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Review

Microalgal hydrogen production – A review

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HIGHLIGHTS

- Critically reviews the bio-hydrogen production from microalgae.
- Recent technological progress towards microalgae based H_2 production.
- Development of photobioreactors for large-scale biomass and H_2 production.
- Genetic and metabolic engineering approaches towards H_2 production from microalgae.

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ABSTRACT

Bio-hydrogen from microalgae including cyanobacteria has attracted commercial awareness due to its potential as an alternative, reliable and renewable energy source. Photosynthetic hydrogen production from microalgae can be interesting and promising options for clean energy. Advances in hydrogen-fuel-cell technology may attest an eco-friendly way of biofuel production, since, the use of H_2 to generate electricity releases only water as a by-product. Progress in genetic/metabolic engineering may significantly enhance the photobiological hydrogen production from microalgae. Manipulation of competing metabolic pathways by modulating the certain key enzymes such as hydrogenase and nitrogenase may enhance the evolution of H_2 from photoautotrophic cells. Moreover, biological H_2 production at low operating costs is requisite for economic viability. Several photobioreactors have been developed for large-scale biomass and hydrogen production. This review highlights the recent technological progress, enzymes involved and genetic as well as metabolic engineering approaches towards sustainable hydrogen production from microalgae.

Contents

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1. Introduction

Microalgae are considered the most primitive and dominant form of photosynthetic life on the Earth's surface. They play an important role in dynamics of energy flow in an ecosystem and are an immense source of several valuable natural products of ecological and economic importance (Skjånes et al., 2013; Rastogi et al., 2017). The use of certain species of microalgae as a nonconventional source of food and pharmaceuticals seems promising. Some members of green algae and cyanobacteria are considered an excellent source of renewable biofuels such as bio-diesel, biogas, bio-oil and bio-hydrogen (Maneeruttanarungroj et al., 2010; Khetkorn et al., 2013; Skjånes et al., 2013).

Hydrogen gas is one of the highly versatile, efficient and sustainable clean energy carriers that may be used to replace the fossil fuels due to its high energy yield when compared to traditional hydrocarbon fuels (Aziz, 2016; Hosseini and Wahid, 2016). A unit weight of hydrogen gas can generate heating value as 141.65 MJ/kg (Perry, 1963). Among various secondary energy resources, hydrogen may widely be used as a clean energy in reciprocating combustion engines and fuel cells. (Fig. 1). A fuel cell can generate electricity continuously by the electrochemical reaction of hydrogen and oxygen from the air. Hydrogen-powered fuels can be used in different types of fuel cells, which emit only water and have virtually no pollutant emissions (Edwards et al., 2008). Since hydrogen oxidation yields water as the main product, it can be used in an eco-friendly manner with the reduction of the global greenhouse gas emissions and the environmental damages generated by combustion of fossil fuels (Awad and Veziroglu, 1984).

Hydrogen gas has been used in a variety of applications; it can generate electricity using fuel cells and it can be used as fuel in rocket engines including applications in transportation (Ramachandran and Menon, 1998). Moreover, hydrogen gas is an important industrial gas and raw material in many applications and processes. However, high production cost, difficulties in storage and transportation and an undeveloped hydrogen infrastructure all create problems in economic viability as a fuel source (Chu and Majumdar, 2012; Rashid et al., 2013; Oey et al., 2016). In addition, storage and transportation methods as well as development of hydrogen infrastructure are essential for sustainable hydrogen economy (Moreno-Benito et al., 2016; Unni et al., 2017). The potential of hydrogen production in green microalgae and cyanobacteria depends on strain specific capacity to synthesize different enzymes responsible for hydrogen metabolism, and environmental conditions providing required energy. Furthermore, several genetic engineering approaches such as targeted engineering of certain enzymes (e.g., hydrogenases and nitrogenases) and metabolic pathways may significantly improve the hydrogen production (Khetkorn et al., 2013). Some strains of microalgae have been developed using a genetic engineering approach to enhance their capability of hydrogen production (Baebprasert et al., 2011; Maneeruttanarungroj et al., 2012; Khetkorn et al., 2012a; Khetkorn et al., 2012b; Nyberg et al., 2015).

There are many processes for hydrogen production, such as steam reforming, electrolysis, photolysis or biohydrogen production and several other methods (Holladay et al., 2009; Yilmaz et al., 2016). The steam reforming method is using high temperature steam to produce hydrogen gas from fossil fuel or natural

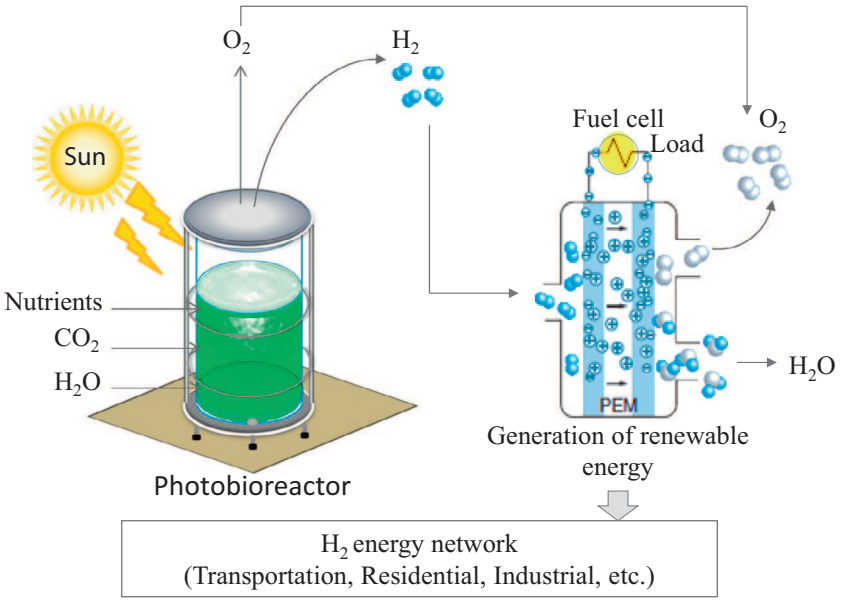

Fig. 1. Photo-biological production of renewable hydrogen energy from microalgae and its application as hydrogen fuel cells that may be employed in transportation, residential and industrial energy networks.

Table 1
Different biological hydrogen production processes in various microorganisms.

Microorganisms	Strains	Different biological H_2 production processes	Advantages and disadvantages
Green algae	*Scenedesmus obliquus* *Chlamydomonas reinhardii* *C. moewusii* *Chlorella vulgaris*	*Direct biophotolysis* $2H_2O + Fd_{(ox)} + light \rightarrow O_2 + 4H + Fd_{(red)}(4e^-)$ $4H^+ + Fd_{(red)}(4e^-) \rightarrow 2H_2 + Fd_{(ox)}$ *Photo-fermentation* $12H_2O + 6CO_2 \rightarrow C_6H_{12}O_6 + 6O_2$ $C_6H_{12}O_6 + 6H_2 \rightarrow 6CO_2 + 12H_2$	*Advantages:* – Can produce H_2 from water and sunlight – Solar conversion energy is increased by 10 folds as compared to trees, crops – Can reduce CO_2 in environment *Disadvantages:* – Inhibition of hydrogen production by oxygen
Cyanobacteria	*Synechocystis* PCC 6803 *Anabaena variabilis* *Anabaena* sp. PCC 7120 *Cyanothece* sp.	*Direct biophotolysis* $2H_2O + Fd_{(ox)} + light \rightarrow O_2 + 4H + Fd_{(red)}(4e^-)$ $4H^+ + Fd_{(red)}(4e^-) \rightarrow 2H_2 + Fd_{(ox)}$ *Indirect biophotolysis* $N_2 + 8H^+ + Fd_{(red)}(8e^-) + 16ATP \rightarrow 2NH_3 + H_2 + Fd_{(ox)} + 16ADP + 16P_i$ $8H^+ + 8e^- + 16ATP \rightarrow 4H_2 + 16ADP + 16P_i$ $12H_2O + 6CO_2 \rightarrow C_6H_{12}O_6 + 6O_2$ $C_6H_{12}O_6 + 6H_2 \rightarrow 6CO_2 + 12H_2$	*Advantages:* – Can produce H_2 from water and sunlight – Can reduce CO_2 in environment – H_2 production is separated from O_2 production *Disadvantages:* – Consumption of H_2 by uptake hydrogenase – Inhibition of hydrogen production by oxygen
Photosynthetic bacteria	*Rhodobater sphaeroides* *R. capsulatus* *R. sulidophilus* *Thiocapsa roseopersicina*	*Photo-fermentation* $CH_3COOH + 2H_2O + light = 4H_2 + 2CO_2$ $N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16P_i$	*Advantages:* – Can use different waste materials like, whey, distillery, etc. – Can use wide spectrum of light *Disadvantages:* – Requires light for the H_2 production – Fermented broth causes water pollution problem
Fermentative bacteria	*Enterobacter aerogenes* *Clostridium butyricum* *Magashaera elsdenii*	*Dark fermentation* $C_6H_{12}O_6 + 6H_2O \rightarrow 12H_2 + 6CO_2$ Pyruvate + CoA → acetyl-CoA + formate Pyruvate + CoA + $2Fd_{(ox)}$ → acetyl-CoA + $CO_2 + 2Fd_{(red)}$	*Advantages:* – Can produce H_2 all day long without light in anaerobic process – Can utilize different carbon sources like, starch, cellobiose, sucrose, etc. – Produces valuable metabolites such as butyric acid, lactic acid *Disadvantages:* – Requires C and N-sources for growth – Fermented broth causes water pollution problem – CO_2 is present in the gas

gas. A number of catalysts have been explored for enhancing the efficiency of steam reforming processes (Obradovic et al., 2013). The electrolysis is an electrolytic process to decompose water molecule into O_2 and H_2. However, these two methods are energy intensive and generate CO/CO_2 and other pollutants during process as by products, which is not always environmentally friendly. Moreover, besides several methods of H_2 production, photobiological hydrogen production may be one of the efficient, environmentally friendly and less energy intensive approaches as compared to thermo-chemical and electrochemical processes (Debabrata and Veziroğlu, 2001; Khetkorn et al., 2013; Nyberg et al., 2015). In addition, hydrogen is considered an important alternative which can secure energy future for the world. In this review emphasize the recent progress and technological development towards a sustainable hydrogen production from microalgae.

2. Biological hydrogen production

Biological hydrogen production is a method for producing hydrogen gas by using microorganisms. There are several photosynthetic and non-photosynthetic microorganisms that can produce hydrogen gas namely, green algae, cyanobacteria, photosynthetic bacteria and dark fermentative bacteria. These microorganisms have very diverse physiology and metabolism that allow them to generate hydrogen using different metabolic pathways (Table 1). The production of hydrogen by microorganisms has attracted public interest due to its potential as a renewable energy carrier which can be produced using nature's plentiful resources, solar energy and water.

Microalgae are one of the groups of photosynthetic microorganisms suitable for the photo-biological production of hydrogen (Hansel and Lindblad, 1998; Nagarajan et al., 2017). A number of microalgae belonging to the genera *Botryococcus*, *Chlamydomonas*, *Chlorococcum*, *Chlorella*, *Scenedesmus*, *Synechocystis*, *Tetraspora*, *Anabaena*, *Nostoc*, etc. may harbor a hydrogenase enzyme for hydrogen production (Eroglu and Melis, 2011). They can grow in the simple nutrient, with the ability to fix CO_2 from atmosphere as carbon source and many strains are able to reduce atmospheric N_2 to ammonia as well as utilize sunlight as only source of energy to produce hydrogen. Biological processes of hydrogen production have several advantages over the conventional chemical and/or physical production processes. A number of microalgae are endowed with powerful photosynthetic machinery required for photobiological hydrogen production (Eroglu and Melis, 2016; He et al., 2017) using water as a source of electrons and solar light as a source of energy (Esper et al., 2006) (Fig. 1). Furthermore, hydrogen can directly be used in a fuel cell to generate electricity with high efficiency. It is important to design efficient hydrogen-powered fuel cells suitable for different energy applications.

3. Biophotolysis and hydrogen metabolism in microalgae

Green microalgae and cyanobacteria (also called blue green algae) are oxygenic photosynthetic microorganisms. They contain pigment molecules which are able to absorb solar energy and convert it into chemical energy by simultaneous splitting of water to molecular oxygen (O_2) and protons (H^+) (Yilmaz et al., 2016). Photosynthesis is a complex redox reaction occurring in the thylakoid membranes of the chloroplast of green algae (Fig. 2), and the photosynthetic membranes (thylakoids) of the cytoplasm of cyanobacteria (Fig. 3) (Singh et al., 2015). The photosynthetic electron transfer constitutes two reactions, namely light and dark reactions. The light reaction is involved in obtaining electrons by splitting water in photosystem II (PSII), transferring the electrons through an electron transport chain from PSII via the plastoquinone (PQ) pool, cytochrome b_6f complex (Cyt b_6f), Photosystem I (PSI) to ferredoxin (Fd), resulting in the generation of ATP and strong reductants (NAD(P)H). For the dark reaction, CO_2 is fixed and

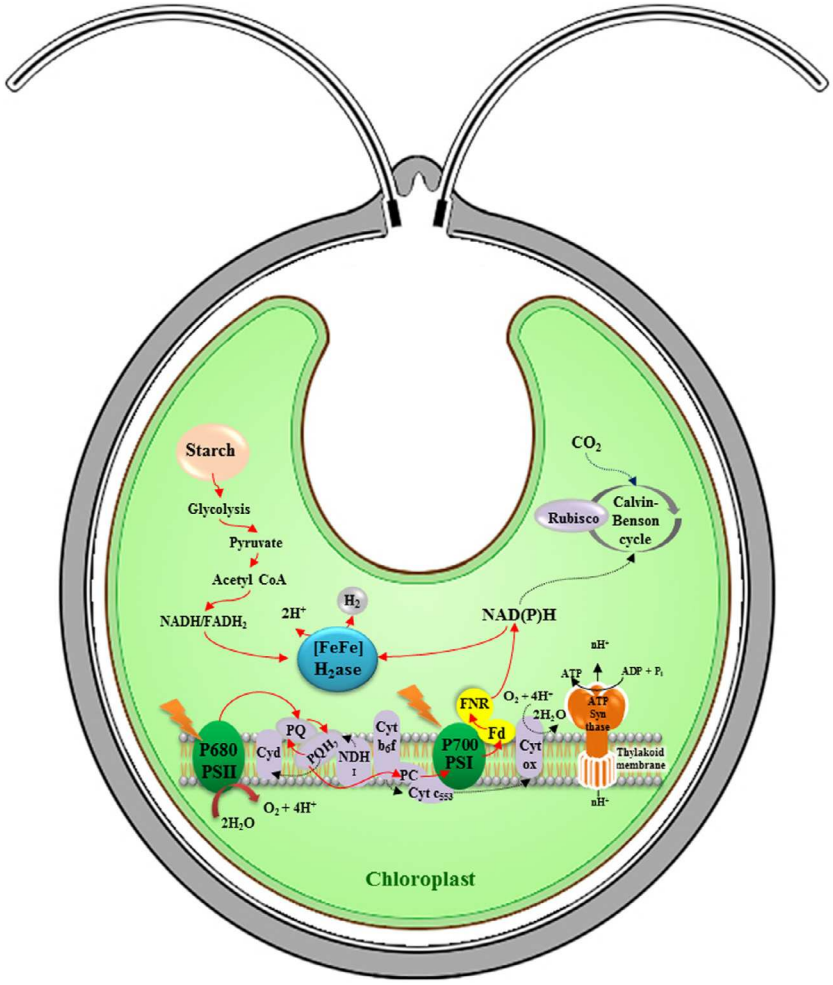

Fig. 2. Simplified view of metabolic pathways related to photosynthesis and hydrogen metabolism in green algae. These metabolic pathways are involved in direct biophotolysis process for hydrogen production in the model green alga *Chlamydomonas reinhardtii*. Abbreviations; [FeFe] H_2ase: [FeFe] hydrogenase, PSII: photosystem II, PSI: photosystem I; PQ/PQH_2: plastoquinone pool, Cyt b_6f: cytochrome b_6f, PC: plastocyanin, Cyt c_{553}: cytochrome c_{553}, Cyd: quinol oxidase, Fd: ferredoxin, FNR: ferredoxin-NADP reductase, SDH: succinate dehydrogenase, NDH-I: NADPH dehydrogenase (complex I), Cyt ox: cytochrome c oxidase, OPP: oxidative pentose phosphate pathway, Rubisco: Ribulose-1,5-bisphosphate carboxylase oxygenase. This view was modified from previous publications (Radakovits et al., 2010; Srirangan et al., 2011; Khetkorn et al., 2013). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

reduced into organic compounds in the form of starch (in green algae) or glycogen (in cyanobacteria) by using chemical energy obtained from the light reaction (Allahverdiyeva et al., 2014). Photobiological hydrogen production is associated with photosynthesis, where the final electron acceptor ferredoxin donates electrons to enzymes involved in hydrogen metabolism (Eroglu and Melis, 2016). Biophotolysis is the initial step of microalgal biohydrogen production. It can be divided into two pathways, i.e., direct and indirect biophotolysis pathways. During direct biophotolysis, H_2 is derived from the electrons and protons generated by water splitting at PSII found in both green algae and cyanobacteria, whereas for indirect biophotolysis, electrons and protons are mainly supplied by the degradation of intracellular carbon compounds. The advantage of indirect biophotolysis is the ability to use other carbon sources to provide protons and electrons for hydrogen generation which is mostly found in cyanobacteria (Mathews and Wang, 2009).

4. Hydrogen synthesizing enzymes in microalgal metabolism

Hydrogenases (H_2ase) and nitrogenases (N_2ase) are considered to be the key cellular machinery responsible for biological hydrogen production in microalgae (Winkler et al., 2009). H_2ase is mainly found in green microalgae and cyanobacteria, but their enzyme activity, enzyme maturation, and structural diversity may vary in different species. Nitrogenase, which can produce significant amounts of hydrogen under anaerobic and nitrogen-deficient conditions, is present only in cyanobacteria (Carrieri et al., 2011; Kim and Kim, 2011). Both enzymes play a major role in the reduction of proton (H^+) to H_2.

4.1. Hydrogenase

Green microalgal and cyanobacterial hydrogenases are diverse classes of enzymes. There are three different classes of hydrogenase, i.e., [FeFe] hydrogenase, [NiFe] hydrogenase, and [Fe] hydrogenase. The last enzyme is a homodimer found only in some methanogenic archaea (Vogt et al., 2008). It has been supposed that both the green microalgal and cyanobacterial hydrogenases evolve separately, but perform the same enzymatic reaction through their respective metallo-clusters. In microalgae, H_2 production results from the coupling of the photosynthetic electron transport chain and the plastidial [FeFe] hydrogenase. The [FeFe] hydrogenase in green microalgae has a unique active center (H-cluster) which produces about 100-fold higher activity than the

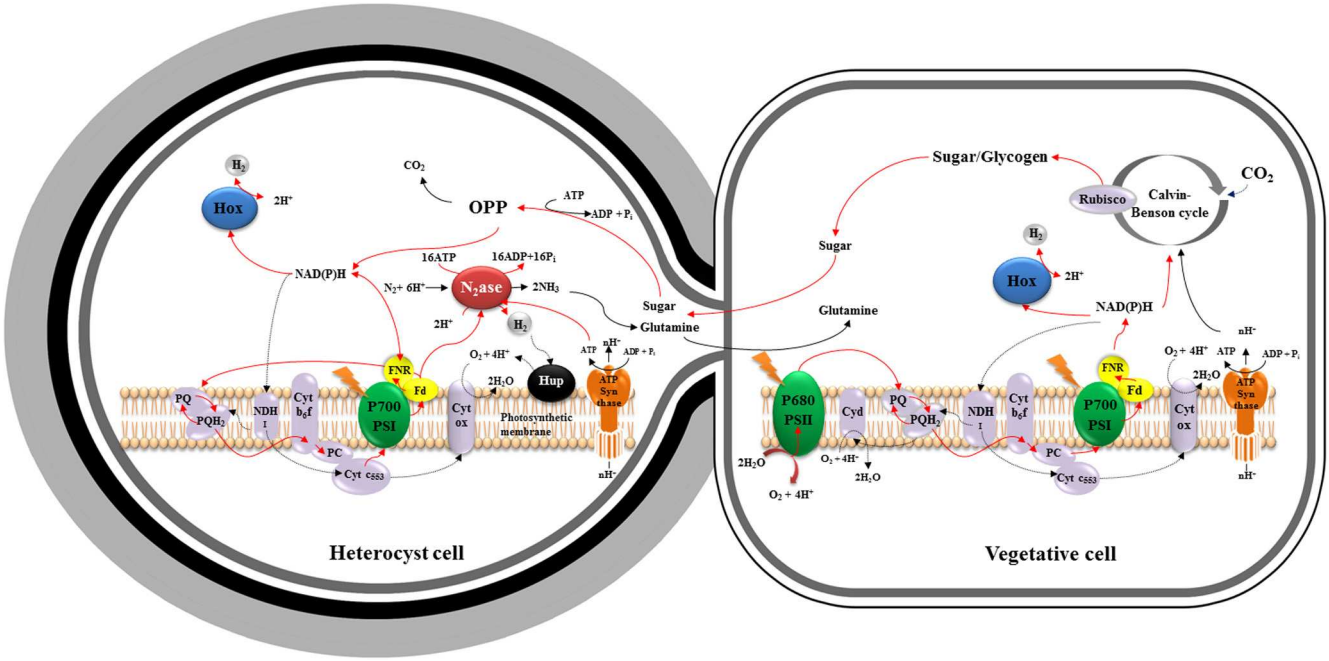

Fig. 3. Simplified view of metabolic pathways related to photosynthesis, nitrogen fixation and hydrogen metabolism in heterocystous filamentous cyanobacteria. One important metabolic pathway in heterocystous cyanobacteria is the heterocyst differentiation for nitrogen fixing. Heterocyst cell imports carbohydrates from vegetative cell to generate energy and supply reducing power for nitrogen fixation. In turn, it exports glutamine to the vegetative cell. The electrons and ATP required for hydrogen production via nitrogenase (N_2ase) and bidirectional Hox-hydrogenase ([NiFe] H_2ase) are gained by either the photosynthetic oxidation of water or carbohydrate catabolism (red line). This view was modified from previous publications (Gutthann et al., 2007; Srirangan et al., 2011; Khetkorn et al., 2013). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

other hydrogenases (Vogt et al., 2008). The [FeFe] hydrogenase can catalyze either the production of H_2 or proton (H^+) released from hydrogen. It is encoded by *hydA* gene in the nucleus and localized in the chloroplast after enzyme maturation (Meyer, 2007). The enzyme has a monomeric composition and a molecular weight of around 45–50 kDa. The catalytic site (called the H-cluster) is composed of [FeFe] bonds with sulfur bridges and 4Fe-4S residue (Meyer, 2007), non-proteinous ligands CN and CO are attached to both Fe atoms (Fig. 4A). However, the active site of the enzyme, where electrons are transferred from ferredoxin, is highly sensitive to oxygen which makes it difficult to produce H_2 naturally under oxygenic conditions. Recently, the structural insight into the complex of ferredoxin and [FeFe] hydrogenase from *Chlamydomonas reinhardtii* was presented (Rumpel et al., 2015).

The [NiFe] hydrogenase constitutes the largest number of hydrogenases. Cyanobacteria may contain [NiFe] hydrogenases as the bidirectional and uptake hydrogenase enzymes. It has been suggested that both ferredoxins and flavodoxins could act as electron donors to the cyanobacterial hydrogenase (Gutekunst et al. 2014, Khanna and Lindblad, 2015). The basic structure of the enzyme consists of two compartments. The active site is the larger subunit (~60 kDa), which has [NiFe] bonds; whereas the small subunit (~30 kDa) only has a Fe-S (4Fe-4S or 3Fe-4S) cluster (Fig.4B). Four cysteine residues are bonded to metallogenic compartments with sulfur bonds. The small subunit transfers electrons to the active site and protons are reduced to hydrogen. Bidirectional Hox-hydrogenase is a heteropentameric, NAD^+-reducing enzyme, encoded by *hoxEFUYH*. It consists of two protein complexes; hydrogenase complex (HoxY and HoxH) and a diaphorase complex (HoxE, HoxF and HoxU). The bidirectional Hox-hydrogenase is commonly found in all cyanobacteria (Tamagnini et al., 2007; Aubert-Jousset et al., 2011) which catalyzes both consumption and production of molecular hydrogen. Uptake Hup-hydrogenase is a heterodimeric enzyme found in almost all nitrogen fixing cyanobacteria. It consists of at least two subunits, HupS (small subunit, encoded by *hupS*) and HupL (large subunit, encoded by *hupL*) which catalyzes consumption of the hydrogen produced by nitrogenase (Tamagnini et al., 2007). Besides the main enzymatic differences between [FeFe] and [NiFe]-enzymes as discussed above, [NiFe] hydrogenases are significantly more oxygen tolerant than the [FeFe] hydrogenases. Moreover, [FeFe] hydrogenases are irreversibly inhibited by O_2, whereas the O_2-inhibition of [NiFe] hydrogenases has a reversible character (English et al. 2009). It has been shown that [NiFe] hydrogenases are constitutively expressed, while the expression of [FeFe] hydrogenases requires anaerobic induction (Ghirardi, 2015).

4.2. Nitrogenase

Many cyanobacteria are able to fix atmospheric N_2 into ammonia (NH_3) and produce H_2 as a by-product. Nitrogenase is a multiprotein enzyme complex consisting of two proteins: the dinitrogenase (MoFe protein or protein I) and dinitrogenase reductase (Fe protein or protein II) as shown in Fig. 4C. The dinitrogenase is a heterotetramer $\alpha_2\beta_2$ (~220–240 kDa) comprising α and β subunits encoded by *nifD* and *nifK* gene, respectively. The function of dinitrogenase is reduction of N_2 bonds leading to the formation of ammonia (NH_3). The dinitrogenase reductase is a homodimer (~60–70 kDa) and encoded by *nifH* gene. It has an important role for transferring electrons from the external electron donor to the dinitrogenase protein (Bothe et al., 2010). In the absence of the substrate N_2, nitrogenase may exclusively catalyze the formation of hydrogen and high potential electrons. However, hydrogen production by nitrogenase requires considerable amount of electrons, reductants and a minimum of 16 ATP molecules (Srirangan et al., 2011) provided from photosynthesis or by carbohydrate degradation in the cell. The turnover rate of nitrogenase is lower than that of [FeFe] and [NiFe] hydrogenases (Meyer, 2007; Srirangan et al., 2011).

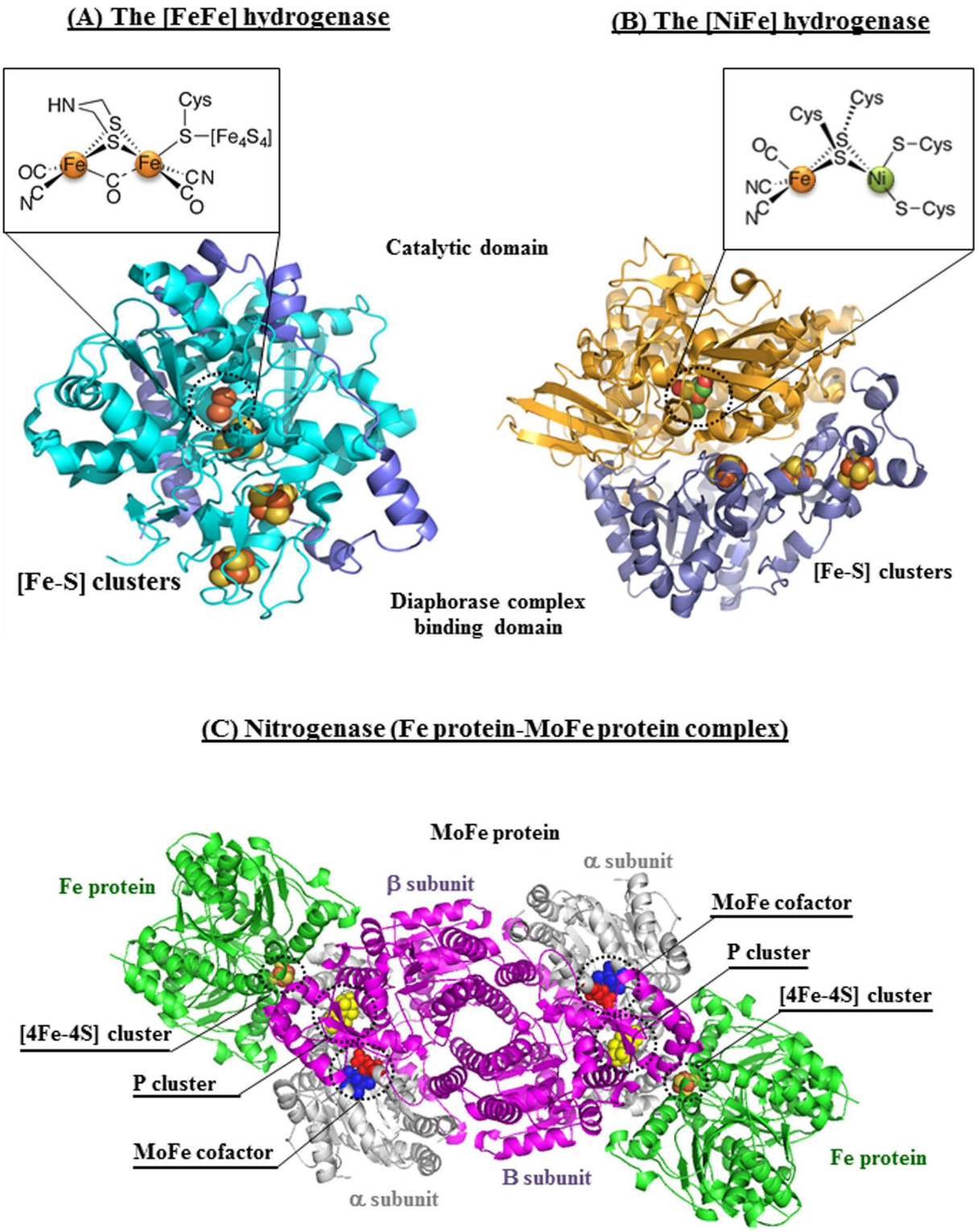

Fig. 4. A ribbon representation of the X-ray-determined standard structure of the hydrogen producing enzymes. (A) [FeFe] hydrogenase, (B) [NiFe hydrogenase and (C) Fe protein-MoFe protein complex of nitrogenase.

5. Hydrogen production by green microalgae and cyanobacteria

Green microalgae have immense potential as renewable fuel sources. A number of green algae such as *Scenedesmus obliguus*, *Chlamydomonas reinhardtii*, *Tetraspora* sp., *Chlorella vulgaris*, etc. have widely been studied for their efficient role in hydrogen production (Maneeruttanarungroj et al., 2010; Rumpel et al., 2014; Hwang et al., 2014; Torzillo et al., 2015). The production of H_2 in green algae is associated with the direct biophotolysis process (Fig. 2), which usually occurs when algal cultures are exposed to light after a period of dark with anaerobic adaptation. Recently, *Chlorella vulgaris* strains YSL01 and YSL16 were found to upregulate the expression of the hydrogenase gene (*HYDA*) under aerobic conditions and to produce hydrogen through photosynthesis, using

CO_2 as the sole source of carbon with continuous illumination (Hwang et al., 2014). The microoxic niches within the thylakoid stroma of air-grown *Chlamydomonas reinhardtii* preserves [FeFe]-hydrogenase activity and supports continuous hydrogen production under completely aerobic environment (Liran et al., 2016). The hydrogen production rate (up to 300 μmol H_2 mg chla^{-1} h^{-1}) depends directly on species and the light intensity. High rate of hydrogen production could be observed in the light for short periods of time. For example, in dark-adapted *Chlorella vulgaris* cultures, the hydrogen production rate achieves the maximum in 2.5 s after exposing cells to about 0.03 W m^2 and the kinetics is linear for at least 1 min (Boichenko et al., 1983). Although hydrogen production in green algae is a short-term phenomenon, its hydrogen conversion efficiency is higher than that in cyanobacteria. The maximum efficiency for the direct water biophotolysis process has been estimated at around 10% (Akkerman et al., 2002). The green microalga *C. reinhardtii* is a model organism for hydrogen production, since its chloroplast, mitrochondrial, and nuclear genomes have been sequenced (Radakovits et al., 2010). The green microalga *C. reinhardtii* has been extensively studied for enhanced hydrogen production using genetic engineering techniques since its chloroplast and nuclear DNA can easily be transformed. The hydrogen production rate of genetically engineered *C. reinhardtii* strain *Stm6* was 5–13 times that of the control wild type strain over an illumination range of 15–1300 μmol m^{-2} s^{-1} with a maximum rate of 4 mL H_2 L^{-1} h^{-1} under sulfur deprivation for 14 days (Kruse et al., 2005). Moreover, *C. reinhardtii* and other green algae can use organic substrates for producing H_2 through photofermentation, but the reaction is not complete. The major by-products are formate, acetate and ethanol. However, the final composition varies significantly among different algal strains under different environmental conditions (Gibbs et al., 1986).

Cyanobacteria (e.g., *Synechocystis* sp. PCC 6803, *Anabaena variabilis*, *Anabaena* sp. PCC 7120 and *Cyanothece* sp.), constitute an extremely diverse group of prokaryotes which have different morphological forms ranging from unicellular to heterocystous and/or non-heterocystous filaments. They are potential microbial species for hydrogen production via direct and indirect photolysis (Pinto et al., 2002; Khanna et al., 2016). Hydrogen production has been studied in a wide variety of cyanobacterial species, since they require the simplest nutritional conditions. Moreover, cyanobacteria have some different strategies to protect O_2-sensitive enzymes from photosynthetically evolved oxygen. In the absence of nitrogen source, several nitrogen fixing filamentous cyanobacteria undergo cellular differentiation to physically separate the oxygenic photosynthesis and nitrogen fixing enzymes by specialized heterocystous cells, which are regularly spaced among the vegetative cells. Development of heterocyst across the vegetative filaments entails incorporation of multiple external as well as internal signals, regulation of genes and several cellular processes (Kumar et al., 2009). Mature heterocysts are unique cells providing a microaerobic environment suitable for the enzymes involved in nitrogen fixation and hydrogen production. Heterocyst cell contains a thick cell wall (Nicolaisen et al. 2009) and lacks active PSII complexes resulting in an absence of photosynthetic O_2 evolution. The vegetative cells perform photosynthetic and CO_2 fixing processes, whereas CO_2 fixation in heterocyst cells is absent since they lack the primary enzyme ribulose bisphosphate carboxylase/oxygenase (Rubisco). The heterocysts import carbohydrates, most likely in the form of sugars, from vegetative cells and use the oxidative pentose phosphate (OPP) pathway for carbohydrate degradation to generate energy and reducing power for nitrogen fixation. In return, the heterocysts export nitrogen in the form of glutamine to the vegetative cells (Fig. 3) (Thomas et al., 1977). Nitrogenase activity is mainly responsible for hydrogen production in heterocystous filamentous cyanobacteria. For example, *Anabaena variabillis* ATCC 29413, *Anabaena* sp. PCC 7120 and *Anabaena siamensis* TISTR 8012 cells grown under nitrogen deprivation showed increased nitrogenase and hydrogen production (Tsygankov et al., 1998; Khetkorn et al., 2010; Yeager et al., 2011). The unicellular and non-heterocystous filamentous cyanobacteria exhibited temporal separation mechanism, with an efficient process to separate oxygen and hydrogen production, by performing oxygenic photosynthesis during the daytime and nitrogen fixation at night (Compaore and Stal, 2010). The energy generated by photosynthesis under light condition is stored in glycogen granules, which are then used as a source of electron and ATP for nitrogenase and/or bidirectional Hox-hydrogenase by carbohydrate degradation through the oxidative pentose phosphate pathway (OPP) under dark condition. In the non-heterocystous cyanobacterium, *Cyanothece* sp. ATCC 51142, thriving in marine environment, nitrogenase-mediated hydrogen production was found to generate high levels of hydrogen under dark condition with the specific rate of over 150–300 μmol H_2 mg chla^{-1} h^{-1} (Bandyopadhyay et al., 2010). Moreover, the maximum hydrogen production rate reached 465 μmol H_2 mg chla^{-1} h^{-1} when using glycerol as a substrate.

Deprivation of certain nutrient such as sulfur could also induce H_2 production from green microalgae (Eroglu and Melis 2011; Gonzalez-Ballester et al. 2015). During sulfur deprivation, oxygenic photosynthesis decreases, whereas respiration induces anaerobic condition suitable for hydrogen production (Torzillo et al., 2015).

6. Metabolic and genetic engineering of microalgae for enhanced hydrogen production

A large number of green algae and cyanobacteria utilize light as the driving force to extract electrons from water and generate strong reductants such as NAD(P)H and reduced ferredoxin. These reductants can be used as substrates for hydrogen production through the functions of nitrogenase and hydrogenase. However, major limitations for sustainable and sufficient hydrogen production are the oxygen sensitivity of the enzymes and H_2-uptake followed by low hydrogen productivity due to the competition for electrons by other assimilatory pathways. Consequently, the improvement of hydrogen production using microalgal cells is a challenging issue. Most of the biohydrogen production has merely been established at the laboratory scale with low yield for commercial application. It is of utmost importance to enhance the bio-production rate of hydrogen for commercial feasibility by addressing the sound scientific research on algae based biofuels.

Recently, several metabolic and genetic engineering approaches have been identified for hydrogen production from microalgae (Baebprasert et al., 2011; Maneeruttanarungroj et al., 2012; Khetkorn et al., 2012a; Khetkorn et al., 2012b; Nyberg et al., 2015). In comparison to the wild type, a significant effect on H_2 production has been observed subsequent to genetic modification of some microalgal species (Baltz et al., 2014; Torzillo et al., 2015; Eroglu and Melis, 2016). Along with several biotechnological approaches, photobiological hydrogen production from green microalgae has been intensively investigated for sustainable biohydrogen production. Some recent activities on genetic engineering of enzymes and metabolic pathways for enhanced hydrogen production are summarized in Table 2.

6.1. Inactivation of uptake hydrogenase

The major obstacle for enhanced hydrogen production in nitrogen fixing cyanobacteria is the function of the uptake Hup-hydrogenase. Therefore, a discernible strategy to enhance hydrogen production from cyanobacteria would be to disrupt the uptake

Table 2
Metabolic and genetic engineering of microalgae using different strategies for enhanced hydrogen production.

Microalgal strains	Strategies	Engineered genes	H_2 production rate	H_2 production assay condition	Ref.
Inactivation of uptake hydrogenase					
Anabaena variabilis strain AVM13	Insertional mutagenisis	*hupSL*	135 μmol H_2 mg chl*a*$^{-1}$ h^{-1}	Ar, 100 μEm^{-2} s^{-1}, N_2-fixing	Happe et al. (2000)
Nostoc punctiforme strain NHM5	Insertional mutagenisis	*hupL*	14 μmol H_2 mg chl*a*$^{-1}$ h^{-1}	Light and N_2-fixing	Lindberg et al. (2002)
Anabaena sp. PCC 7120	Insertional mutagenisis	*hupL/hoxH*	53 μmol H_2 mg chl*a*$^{-1}$ h^{-1}	Ar, 10 Wm^{-2}, N_2-fixing	Masukawa et al. (2002)
Anabaena sp. PCC 7120	Insertional mutagenisis	*hupW*	3.3 μmol H_2 mg chl*a*$^{-1}$ h^{-1}	Ar, 10 Wm^{-2}, N_2-fixing	Lindberg et al. (2012)
Anabaena siamensis TISTR 8012	Insertional mutagenisis	*hupS*	29.7 μmol H_2 mg chl*a*$^{-1}$ h^{-1}	Ar, 200 μEm^{-2} s^{-1}, N_2-fixing	Khetkorn et al. 2012a)
Eliminating pathways competing for electrons					
Synechocystis strain M55	Insertional mutagenisis	*ndhB*	200 nmol H_2 mg chl*a*$^{-1}$ min^{-1}	Anaerobic and nitrogen deprivation	Ekman et al. (2011)
Synechocystis sp. PCC 6803	Insertional mutagenisis	*ctaI/cyd*	190 nmol H_2 mg chl*a*$^{-1}$ min^{-1}	Anaerobic and nitrogen deprivation	Cournac et al. (2004)
Synechocystis sp. PCC 6803	Insertional mutagenisis	*ctaII/cyd*	115 nmol H_2 mg chl*a*$^{-1}$ min^{-1}	Anaerobic and nitrogen deprivation	Gutthann et al. (2007)
Synechocystis sp. PCC 6803	Insertional mutagenisis	*narB/nirA*	300 nmol H_2 mg chl*a*$^{-1}$ h^{-1}	Ar, darkness, nitrogen deprivation	Baebprasert et al. (2011)
Overcoming oxygen sensitivity of hydrogen producing enzymes					
Chlamydomonas reinhardtii mutant strain L159I-N230Y	Amino acid deletions in D1 protein	*psbA*	5.77 mL H_2 L^{-1} h^{-1}	70 μEm^{-2} s^{-1}, sulfur deprivation	Torzillo et al. (2009)
C. reinhardtii	Antisense transformation of sulfur uptake genes	*sulP/sulP2*	Promoted H_2 accumulation but not reported H_2 amount	Anaerobic, sulfur containing condition	Melis and Chen (2005)
Synechococcus PCC7942	Gene overexpression	*hydA* from *Clostridium acetobutylicum*	6.8 μmol H_2 mg chl*a*$^{-1}$ h^{-1}	Ar, dark adaptation	Asada et al. (2000)

hydrogenase. The advanced molecular tools for cyanobacteria, targeted inactivation and site directed mutagenesis studies have been carried out. Previous studies have reported that hydrogen uptake-deficient mutants of *Anabaena variabilis* AVM13 (Δ*hupSL*) (Happe et al., 2000), *Nostoc punctiforme* NHM5 (Δ*hupL*) (Lindberg et al., 2002), *Anabaena* PCC 7120 (Δ*hupL*/Δ*hoxH*, Δ*hupW*) (Masukawa et al., 2002; Lindberg et al., 2012), and *Anabaena siamensis* TISTR 8012 (Δ*hupS*)] (Khetkorn et al., 2012a), all have an ability to produce hydrogen with significantly higher rate compared to the respective wild types (Table 2). In a recent study Raleiras et al. (2016) introduced a designed C12P mutation in the small subunit HupS from the uptake hydrogenase of *Nostoc punctiforme*. The modification caused the conversion of the original low-potential [4Fe-4S] cluster to a species that presents properties consistent with a higher potential [3Fe-4S] cluster. The introduction of the C12P mutation in HupSL in *N. punctiforme* converted the cyanobacterium to a sustained light-driven hydrogen producer under nitrogen-fixing conditions. It was suggested that HupSL effectively turned its catalytic bias from being a hydrogen uptake hydrogenase to a hydrogen evolving enzyme, likely due to a drastic increase of the reduction potential of the proximal cluster [Fe-S] cluster. Ekman et al. (2011) reported that proteins encoded by the genes involved in the oxidative pentose phosphate (OPP) pathway are more abundant in heterocysts of hydrogen uptake deficient mutant *N. punciforme*, suggesting that the deletion of uptake hydrogenase may increase production of the substrates for the OPP pathway. It may be interesting to note that the deletion of the uptake Hup-hydrogenase results in significantly higher hydrogen production.

6.2. Elimination of pathways competing for electrons

The electrons from primary electron donors such as reduced ferredoxin and NAD(P)H are generally required in combinations with protons to produce hydrogen through the function of nitrogenase and/or bidirectional Hox-hydrogenase. By utilizing targeted ferredoxin and ferredoxin-NADP$^+$-oxidoreductase (FNR) variants in a light-dependent competition assay, electrons can be redirected to the hydrogenase producing a fivefold enhanced hydrogen evolution (Rumpel et al., 2014). Nevertheless, electrons are mainly transferred to other assimilatory or competing pathways, namely the respiratory electron transport system, nitrate assimilation and carbon fixation *via* Calvin-Benson cycle. Therefore, a genetic engineering strategy for enhanced hydrogen production by re-directing the electron flow towards hydrogen metabolism has received recent attention. In *Synechocystis* PCC 6803, inactivation of the respiratory complex I, NADPH-dehydrogenase (NDH-1) by deleting the large subunit NdhB resulted in lower level of O_2 produced under light condition. The engineered *Synechocystis* M55 cells were able to sustain hydrogen production for 30 min in the presence of glucose (Cournac et al., 2004). Moreover, re-directing the electron flow to hydrogen production by interruption of all respiratory terminal oxidases (Δ*ctaI*, Δ*ctaII* and Δ*cyd*) in *Synechocystis* PCC 6803 showed that the absence of the quinol oxidase induces higher activity of bidirectional Hox-hydrogenase and hydrogen production rates under light condition when compared to those in the wild type (Gutthann et al., 2007). The nitrate assimilation pathway is also a potential competitive pathway that may reduce the electron flow to hydrogen metabolism. Engineered *Synechocystis* PCC 6803 strains with disrupted nitrate assimilation in either nitrate reductase (Δ*narB*) or nitrite reductase (Δ*nirA*) or both genes (Δ*narB*/Δ*nirA*) in *Synechocystis* PCC 6803 showed that the deletion of both Δ*narB* and Δ*nirA* resulted in higher hydrogen production than wild type strain (Baebprasert et al., 2011). Accordingly, an engineering approach by eliminating competitive electron pathways can be a very effective and promising method to improve and optimize hydrogen production in cyanobacteria. This genetic strategy deserves more attention for future algal hydrogen production.

6.3. Overcoming oxygen sensitivity of hydrogen enzymes

One of the major problems for hydrogen production using biological systems is the extreme sensitivity of hydrogen synthesizing enzymes to oxygen (Ghirardi, 2015). Green algae and cyanobacte-

ria are oxygenic photosynthetic microorganisms; oxygen generation due to the water splitting in PSII may inhibit the hydrogenase activities. Moreover, in photosynthetic microorganisms, prompt inactivation of hydrogenases by O_2 is considered to be a major limiting factor for sustained and efficient H_2 production (Lambertz et al. 2011; Ghirardi, 2015).

Under normal growth conditions, the photosynthetic rate is 4–7 folds higher than the respiration rate, whereas sulfur deprived condition could decrease oxygenic photosynthesis and induce respiration rate under anaerobic condition, suitable for hydrogen production. It has been reported that *C. reinhardtii* D1 mutant strain that carried a double amino acid substitution, the leucine residue L159 was replaced by isoleucine, and the asparagine residue N230 was replaced by tyrosine (L159I-N230Y) showed higher rate of hydrogen production under sulfur deprivation (Torzillo et al., 2009). This strain is very efficient for prolonged H_2 production with lower chlorophyll content and higher respiration rate. In addition, antisense transformation of sulfate uptake genes (*sulP*/*sulP2*) in *C. reinhardtii* has been shown to increase the oxygen tolerance of hydrogen producing enzymes, especially hydrogenase (Melis and Chen, 2005). Furthermore, heterologous overexpression of an efficient hydrogenase enzyme into the cells was also of interest. Cyanobacteria with a bidirectional [NiFe] hydrogenase may be inefficient for hydrogen production as compared to [FeFe] hydrogenase; moreover, hydrogen is mostly produced by nitrogenase. Interestingly an [FeFe] hydrogenase encoded by *hydA* from *Clostridium acetobutylicum* was expressed without the co-expression of maturation proteins in the cyanobacterium *Synechococcus* PCC7942 (Asada et al., 2000). In addition, Clostridial *hydA* has been cloned into *Rhodospirillum rubrum* and the native hydrogenase of *R. rubrum* (HydC) has been overexpressed. In both cases pyruvate is the electron donor for hydrogen production (Kim et al., 2008). Moreover, in cyanobacteria, the heterologous expression of O_2-tolerant hydrogenases is extremely challenging, due to the fact that the maturation of [NiFe]-hydrogenases requires a large number of specific maturation enzymes (Ghirardi et al., 2014; Ghirardi, 2015). Furthermore, until recently, it was considered that hydrogenase could not be active in air-grown microalgal cells, and sensitivity of [FeFe]-hydrogenase was supposed to be main obstacle towards metabolic engineering for photosynthetic hydrogen production. However, recent findings indicated that the microoxic/anaerobic locality at the thylakoid stroma in the chloroplast may preserve [FeFe]-hydrogenase activity facilitating a continuous production of hydrogen in air-grown microalgal cells (Liran et al., 2016). As mentioned above, H_2 is mostly produced by nitrogenase enzyme in cyanobacteria. Recently, it has been found that a heterocyst-specific flavodiiron protein (Flv3B) can protect nitrogenase by performing light-induced O_2 uptake, which maintains microoxic conditions inside of the heterocysts of the filamentous cyanobacterium *Anabaena* sp. PCC 7120 (Ermakova et al., 2014).

7. Photobioreactor for the improvement of hydrogen production

Economic hydrogen production requires high H_2 production efficiencies at low capital and operating costs. Large-scale production of biohydrogen mediated by microalgae requires specific bioreactors (Skjånes et al., 2016). Since light is an essential parameter for green algae or cyanobacterial growth, thus bioreactors must be transparent and hence, are called photobioreactors (PBRs). In general, large scale algae cultivation is done in an open/raceway pond; however, this method of algae cultivation is not suitable for generation and collection of hydrogen gas. Several types of closed photobioreactors have been developed for commercial scale microalgae cultivation to offer a greater process control for biohydrogen production (Akkerman et al., 2002; Skjånes et al., 2016; Geada et al., 2017).

All photobioreactors require adequate entry of light, which is usually acquired by sunlight. However, in some photobioreactors other artificial sources of light are also used for providing controlled light. The optimal design of microalgal photobioreactor

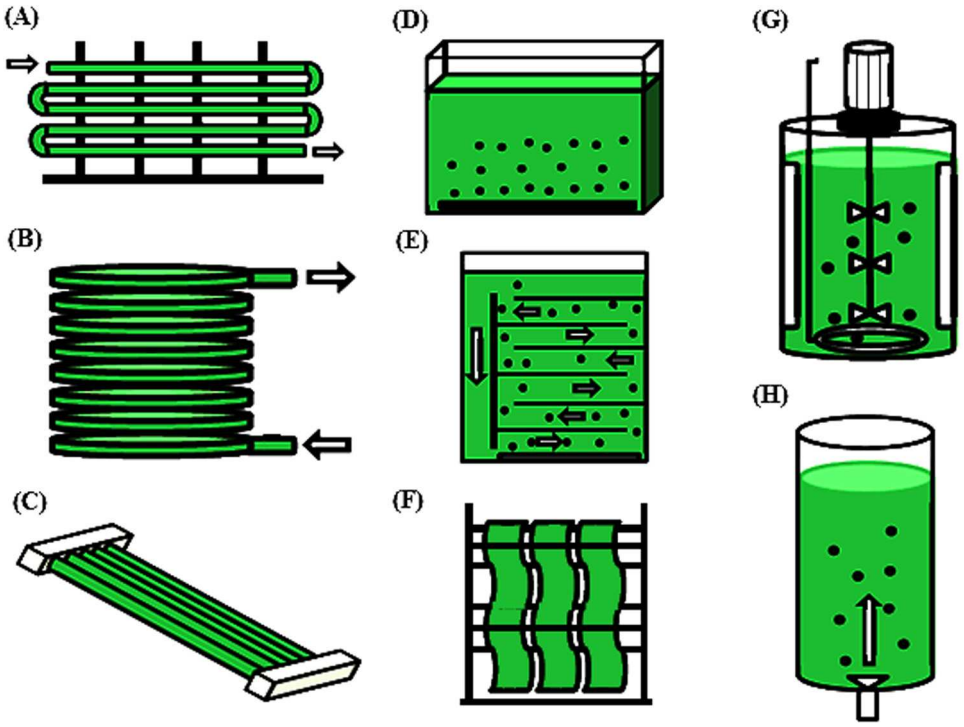

Fig. 5. Schematic representation of the different photobioreactors (PBRs) for biomass and biological hydrogen production. Fence tubular (A), helical tubular (B), horizontal tubular (C), vertical flat panel, (D), air lift type (E), accordion type (F), stirred tank (G), bubble column (H). Modified from (Oncel, 2015).

Table 3
Comparison of biological hydrogen production of microalgae by culturing in different photobioreactors.

Microalgal strains	Cultivation	Photobioreactor types	H_2 production rate	H_2 production assay condition	Ref.
Anabaena sp. PCC 7120 and its mutant AMC 414	Continuous photoautotrophic	Helical tubular (4.35 L)	14.9 mL H_2 L^{-1} h^{-1} (373 mL total) with the mutant	Nitrogen free BG11 medium, under argon atmosphere, 7 days	Lindblad et al. (2002)
Anabaena variabilis (ATCC 29413)	Batch photoautotrophic	Vertical flat panel (450 mL)	4.1 mL H_2 g dcw^{-1} h^{-1}	Nitrogen free BG11 medium, under anaerobic conditions, 120–140 μEm^{-2} s^{-1}, 40 h	Yoon et al. (2006)
Chlamydomonas reinhardtii (non-motile mutant CC-1036 pf18 mt+)	Batch Photomixotrophic, using immobilized cells	Panel (160 mL)	45 mL day^{-1}	Sulfur limitting TAP medium, under anaerobic conditions, 23 days	Laurinavichene et al. (2006)
C. reinhardtii (CC124)	Batch photomixotrophic	Fence tubular (110 L)	0.6 mL H_2 L^{-1} h^{-1}	Sulfur free TAP medium, modified with silica nanoparticle to enhance scattering, 48 h	Giannelli and Torzillo (2012)
C. reinhardtii (CC124)	Batch photomixotrophic	Helical tubularand flat panel (5 L)	1.3 ± 0.05 mL H_2 L^{-1} h^{-1} with flat panel 1.05 ± 0.05 mL H_2 L^{-1} h^{-1} with tubular	Sulfur free TAP medium, mixing time and light intensity as the comparison factors, 120 h	Oncel and Kose (2014)
C. reinhardtii (CC124)	Batch photomixotrophic	Horizontal tubular (50 L)	930 mL H_2 under solar light with acclimated cultures	Sulfur free TAP medium, 75 h	Scoma et al. (2012)
C. reinhardtii (CC124)	Semi-continuous photomixotrophic	Stirred tank (2.5 L)	1108 mL	Sulfur free TAP medium, under anaerobic conditions, 127 days	Oncel and Vardar-Sukan (2009)

depends upon the characteristics of the strain and a major limiting factor for large scale production of hydrogen is the restricted light penetration into the deeper regions of the reactor. Other factors limiting the performance of the bioreactors include area/volume ratio, agitation, temperature and gas exchange. Several types of photobioreactors have been used for hydrogen production. These can be mainly divided into three types of photobioreactors (PBRs): Vertical column reactor, tubular type and flat panel photobioreactors (Fig. 5). Vertical PBR consists of a transparent column usually made up of high quality glass and surrounded by a water jacket that while allowing maintenance of the temperature with circulating water allows adequate entry of light (Miron et al., 1999). Tubular type photobioreactor consists of long transparent tubes, the algal culture is pumped through these tubes by mechanical or air-lift pumps (Slegers et al., 2013; Oncel and Kose, 2014). There is flexibility in volume to surface area ratio and flexibility in shifting the place receiving light (Molina et al., 2001). Flat panel photobioreactor consists of a stainless-steel frame and three polycarbonate panels, the reactor comprises of two compartments placed side by side. The flat plate photobioreactor system is controlled by a specially made control system that can monitor and control pH, temperature, optical density, and amount of produced hydrogen and dissolved oxygen concentration as well (Skjånes et al., 2016). Moreover, high photosynthetic efficiencies and effective control of gas pressure can be achieved in flat-plate photobioreactors and has been found more economic compared to other bioreactors. However, difficulty arises to maintain the specific culture temperature and suitable agitation system during hydrogen production (Dasgupta et al., 2010). Overall, based on a comparative analysis of photobioreactors, it was concluded that the flat panel photobioreactor is more suitable for hydrogen production, due to the fact that backpressure of accumulated hydrogen could be avoided in flat photobioreactors (Oncel and Kose, 2014; Nyberg et al., 2015). Moreover, various photobioreactors have been developed (Oncel and Kose, 2014) for biomass and hydrogen production from microalage as shown in Table 3. Development of a suitable photobioreactor for microalgal hydrogen production is still challenging (Sevda et al., 2017; Kroumov et al., 2017). Some stringent parameters that should be considered for a specific reactor are i) photobioreactor should be an enclosed system so that the produced hydrogen may be collected without any loss. ii) the reactor design must allow sterilization with convenience and ease. iii) maximizing the area of incident light, thus allowing high growth and hydrogen production, photobioreactor design should provide high surface to volume ratio. Over the last few years, research on microalgae based hydrogen production has been effectively applied; however, further extensive research is still needed towards performance of photobioreactors for commercial cultivation of microalgae with decreased costs of biohydrogen production.

8. Future perspectives

Currently, about 20% of global energy is utilized as electricity, while 80% is utilized as fuel. Hydrogen energy is a clean and alternative energy that has been suggested as the energy carrier of the future. Solar-driven microalgal hydrogen production is both a promising and challenging biotechnology, which play an important role in the global drive to reduce GHG emissions. One of the major barriers with regard to hydrogen economy is its production cost and inefficient storage methods, which need to be resolved. Current research efforts are focused on strain improvement by systems metabolic engineering and finding suitable conditions to increase the levels of hydrogen production. Systems biology using various genome-wide tools, including high-throughput analytical

techniques, computational analyses and omics analyses that cover genomic, transcriptomic, proteomic and metabolomics, have enabled the analysis of large amount of data for investigating cellular metabolism and physiology at systems-level. Information obtained from such studies can be applied in an integrated manner during the strain development by metabolic engineering. In the near future, it may be possible to perform knockouts and insertions based on the data available by modeling previous studies. The advent of synthetic biology necessitates such models, since it aims at standardizing biology, which should give predicted responses. With all these advancements, the commercial feasibility of H_2 production may rely on efficient production strategies with elevated yield, well-organized transport and storage systems ensuring the secured supply of hydrogen. Moreover, the future of biological hydrogen production depends not only on research advances such as improvement in efficiency through genetically engineered microorganisms and/or the development of bioreactors, but also on economic considerations like cost of fossil fuels, social acceptance, and use of hydrogen energy systems in our society. Today, hydrogen is being used to power a fleet of buses in some countries. More industries will accept hydrogen energy when a renewable economically viable process of hydrogen production is achieved. Last but not least, the integrated effort of both scientists and engineers is needed to fully implement hydrogen energy as the energy for the future.

9. Conclusions

Global crisis of fossil fuels has greatly stimulated worldwide interest to develop sustainable sources of energy carriers. Microalgae can be used as a potential source of hydrogen energy due to their inherent capacity to split water into H_2 and O_2 using the solar energy. Photo-biological hydrogen production is considered as a more efficient and less energy intensive process. Hydrogen-powered fuels can be used in different types of fuel cells as a clean energy to generate electricity with high efficiency. At present, hydrogen energy from microalgae is economically less feasible due to its high production cost. Nevertheless, efficient biohydrogen production from microalgae may be accelerated by recent technological advancements with metabolic and genetic engineering approaches.

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