_2 (38.5 mLH_2/g DW) was obtained as compared to ultrasonication pretreatment (55.9 mL H_2/g DW).

Thermal pretreatment utilizes heat energy for the solubilization of microalgal biomass. Optimal range of temperature for the disintegration of organic matter varies according to substrate characteristics. For microalgal biomass, pretreatment temperature and time duration range from 65 to 180 °C and 15–60 min, respectively (Wang and Yin 2017). Thermal pretreatment by microwave heating is favoured for uniform distribution of heat and for achieving higher temperature in less time. Hydrothermal pretreatment (steam heating) at 100 and 121 °C led to increase in carbohydrates and proteins solubilization from lipid-extracted *Scenedesmus* biomass (Yang et al. 2010). However, thermal pretreatment alone is not sufficient for efficiently hydrolysing the microalgal biomass. Combination of heat and chemical pretreatment is commonly applied to improve the hydrogen yield from algal substrate. For instance, microwave and steam heating with dilute acid efficiently pretreated the biomass of *C. pyrenoidosa* with 8.6- and 9.5-fold increase in H_2 yields, respectively (Xia et al. 2013). Similarly, Roy et al. (2014) obtained high H_2 production (1.33 m^3/m^3) and reducing sugar concentration (9.6 kg/m^3) from HCl-heat pretreated biomass of *Chlorella sorokiniana*. Thermal–alkaline pretreatment of lipid-extracted *Scenedesmus* sp. biomass enhanced the H_2 production up to 168% (Yang et al. 2010).

Chemical pretreatment method involves the use of acid, alkali, solvents and oxidizing agent for the cell wall disintegration and saccharification of microalgal carbohydrates. Among the chemicals, acid and alkali reagents, generally in combination with heat, are used for the solubilization of organic matter. Liu et al. (2012) reported H_2 production of 1.42 L/L from acid (HCl)-pretreated hydrolysate of *Chlorella vulgaris* ESP6. In contrast, alkaline (NaOH)-pretreated hydrolysate was found to be less efficient for biohydrogen production. However, strong acidic conditions during the pretreatment may generate fermentative inhibitors such as furfural and HMF due to the dehydration of sugars. Moreover, the formation of inhibitory by-products can occur during the neutralization of the hydrolysate after acidic or alkaline pretreatment (Liu et al. 2012; Harun et al. 2014). Oxidizing agent such as H_2O_2 generates the nascent oxygen which helps in breaking the glycosidic bonds of complex sugars and converts it into simpler fermentable form. Roy et al. (2014) observed better H_2 production from H_2O_2-pretreated algal biomass than

autoclaved and sonicated algal biomass. Diluted acid in combination with autoclaving is the most commonly used method for the pretreatment of microalgal feedstock due to its simple operation and high yield of reducing sugar (Nguyen et al. 2010; Liu et al. 2012; Kumar et al. 2013; Roy et al. 2014).

Biological pretreatment by enzymes is considered as an efficient method for the hydrolysis of microalgal biomass due to the high substrate specificity, milder operating conditions, less energy consumption and no generation of inhibitory by-products. The effectiveness of this method depends upon the substrate characteristics, enzyme dosage, temperature, pH and treatment duration. For hydrolysis, selection of enzyme is based on the microalgal cell wall composition. The commonly used enzymes for microalgal pretreatment include cellulases, α-amylases, amyloglucosidases, xylanases and proteases (Hom-Diaz et al. 2016). The pretreatment of biomass by biological method is usually carried out after physical or chemical method. Cheng et al. (2014) studied the combined effect of cellulase and glucoamylase on the reducing sugar yield from acid–heat and alkali–heat pretreated algal biomass. In combination both enzymes gave better sugar yield than cellulase alone. To increase the conversion of starch, Nguyen et al. (2010) carried out the enzymatic hydrolysis of *C. reinhardtii* biomass by utilizing Termamyl (endoglucanase) enzyme. Under the optimized enzymatic hydrolysis condition, maximum H_2 yield of 2.5 mol/mol glucose equivalent was achieved via separate hydrolysis and fermentation (SHF) process. However, pure enzymes are expensive and use of such enzymes for biomass pretreatment makes the H_2 production process economically unattractive. Therefore, beside commercial enzymes, bacterial or fungal crude enzymes can be used as cheaper alternative for microalgal biomass pretreatment. Many bacterial and fungal species possess the unique ability of producing wide variety of extracellular hydrolytic enzymes. Prajapati et al. (2015) reported that crude enzyme obtained from *Aspergillus lentulus* can efficiently solubilize the microalgal sugars. Soluble sugar concentration of 57 mg/L and 29% COD solubilization were obtained when biomass of *Chroococcus* sp. was pretreated by the fungal crude enzyme concentration of 20% v/v. Nevertheless, biological pretreatment is a green approach of obtaining high sugar yield from microalgal biomass, and the lower rate of hydrolysis makes this process time consuming and unsuitable for commercialization. Research on fermentative hydrogen production from microalgal feedstock has just started, and most of the studies have been conducted in batch systems. The main findings on biohydrogen production using microalgal biomass as substrate are presented in Table 2.

2.3 *Molecular Approaches Towards Improvement in Biohydrogen Production from Microalgae*

Production of H_2 from microalgae is an attractive process, although this renewable energy system is limited by low H_2 yield and productivity. There are several

Table 2 Biohydrogen production using microalgal biomass as substrate

Microalgae	Carbohydrate content (% w/w)	Biomass (g/L)	Pretreatment	Inoculum	Operational conditions[a]	H_2 yield	Reference
Chlamydomonas reinhardtii	–	–	Enzymatic	*Thermotoga neapolitana* DSM 4359	Serum bottles, T = 75 °C	311.1 mL/g glucose	Nguyen et al. (2010)
Lipid-extracted *Scenedesmus obliquus*	24.7	18	Steam heating with dilute NaOH	Anaerobic digested sludge	Serum bottles, T = 37 °C, pH = 6.3	45.54 mL/gVS	Yang et al. (2010)
Chlorella vulgaris	8.0	–	No pretreatment	Satellite bacteria associated with algal biomass	Glass bottles, T = 37 °C, pH = 8.0	10.8 mL/gVS	Lakaniemi et al. (2011)
Dunaliella tertiolecta	4.0					12.6 mL/gVS	
Arthrospira platensis	44.4	20	Microwave heating with dilute H_2SO_4 + Enzymatic	Anaerobic activated sludge	Serum bottles, T = 35°C, pH = 6.5	96.6 mL/gTS	Cheng et al. (2012)
Chlorella vulgaris ESP6	57.0		Steam heating with dilute HCl	*Clostridium butyricum* CGS5	Serum bottles, T = 37°C, pH = 7.0	81.0 mL/g TS	Liu et al. (2012)
Chlorella sorokiniana	–	10	Steam heating with dilute HCl	*Enterobacter cloacae* IIT-BT 08	Double jacketed reactor, T = 37 °C, pH = 6.8	201.6 mL/g COD	Kumar et al. (2013)
Nannochloropsis oceanica	21.9	25	Microwave heating with dilute H_2SO_4	Anaerobic digested sludge	Glass bottles, T = 35 °C, pH = 6.0	39.0 mL/gVS	Xia et al. (2013)

(continued)

Table 2 (continued)

Microalgae	Carbohydrate content (% w/w)	Biomass (g/L)	Pretreatment	Inoculum	Operational conditions[a]	H_2 yield	Reference
Lipid and pigment extracted-*Nannochloropsis* sp.	–	2.5	Milling + supercritical fluid extraction	*Enterobacter aerogenes* ATCC 13048	Glass bottles, T = 35 °C	60.6 mL/g TS	Nobre et al. (2013)
Scenedesmus obliquus	30.7	2.5	Autoclave	*Enterobacter aerogenes* ATCC 13048	Serum bottles, T = 30°C	45.1 mL/gTS	Batista et al. (2014)
		50	Autoclave	*Clostridium butyricum* DSM 10702	Serum bottles, T = 37 °C	90.3 mL/gTS	
Anabaena sp. PCC 7120	–	12	Enzymatic	Enriched thermophilic mixed culture	Double jacketed reactor, T = 60 °C, pH = 5.5	143.8 mL/g COD	Nayak et al. (2014)
Chlorella sorokiniana	14.5	14	Steam heating with dilute HCl	Enriched thermophilic mixed culture	Double jacketed reactor, T = 60 °C, pH = 6.5	333.5 mL/g hexose	Roy et al. (2014)

[a]All the hydrogen production studies were carried out in batch mode

research challenges that must be addressed to prove the technological feasibility of the process. Understanding the molecular fundamentals of H_2 production pathway and application of genetic and metabolic engineering approaches could enhance the microalgal biohydrogen production. Significant advances in the development of genetic tools have been made to overcome some of the major bottlenecks associated with microalgal H_2 production system and to improve the product yield.

2.3.1 Oxygen Sensitivity of Hydrogen-Producing Enzymes

Biophotolysis method exploits highly active microalgal hydrogenases for H_2 production. However, the extreme O_2 sensitivity of these enzymes presents a challenge for achieving sustained evolution of H_2. It has been found that the presence of O_2 irreversibly inhibits the [FeFe]-hydrogenases by attacking the [4Fe–4S] domain of the H-cluster (Stripp et al. 2009). Even O_2 not only inactivates the hydrogenases but also imposes inhibitory effect on transcription and protein maturation (Oey et al. 2016). Therefore, several studies have been conducted to increase the O_2 tolerance of the microalgal hydrogenases. Random and site-directed mutagenesis helped in obtaining mutants of *C. reinhardtii* having tenfold high O_2 tolerance (Ghirardi et al. 2000). Xu et al. (2005) developed a recombinant cyanobacterial system by transferring the O_2-tolerant hydrogenase genes from *T. roseopersicina* into *Synechococcus* sp. PCC 7942. In a different approach for O_2 sequestration, leghaemoglobin proteins (having high affinity to O_2) from legume plant (soybean) were transformed into the chloroplast of *C. reinhardtii.* This method helped in rapid consumption of O_2 and facilitated fourfold increase of H_2 production in transgenic microalgal cultures (Chen et al. 2013; Wu et al. 2010).

2.4 Photon Conversion Efficiency

For the biofuel production by utilizing the photosynthetic machinery, quantum efficiency holds paramount importance. Microalgal H_2 production system is greatly limited by the low solar conversion efficiency. Under the controlled conditions and low light intensities, algal cultures could achieve light-to-hydrogen energy conversion efficiency of up to 10% which is comparatively higher than obtained under similar conditions with solar light (<4%). In bright light, the pigments of the huge light harvesting complex (antenna system) capture more photons that can be utilized by the photosynthetic system. In such case, microalgal cells protect themselves from photodamage by dissipating (wasting) excess photons ($\sim$90%) as heat and fluorescence via a process known as 'energy-dependent non-photochemical quenching' (NPQ). This occurs at the upper layer of the algal culture; however, the cells present at lower surface may not receive sufficient light due to the 'self-shading effect' imposed by dense culture. Thus, NPQ at the top layer and the self-shading effect at lower surface result in low photon conversion efficiency.

This efficiency can be improved by modifying the antenna complex through genetic engineering. Reduction in the antenna size can minimize the energy wastage and improve the penetration of light inside the reactor. Polle et al. (2002) developed *C. reinhardtii* strain having truncated Chl antenna size of PSII, which showed better H_2 production and cellular productivity than wild strain. In other study, a truncated antenna mutant of *C. reinhardtii* under high light conditions showed 8.5-fold higher solar-to-hydrogen conversion efficiency than parent strain (Kosourov et al. 2011). To reduce the energy losses, recently simultaneous down regulation of entire LHC gene family in *C. reinhardtii* Stm3LR3 was carried out by applying RNAi technology. The mutant exhibited high photosynthetic efficiency under elevated light intensity (Mussgnug et al. 2007). Cyanobacteria (*Synechocystis* PCC 6803) lacking phycocyanin or whole phycobilisome expected to produce H_2 efficiently under photoautotrophic condition (Bernát et al. 2009).

2.5 Elimination of Uptake Hydrogenases

Another main concern to obtain adequate amount of hydrogen is the elimination of uptake hydrogenase present in the heterocyst of the nitrogen-fixing cyanobacteria. These hydrogenases catalyse the oxidation of H_2 to recover the energy lost during nitrogen fixation. In several studies, mutants developed by knockout of the uptake hydrogenase genes (*hupL* or *hupS*) resulted in higher H_2 yield. Significantly, higher amount of H_2 was obtained by the mutants of *Anabaena variabilis* developed by disruption of hup genes (Mikheeva et al. 1995; Happe et al. 2000). Khetkorn et al. (2012) demonstrated fourfold increase in hydrogen production of *A. siamensis* TISTR 8012 by the disruption of *hupS* gene. Although deletion of uptake hydrogenase helps in improving the H_2 production, *hox*-encoded [NiFe]-bidirectional hydrogenases may still reabsorb the H_2 produced by the nitrogenase due to its small Km value for H_2. In this regard, Masukawa et al. (2002) studied the effect of *hupL*, *hoxH* and *hupL*/*hoxH* deletions on photobiological H_2 production by *Anabaena* sp. PCC 7120. Compared to wild strain, the *hupL*$^-$ mutant produced H_2 at 4–7 times high rate. However, the *hoxH*$^-$ mutant did not show any improvement in H_2 production.

2.6 Substrate Utilization

In indirect biophotolysis, for H_2 production the e^- is supplied via external substrate. Strategies for improving the utilization of different substrate (sugars) by microalgae might be helpful in enhancing the biomass and biohydrogen production. In this view, modification in the transporter protein may assist the efficient transfer of external substrate inside the cell. Recently, hexose symporter (*HUP1*) gene from *C. kessleri* was heterologously expressed in *C. reinhardtii* stm6 cells, lacking the

glucose transporter. The insertion of *HUP1* facilitated the import of glucose (1 mM) inside the stm6 cells. The transformed *C. reinhardtii* stm6Glc4 produced H_2 by simultaneously utilizing the water (66%) and glucose (33%) and showed fivefold increase in H_2 production than wild type (Doebbe et al. 2007).

2.7 Carbohydrate Metabolism of Microalgae

Carbohydrate-rich microalgal biomass is a suitable substrate for the fermentative H_2 production. The synthesis and accumulation of carbohydrates in microalgae occur due to CO_2 fixation, through a cyclic metabolic pathway known as Calvin cycle. CO_2 is reduced at the expense of ATP and NADPH generated during the light-dependent reaction of photosynthesis. In microalgae, the biosynthetic and catabolic pathways of energy storage molecules (starch and lipid) are closely linked. Some research findings suggest that a competition exist for the allocation of microalgal carbon between the carbohydrate and lipid synthesis (Rismani-Yazdi et al. 2011; Ho et al. 2012). Moreover, starch degradation provides main precursor (glycerol-3-phosphate, G3P) for triacylglycerol (TAG) synthesis. Thus, to enhance the biohydrogen production from microalgal feedstock, understanding and manipulating the starch metabolism become vital. The rate-limiting step in carbohydrate synthesis is catalysed by the enzyme ADP-glucose pyrophosphorylase (AGPase). An allosteric activator of AGPase is 3-phosphoglyceric acid (3-PGA) which is the intermediate product of CO_2 fixation reaction. Therefore, enhancing the photosynthetic efficiency might prove helpful to improve the carbohydrate synthesis and accumulation. In some studies, genetic modification in the RuBisCO subunits increased the photosynthetic efficiency of *Chlamydomonas* (Genkov et al. 2010; Zhu et al. 2010). An alternative strategy to enhance the microalgal starch accumulation is to decrease the starch degradation. The mechanism of microalgal carbohydrate catabolism is not completely understood, but it is well inferred in *Arabidopsis thaliana*. Phosphorolytic and/or hydrolytic enzymes play major role in starch degradation mechanism. Targeting these enzymes for gene knockout probably helps in developing microalgae with desirable phenotype (high carbohydrate content) (Radakovits et al. 2010). Except the starch stored in plastids, carbohydrates in algae are also found entrapped within the cell wall mainly in form of cellulose. The process of cellulose biosynthesis is complicated and involves several enzymatic reactions. It is synthesized by cellulase synthase utilizing UDP-glucose as precursor (Chen et al. 2013).

Due to the poor understanding of carbon partitioning between the biosynthetic pathways of energy-rich molecules, in comparison with genetic engineering, process engineering methodologies have greatly helped in the increment of microalgal carbohydrate content. However, few studies with molecular approaches have been carried out in cyanobacteria. In one such study, to enhance the cellulose yield, the genes for cellulose synthesis (*acsAB*) were transferred from *A. xylinum* into *Anabaena* sp. PCC 7120 via conjugation (Su et al. 2011). The mutant produced

total extractable glucose of 0.53–0.66 mg/mL/OD750 which could be used for biohydrogen production. Recently, Patel et al. (2016) applied random mutagenesis on *Synechocystis* PCC 6803 to develop strain with high biomass and carbohydrate productivity. The mutant produced 3.6-fold more biomass and carbohydrate yield of 225 mg/L, indicating its potential to be used as fermentative feedstock.

Finally, it could be inferred that advances made in genetic and metabolic engineering have brought a major breakthrough in microalgal H_2 production process by overcoming several barriers associated with the low hydrogen yield. Indeed, there are some other problems that must be resolved to increase the overall feasibility of the process. For instance, most of the studies on photobiological H_2 production are carried out at bench-scale photobioreactors (PBRs). Due to the data scarcity, addressing several engineering issues for the scaling up of the PBR becomes challenging (Fernández-Sevilla et al. 2014). Another major problem in H_2 production is the incomplete conversion of organic substrate into H_2 and CO_2 via dark fermentation. H_2 production through this process is associated with the production of some soluble metabolites (volatile fatty acids and alcohols). This leads to low gaseous energy recovery, and the spent media rich in organic acids may pose threat to environment. To overcome this problem, an integrative system can be devised where the effluent of dark fermentation can be integrated with anaerobic digestion, photofermentation and bioelectrochemical systems (Sambusiti et al. 2015). Interestingly, volatile fatty acids rich spent media can be efficiently utilized as substrate for the mixotrophic cultivation of microalgae (Ghosh et al. 2017). Furthermore, utilization of wastewater grown and lipid/value-added product extracted microalgae as feedstock for biohydrogen production and could make the process more economically alluring.

3 Conclusion

Hydrogen production through biological routes is considered as the cleanest way of renewable energy generation. Most of the green microalgae and cyanobacteria possess novel metabolic features to carry out photobiological hydrogen evolution. Moreover, microalgal biomass has great potential to be used as substrate for fermentative biohydrogen production. Nevertheless, an efficient and economical method of biomass pretreatment is critical for carbohydrate saccharification and its utilization by hydrogen-producing bacteria. Oxygen sensitivity of hydrogenases and low photon conversion efficiency are two major bottlenecks of microalgal hydrogen production via biophotolysis, while incomplete knowledge of carbohydrate metabolism presents a challenge for developing sugar-enriched microalgal feedstock for dark fermentation. Although the application of genetic tools to enhance the biohydrogen production from microalgae is currently in its infancy, promising advances have been made to develop the genetically engineered microalgae with unprecedented precision. It is likely that further research in this

direction might help in developing industrially relevant microalgal species for carbon-neutral hydrogen generation.

References

Almon, H., & Böger, P. 1988. Nitrogen and hydrogen metabolism: induction and measurement. *Methods in Enzymology*, Academic Press, 167.

Baebprasert, W., Lindblad, P., & Incharoensakdi, A. (2010). Response of H_2 production and Hox-hydrogenase activity to external factors in the unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803. *International Journal of Hydrogen Energy, 35*(13), 6611–6616.

Batista, A. P., Moura, P., Marques, P. A. S. S., Ortigueira, J., Alves, L., & Gouveia, L. (2014). *Scenedesmus obliquus* as feedstock for biohydrogen production by *Enterobacter aerogenes* and *Clostridium butyricum*. *Fuel, 117,* 537–543.

Benemann, J. R. (2000). Hydrogen production by microalgae. *Journal of Applied Phycology, 12,* 291–300.

Bernát, G., Waschewski, N., & Rögner, M. (2009). Towards efficient hydrogen production: The impact of antenna size and external factors on electron transport dynamics in *Synechocystis* PCC 6803. *Photosynthesis Research, 99*(3), 205–216.

Borodin, V. B., Tsygankov, A. A., Rao, K. K., & Hall, D. O. (2000). Hydrogen production by *Anabaena variabilis* PK84 under simulated outdoor conditions. *Biotechnology and Bioengineering, 69*(5), 478–485.

Burrows, E. H., Chaplen, F. W. R., & Ely, R. L. (2008). Optimization of media nutrient composition for increased photofermentative hydrogen production by *Synechocystis* sp. PCC 6803. *International Journal of Hydrogen Energy, 33*(21), 6092–6099.

Chen, C. Y., Zhao, X. Q., Yen, H. W., Ho, S. H., Cheng, C. L., Lee, D. J., et al. (2013). Microalgae-based carbohydrates for biofuel production. *Biochemical Engineering Journal, 78,* 1–10.

Cheng, J., Liu, Y., Lin, R., Xia, A., Zhou, J., & Cen, K. (2014). Cogeneration of hydrogen and methane from the pretreated biomass of algae bloom in Taihu Lake. *International Journal of Hydrogen Energy, 39*(33), 18793–18802.

Cheng, J., Xia, A., Liu, Y., Lin, R., Zhou, J., & Cen, K. (2012). Combination of dark- and photo-fermentation to improve hydrogen production from *Arthrospira platensis* wet biomass with ammonium removal by zeolite. *International Journal of Hydrogen Energy, 37*(18), 13330–13337.

Das, D., & Veziroğlu, T. N. (2001). Hydrogen production by biological proceses: A survey of literature. *International Journal of Hydrogen Energy, 26,* 13–28.

Das, D., Khanna, N., & Dasgupta, C. N. (2014). *Biohydrogen production: Fundamentals and technology advances*. CRC Press, Taylor and Francis Group, LLC.

Doebbe, A., Rupprecht, J., Beckmann, J., Mussgnug, J. H., Hallmann, A., Hankamer, B., et al. (2007). Functional integration of the HUP1 hexose symporter gene into the genome of *C. reinhardtii*: Impacts on biological H_2 production. *Journal of Biotechnology, 131*(1), 27–33.

Eroglu, E., & Melis, A. (2011). Photobiological hydrogen production: Recent advances and state of the art. *Bioresource Technology, 102*(18), 8403–8413.

Fernández-Sevilla, J. M., Acién-Fernández, F. G., & Molina-Grima, E. (2014). Microbial bioenergy: Hydrogen production. *Advances in Photosynthesis and Respiration, 38,* 291–320.

Forestier, M., King, P., Zhang, L., Posewitz, M., Schwarzer, S., Happe, T., et al. (2003). Expression of two [Fe] -hydrogenases in *Chlamydomonas reinhardtii* under anaerobic conditions. *European Journal of Biochemistry, 270,* 2750–2758.

Gaffron, H., & Rubin, J. (1942). Fermentatinve and photochemical production of hydrogen in algae. *The Journal of General Physiology, 26*(2), 219–240.

Genkov, T., Meyer, M., Griffiths, H., & Spreitzer, R. J. (2010). Functional hybrid rubisco enzymes with plant small subunits and algal large subunits: Engineered rbcS cDNA for expression in *Chlamydomonas*. *Journal of Biological Chemistry, 285*(26), 19833–19841.

Ghimire, A., Frunzo, L., Pirozzi, F., Trably, E., Escudie, R., Lens, P. N. L., et al. (2015). A review on dark fermentative biohydrogen production from organic biomass: Process parameters and use of by-products. *Applied Energy, 144,* 73–95.

Ghirardi, M. L., Togasaki, R. K., & Seibert, M. (1997). Oxygen sensitivity of Algal H_2-production. *Applied Biochemistry and Biotechnology, 63–65,* 141–151.

Ghirardi, M. L., Zhang, L., Lee, J. W., Flynn, T., Seibert, M., Greenbaum, E., et al. (2000). Microalgae: A green source of renewable H_2. *Trends in Biotechnology, 18*(12), 506–511.

Ghosh, S., Roy, S., & Das, D. (2017). Enhancement in lipid content of *Chlorella* sp. MJ11/11 from the spent medium of thermophilic biohydrogen production process. *Bioresource Technology, 223,* 219–226.

Greenbaum, E. (1982). Photosynthetic hydrogen and oxygen production: Kinetic studies. *Science (New York), 215*(4530), 291–293.

Happe, T., Hemschemeier, A., Winkler, M., & Kaminski, A. (2002). Hydrogenases in green algae: Do they save the algae's life and solve our energy problems? *Trends in Plant Science, 7*(6), 246–250.

Happe, T., Schütz, K., & Böhme, H. (2000). Transcriptional and mutational analysis of the uptake hydrogenase of the filamentous cyanobacterium *Anabaena variabilis* ATCC 29413. *Journal of Bacteriology, 182*(6), 1624–1631.

Harun, R., Yip, J. W. S., Thiruvenkadam, S., Ghani, W. A. W. A. K., Cherrington, T., & Danquah, M. K. (2014). Algal biomass conversion to bioethanol-a step-by-step assessment. *Biotechnology Journal, 9*(1), 73–86.

Hernández, D., Riaño, B., Coca, M., & García-González, M. C. (2015). Saccharification of carbohydrates in microalgal biomass by physical, chemical and enzymatic pre-treatments as a previous step for bioethanol production. *Chemical Engineering Journal, 262,* 939–945.

Ho, S. H., Chen, C. Y., & Chang, J. S. (2012). Effect of light intensity and nitrogen starvation on CO_2 fixation and lipid/carbohydrate production of an indigenous microalga *Scenedesmus obliquus* CNW-N. *Bioresource Technology, 113,* 244–252.

Ho, S. H., Huang, S. W., Chen, C. Y., Hasunuma, T., Kondo, A., & Chang, J. S. (2013). Bioethanol production using carbohydrate rich micraolgae biomass as feedstock. *Bioresource Technology, 135,* 191–198.

Holladay, J. D., Hu, J., King, D. L., & Wang, Y. (2009). An overview of hydrogen production technologies. *Catalysis Today, 139*(4), 244–260.

Hom-Diaz, A., Passos, F., Ferrer, I., Vicent, T., & Blánquez, P. (2016). Enzymatic pretreatment of microalgae using fungal broth from *Trametes versicolor* and commercial laccase for improved biogas production. *Algal Research, 19,* 184–188.

Khan, M. I., Lee, M. G., Shin, J. H., & Kim, J. D. (2017). Pretreatment optimization of the biomass of Microcystis aeruginosa for efficient bioethanol production. *AMB Expr*, *7*(19), 1–9.

Khetkorn, W., Lindblad, P., & Incharoensakdi, A. (2012). Inactivation of uptake hydrogenase leads to enhanced and sustained hydrogen production with high nitrogenase activity under high light exposure in the cyanobacterium *Anabaena siamensis* TISTR 8012. *Journal of Biological Engineering, 6*(19), 1–11.

Kim, D.-H., & Kim, M.-S. (2011). Hydrogenases for biological hydrogen production. *Bioresource Technology, 102*(18), 8423–8431.

Kosourov, S., Makarova, V., Fedorov, A. S., Tsygankov, A., Seibert, M., & Ghirardi, M. L. (2005). The effect of sulfur re-addition on H_2 photoproduction by sulfur-deprived green algae. *Photosynthesis Research, 85,* 295–305.

Kosourov, S., Tsygankov, A., Seibert, M., & Ghirardi, M. L. (2002). Sustained hydrogen photoproduction by *Chlamydomonas reinhardtii*: Effects of culture parameters. *Biotechnology and Bioengineering, 78*(7), 731–740.

Kosourov, S. N., Ghirardi, M. L., & Seibert, M. (2011). A truncated antenna mutant of *Chlamydomonas reinhardtii* can produce more hydrogen than the parental strain. *International Journal of Hydrogen Energy, 36*(3), 2044–2048.

Kumar, K., Roy, S., & Das, D. (2013). Continuous mode of carbon dioxide sequestration by *C. sorokiniana* and subsequent use of its biomass for hydrogen production by *E. cloacae* IIT-BT 08. *Bioresource Technology, 145,* 116–122.

Lakaniemi, A. M., Hulatt, C. J., Thomas, D. N., Tuovinen, O. H., & Puhakka, J. A. (2011). Biogenic hydrogen and methane production from *Chlorella vulgaris* and *Dunaliella tertiolecta* biomass. *Biotechnology for Biofuels, 4*(1), 34.

Laurinavichene, T. V., Fedorov, A. S., Ghirardi, M. L., Seibert, M., & Tsygankov, A. A. (2006). Demonstration of sustained hydrogen photoproduction by immobilized, sulfur-deprived *Chlamydomonas reinhardtii* cells. *International Journal of Hydrogen Energy, 31*(5), 659–667.

Lay, C. H., Sen, B., Chen, C. C., Wu, J. H., Lee, S. C., & Lin, C. Y. (2013). Co-fermentation of water hyacinth and beverage wastewater in powder and pellet form for hydrogen production. *Bioresource Technology, 135,* 610–615.

Levin, D. B., Pitt, L., & Love, M. (2004). Biohydrogen production: Prospects and limitations to practical application. *International Journal of Hydrogen Energy, 29*(2), 173–185.

Lindblad, P., Christensson, K., Lindberg, P., Fedorov, A., Pinto, F., & Tsygankov, A. (2002). Photoproduction of H2 by wildtype Anabaena PCC 7120 and a hydrogen uptake deficient mutant: From laboratory experiments to outdoor culture. *International Journal of Hydrogen, 27,* 1271–1281.

Liu, C. H., Chang, C. Y., Cheng, C. L., Lee, D. J., & Chang, J. S. (2012). Fermentative hydrogen production by *Clostridium butyricum* CGS5 using carbohydrate-rich microalgal biomass as feedstock. *International Journal of Hydrogen Energy, 37*(20), 15458–15464.

Márquez-Reyes, L. A., Sánchez-Saavedra, M. D. P., & Valdez-Vazquez, I. (2015). Improvement of hydrogen production by reduction of the photosynthetic oxygen in microalgae cultures of *Chlamydomonas gloeopara* and *Scenedesmus obliquus*. *International Journal of Hydrogen Energy, 40*(23), 7291–7300.

Markou, G., Angelidaki, I., & Georgakakis, D. (2012a). Microalgal carbohydrates: An overview of the factors influencing carbohydrates production, and of main bioconversion technologies for production of biofuels. *Applied Microbiology and Biotechnology, 96*(3), 631–645.

Markou, G., Chatzipavlidis, I., & Georgakakis, D. (2012b). Cultivation of *Arthrospira* (*Spirulina*) platensis in olive-oil mill wastewater treated with sodium hypochlorite. *Bioresource Technology, 112,* 234–241.

Masukawa, H., Mochimaru, M., & Sakurai, H. (2002). Disruption of the uptake hydrogenase gene, but not of the bidirectional hydrogenase gene, leads to enhanced photobiological hydrogen production by the nitrogen-fixing cyanobacterium *Anabaena* sp. PCC 7120. *Applied Microbiology and Biotechnology, 58*(5), 618–624.

McKinlay, J. B., & Harwood, C. S. (2010). Photobiological production of hydrogen gas as a biofuel. *Current Opinion in Biotechnology, 21*(3), 244–251.

Melis, A., Zhang, L., Forestier, M., Ghirardi, M. L., & Seibert, M. (2000). Sustained photobiological hydrogen gas production upon reversible inactivation of oxygen evolution in the green Alga *Chlamydomonas reinhardtii*. *Plant Physiology, 122,* 127–135.

Mikheeva, L. E., Schmitz, O., Shestakov, S. V., & Bothe, H. (1995). Mutants of the cyanobacterium *Anabaena variabilis* altered in hydrogenase activities. *Z. Natutforsch, 50,* 505–510.

Miranda, J. R., Passarinho, P. C., & Gouveia, L. (2012). Pre-treatment optimization of *Scenedesmus obliquus* microalga for bioethanol production. *Bioresource Technology, 104,* 342–348.

Miura, Y., Akano, T., Fukatsu, K., Miyasaka, H., Mizoguchi, T., Yagi, K., et al. (1997). Stably sustained hydrogen production by biophotolysis in natural day/night cycle. *Energy Conservation Management, 38,* 533–537.

Monlau, F., Sambusiti, C., Barakat, A., Quéméneur, M., Trably, E., Steyer, J. P., et al. (2014). Do furanic and phenolic compounds of lignocellulosic and algae biomass hydrolyzate inhibit anaerobic mixed cultures? A comprehensive review. *Biotechnology Advances, 32*(5), 934–951.

Montingelli, M. E., Tedesco, S., & Olabi, A. G. (2015). Biogas production from algal biomass: A review. *Renewable and Sustainable Energy Reviews, 43,* 961–972.

Mussgnug, J. H., Klassen, V., Schlüter, A., & Kruse, O. (2010). Microalgae as substrates for fermentative biogas production in a combined biorefinery concept. *Journal of Biotechnology, 150*(1), 51–56.

Mussgnug, J. H., Thomas-Hall, S., Rupprecht, J., Foo, A., Klassen, V., & McDowall, A., et al. (2007). Engineering photosynthetic light capture: Impacts on improved solar energy to biomass conversion. *Plant Biotechnology Journal*, *5*(6), 802–814.

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

Nguyen, T. A. D., Kim, K. R., Nguyen, M. T., Kim, M. S., Kim, D., & Sim, S. J. (2010). Enhancement of fermentative hydrogen production from green algal biomass of Thermotoga neapolitana by various pretreatment methods. *International Journal of Hydrogen Energy, 35* (23), 13035–13040.

Nobre, B. P., Villalobos, F., Barragán, B. E., Oliveira, A. C., Batista, A. P., Marques, P. A. S. S., et al. (2013). A biorefinary from *Nannchloropsis* sp. microalga - Extraction of oils and pigments. Production of biohydrogen from the leftcover biomass. *Bioresource Tecnology, 135,* 128–136.

Nyberg, M., Heidorn, T., & Lindblad, P. (2015). Hydrogen production by the engineered cyanobacterial strain *Nostoc* PCC 7120 δhupW examined in a flat panel photobioreactor system. *Journal of Biotechnology, 215,* 35–43.

Ortigueira, J., Alves, L., Gouveia, L., & Moura, P. (2015). Third generation biohydrogen production by *Clostridium butyricum* and adapted mixed cultures from *Scenedesmus obliquus* microalga biomass. *Fuel, 153,* 128–134.

Oey, M., Sawyer, A. L., Ross, I. L., & Hankamer, B. (2016). Challenges and opportunities for hydrogen production from microalgae. *Plant Biotechnology Journal, 14*(7), 1487–1499.

Vignais, P. M., & Colbeau, A. (2004). Molecular biology of microbial hydrogenases. *Current Issues in Molecular Biology, 6,* 159–188.

Pancha, I., Chokshi, K., & Mishra, S. (2015). Enhanced biofuel production potential with nutritional stress amelioration through optimization of carbon source and light intensity in *Scenedesmus* sp. CCNM 1077. *Bioresource Technology, 179,* 565–572.

Patel, V. K., Maji, D., Pandey, S. S., Rout, P. K., Sundaram, S., & Kalra, A. (2016). Rapid budding EMS mutants of Synechocystis PCC 6803 producing carbohydrate or lipid enriched biomass. *Algal Research, 16,* 36–45.

Peters, J. W., Lanzilotta, W. N., Lemon, B. J., & Seefeldt, L. C. (1998). X-ray crystal structure of the Fe-only hydrogenase (CpI) from *Clostridium pasteurianum* to 1.8 Angstrom resolution. *Science (New York), 282*(5395), 1853–1858.

Peters, J. W., Schut, G. J., Boyd, E. S., Mulder, D. W., Shepard, E. M., Broderick, J. B., et al. (2015). [FeFe] - and [NiFe] -hydrogenase diversity, mechanism, and maturation. *Biochimica et Biophysica Acta, 1853,* 1350–1369.

Polle, J. E. W., Kanakagiri, S., Jin, E., Masuda, T., & Melis, A. (2002). Truncated chlorophyll antenna size of the photosystems—A practical method to improve microalgal productivity and hydrogen production in mass culture. *International Journal of Hydrogen Energy, 27*(11–12), 1257–1264.

Prajapati, S. K., Bhattacharya, A., Malik, A., & Vijay, V. K. (2015). Pretreatment of algal biomass using fungal crude enzymes. *Algal Research, 8,* 8–14.

Radakovits, R., Jinkerson, R. E., Darzins, A., & Posewitz, M. C. (2010). Genetic engineering of algae for enhanced biofuel production. *Eukaryotic Cell, 9*(4), 486–501.

Randt, C., & Senger, H. (1985). Participation of the two photosystems in light dependent hydrogen evolution in *Scenedesmus obliquus*. *Photochemistry and Photobiology, 42*(5), 553–557.

Rismani-Yazdi, H., Haznedaroglu, B. Z., Bibby, K., & Peccia, J. (2011). Transcriptome sequencing and annotation of the microalgae *Dunaliella tertiolecta*: Pathway description and gene discovery for production of next-generation biofuels. *BMC Genomics, 12*(1), 148.

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

Sambusiti, C., Bellucci, M., Zabaniotou, A., Beneduce, L., & Monlau, F. (2015). Algae as promising feedstocks for fermentative biohydrogen production according to a biorefinery approach: A comprehensive review. *Renewable and Sustainable Energy Reviews, 44,* 20–36.

Shi, X. Y., & Yu, H. Q. (2016). Simultaneous metabolism of benzoate and photobiological hydrogen production by *Lyngbya* sp. *Renewable Energy, 95,* 474–477.

Stripp, S. T., Goldet, G., Brandmayr, C., Sanganas, O., Vincent, K. A., Haumann, M., et al. (2009). How oxygen attacks [FeFe] hydrogenases from photosynthetic organisms. *Proceedings of the National Academy of Sciences of the United States of America, 106*(41), 17331–17336.

Su, H. Y., Lee, T. M., Huang, Y. L., Chou, S. H., Wang, J. B., Lin, L. F., et al. (2011). Increased cellulose production by heterologous expression of cellulose synthase genes in a filamentous heterocystous cyanobacterium with a modification in photosynthesis performance and growth ability. *Botanical Studies, 52,* 265–275.

Taikhao, S., Junyapoon, S., Incharoensakdi, A., & Phunpruch, S. (2013). Factors affecting biohydrogen production by unicellular halotolerant cyanobacterium *Aphanothece halophytica*. *Journal of Applied Phycology, 25*(2), 575–585.

Tamagnini, P., Axelsson, R., Lindberg, P., Oxelfelt, F., Wünschiers, R., & Lindblad, P. (2002). Hydrogenases and hydrogen metabolism of cyanobacteria. *Microbiology and Molecular Biology Reviews, 66*(1), 1–20.

Tiwari, A., & Pandey, A. (2012). Cyanobacterial hydrogen production—A step towards clean environment. *International Journal of Hydrogen Energy, 37*(1), 139–150.

Torzillo, G., Scoma, A., Faraloni, C., Ena, A., & Johanningmeier, U. (2009). Increased hydrogen photoproduction by means of a sulfur-deprived *Chlamydomonas reinhardtii* D1 protein mutant. *International Journal of Hydrogen Energy, 34*(10), 4529–4536.

Torzillo, G., Scoma, A., Faraloni, C., & Giannelli, L. (2015). Advances in biotechnology of hydrogen production with the microalga *Chlamydomonas reinhardtii*. *Critical reviews in Biotecnology, 35*(4), 485–496.

Torzillo, G., & Seibert, M. 2013. Hydrogen production by *Chlamydomonas reinhardtii.* In *Handbook of microalgal culture: Applied phycology and biotechnology*, pp. 417–432.

Troshina, O., Serebryakova, L., Sheremetieva, M., & Lindblad, P. (2002). Production of H_2 by the unicellular cyanobacterium *Gloeocapsa alpicola* CALU 743 during fermentation. *International Journal of Hydrogen Energy, 27*(11–12), 1283–1289.

Tsygankov, A. A., Fedorov, A. S., Kosourov, S. N., & Rao, K. K. (2002). Hydrogen production by cyanobacteria in an automated outdoor photobioreactor under aerobic conditions. *Biotechnology and Bioengineering, 80*(7), 777–783.

Tsygankov, A. A., Kosourov, S. N., Tolstygina, I. V., Ghirardi, M. L., & Seibert, M. (2006). Hydrogen production by sulfur-deprived Chlamydomonas reinhardtii under photoautotrophic conditions. *International Journal of Hydrogen Energy*, *31*, 1574–1584.

Vitova, M., Bisova, K., Kawano, S., & Zachleder, V. (2015). Accumulation of energy reserves in algae: From cell cycles to biotechnological applications. *Biotechnology Advances, 33*(6), 1204–1218.

Wang, J., & Yin, Y. (2017*).* Bihydrogen production from organic wastes. In *Green energy and technology* (pp. 123–195), Springer Nature.

Wang, Y., Ho, S.-H., Yen, H.-W., Nagarajan, D., Ren, N.-Q., & Li, S. et al. (2017). Current advances on fermantative biobutanol production using third generation feedstock. *Biotechnology Advances*. https://doi.org/10.1016/j.biotechadv.2017.06.001.

Wu, S., Huang, R., Xu, L., Yan, G., & Wang, Q. (2010). Improved hydrogen production with expression of *hemH* and *lba* genes in chloroplast of *Chlamydomonas reinhardtii*. *Journal of Biotechnology, 146,* 120–125.

Xia, A., Cheng, J., Ding, L., Lin, R., Song, W., Zhou, J., et al. (2014). Enhancement of energy production efficiency from mixed biomass of *Chlorella pyrenoidosa* and cassava starch through combined hydrogen fermentation and methanogenesis. *Applied Energy, 120,* 23–30.

Xia, A., Cheng, J., Lin, R., Lu, H., Zhou, J., & Cen, K. (2013). Comparison in dark hydrogen fermentation followed by photo hydrogen fermentation and methanogenesis between protein and carbohydrate compositions in *Nannochloropsis oceanica* biomass. *Bioresource Technology, 138,* 204–213.

Xia, A., Cheng, J., Song, W., Su, H., Ding, L., Lin, R., et al. (2015). Fermentative hydrogen production using algal biomass as feedstock. *Renewable and Sustainable Energy Reviews, 51,* 209–230.

Xu, Q., Yooseph, S., Smith, H.O., Venter, C.J. 2005. *Development of a novel recombinant cyanobacterial system for hydrogen production from water*. Paper presented at Genomics: GTL Program Projects, Rockville.

Yang, Z., Guo, R., Xu, X., Fan, X., & Li, X. (2010). Enhanced hydrogen production from lipid-extracted microalgal biomass residues through pretreatment. *International Journal of Hydrogen Energy, 35,* 9618–9623.

Yu, J., & Takahashi, P. (2007). Biophotolysis-based hydrogen production by cyanobacteria and green microalgae. *Trends in Applied Microbiology, 1,* 79–89.

Zhu, X.-G., Long, S. P., & Ort, D. R. (2010). Improving photosynthetic efficiency for greater yield. *Annual Review of Plant Biology, 61*(1), 235–261.