Biomass Conversion and Biorefinery (2023) 13:8575–8592
https://doi.org/10.1007/s13399-021-01414-z

REVIEW ARTICLE

Current prospects and future developments in algal bio-hydrogen production: a review

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Received: 9 September 2020 / Revised: 25 February 2021 / Accepted: 1 March 2021 / Published online: 11 March 2021
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Abstract

The use of fossil fuels for energy demands is insufficient for escalating demands as they are limited in nature and are not environment friendly. Various microbial sources are under scanner for clean energy production out of which algae comes as a promising one. Production of biohydrogen from algae has brought a lot of hopes in the energy production sector. Algae are powerhouse of renewable chemicals including biofuel precursors. Biohydrogen is one of such biofuel precursors; it proves to be a clean and sustainable fuel and can be produced in industries. Currently however, it cannot stand up to the present energy demands of the world due to certain obstacles, such as cost effectiveness, storage and transportation of hydrogen. One of the main hurdles in algal biofuel technology is the rigid nature of algal cell wall. To get around this, pretreatment of algal biomass is imperative to overcome productivity issues, so as not to compromise biofuel yield. Improving the biofuel production at every step can make a huge difference in outcomes and thus comes up as a promising tool. Therefore, in the present state of art review, various methods of algal biomass pretreatment, different enzymes involved in hydrogen production, various factors influencing hydrogen production from algae and genetic engineering avenues have been discussed in brief.

Keywords Algae · Biohydrogen · Hydrogenase · Photosynthesis · Bioenergy · Cyanobacteria

1 Introduction

The current scenario regarding energy poses a dismal future as present sources have several adverse effects on the environment and are gradually depleting. Attempts are being made to find a sustainable, suitable and cost-effective source to meet the rising demands of several consuming sectors [1–3]. Though, in the recent past, attempts to substitute the fossil fuels have been made but the results deviated from the standard norm due to some limitation of supply chain and cost effectiveness [4]. Currently micro- and macroalgae are coming up as extremely promising sustainable feedstocks for biofuels [5–7]. Biofuels are generally categorized into first, second, third and fourth generations on the basis of their source. First-generation biofuels are ethanol and biodiesel, derived from vegetable oil, sugar, starch and animal fats. As they are mainly derived from food crops, they compete for a lot of arable land used for food production. Second-generation biofuels, on the other hand, come from non-food agriculture crops and byproducts (lignocellulosic biomass). Microalgae-derived biofuels come under third-generation biofuels that do not require arable land for feedstock generation, whereas biofuels derived from genetically engineered algae come under fourth-generation biofuels (Fig. 1) [2, 8].

Among variety of biofuels produced from microalgal biomass, biohydrogen has peculiar properties to be used as a fuel [9–11]. Hydrogen gas as a fuel is a great hope as it does not produce toxic by-products such as CO, NOx or CO_2 as in the case of other conventional fuels [12, 13]. It is the lightest element; even lighter than the air, as mass of 1 L of hydrogen is 0.09 g [14]. Its supremacy over other sources is due to the following properties: (i) clean fuel, (ii) highest energy density,

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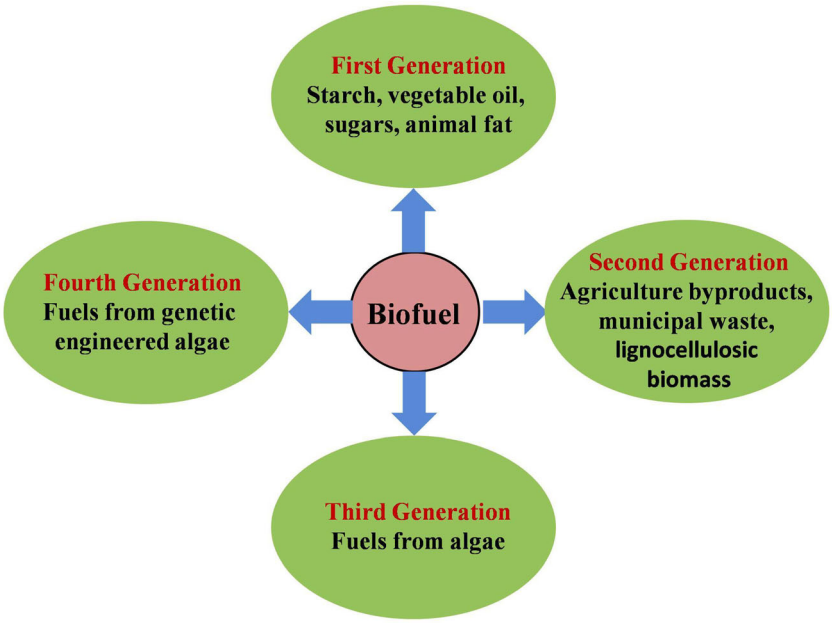

Fig. 1 Different generations of biofuel

(iii) low carbon emission, (iv) can be stored and used when desired, (v) can be produced in circumambient temperatures and pressures, (vi) can be used straight in fuel cell for electricity production [1, 2, 4, 15–17]. It has been used commercially for various purposes like margarine production, production of chemicals, hydrogenation of fats and steel processing [18]. Hydrogen gas is used as fuel for rockets, submarines, ships and buses [19, 20]. Hydrogen has an enormous energy potential and can be sourced through various paths like water electrolysis and thermo-catalytic reformation. At present, large portion of hydrogen gas is generated by means of electricity generation through the thermo chemical reaction using fossil fuels. Biological methods, on the other hand, were considered favourable for hydrogen production, as it does not complicate the waste management process and unlike the current hydrogen production which involve fossil fuels [21–23]. Use of microorganisms for production of hydrogen is called bio-hydrogen production and such microorganisms include cyanobacteria, green algae and certain types of bacteria which through their different metabolic pathways generate hydrogen [19]. Algal biomass has come up as a promising tool for hydrogen production because of its high growth rate, easy availability, no lignin and no land requirement [24–26]. Hydrogenase and nitrogenase enzymes are the key hydrogen producing proteins. These enzymes work in both prokaryotic (cyanobacteria) and eukaryotic (green algae) algal systems, and the function of both of these enzymes are closely associated with the consumption of metabolic outcomes of photosynthetic reaction. Moreover, the efficacy of hydrogen production is greatly affected by the strain specific potential of expressing different enzymes as well as the environmental factors. In addition to this, genetic engineering approaches play a vital role in modifying key enzymes and certain metabolic pathways to improve photosynthetic capacity. Development of oxygen-tolerant algal strains is also in progress to enhance hydrogen production capability [17].

Until now, a lot of research and innovation has been done in the field of algal bio-hydrogen production. But, in spite of diverse through attempts, several limitations still persist that need to be thoroughly addressed to make this approach economically viable. Therefore, in the present review, various crucial aspects of algal bio-hydrogen production, for instance, pretreatment of algal biomass, enzymes involved, factors influencing hydrogen production and scope of genetic engineering for bio-hydrogen production have been discussed in depth.

2 Algae as a feedstock for biohydrogen production

Initially the algae have been identified for its nutritional values, since long it has been consumed as a rich source of nutrients, especially protein in various Asian countries [27]. It was only in the 1970s when the global oil crises led the researchers to explore renewable and sustainable sources of energy. Consequently, algae have emerged as a potential feedstock for biofuel due to their higher lipid and carbohydrates content [28]. Algae are the most primitive organisms on the Earth. Most of them are photoautotrophic (need CO_2 and sunlight), while few are heterotrophic (need organic carbon source) in nature. Their photosynthetic efficiency is 10–50 times higher than other terrestrial plants. Higher productivity, short life cycle and year round supply make algae an ideal biofuel feedstock [29]. One of the major advantages of using algae for biofuel production is the requirement of

mild pretreatment. It is due to the fact that, unlike other terrestrial crops, the cellular structure of algae does not require lignin and hemicelluloses. Hydrogen production by microalgae can be achieved via specific physiological environments like absence of oxygen (anoxia) and higher light intensities. Apart from this, carbohydrate-rich algal biomass can be utilized by anaerobic bacteria for fermentative hydrogen production. Based on the evolution, the algae are differentiated into eukaryote (green algae) and prokaryote (blue-green or cyanobacteria); both of them have unique bio-hydrogen evolving mechanism.

2.1 Bio-hydrogen production in green algae and cyanobacteria

Green algae and cyanobacteria are oxygenic photosynthetic organisms. They both contain unique photosynthetic pigments that absorb solar radiation, which in turn converted into chemical energy. During this complex reaction, splitting of water molecule occurs with the evolution of oxygen and protons. Photosynthetic reaction takes place in the thylakoid membrane of chloroplast and photosynthetic membrane of the cytoplasm of green algae and cyanobacteria respectively. The chief site of photosynthetic light reaction is photosystem II which is involved in the splitting of water molecule. As a result, the electrons evolved are transferred through electron transport chain to final electron acceptor ferredoxin via plastoquinone, cytochrome b_6f and photosystem I, resulting in the generation of NADPH and ATP [30].

Bio-hydrogen production in cyanobacteria is carried out by three distinct pathways. In the first, water molecule acts as an electron donor, and the electrons are finally supplied to bidirectional [NiFe] hydrogenases via light-dependent electron transfer as discussed above. In the second pathway (fermentative pathway), electrons are transferred from light-dependent photosynthetic carbon storage (glycogen in the case of cyanobacteria) to [NiFe] hydrogenases. However, the water molecule is the indirect electron donor in this case, since the glycogen is formed via CO_2 fixation carried out by the splitting of water at photosystem II. Third reaction of hydrogen production is carried out in nitrogen-fixing cyanobacteria, carrying specialized structures called heterocyst. As the nitrogenase enzyme is sensitive to oxygen, the heterocystous cells help in separating oxygenic photosynthesis from nitrogen fixation reaction [31]. During the reaction electrons either come from photosynthetic electron transport chain or from the catabolism of glycogen. The electrons are then transferred to plastoquinone via NAD(P) plastoquinone oxidoreductase (NPQR) or directly to nitrogenase enzyme via ferredoxin-NAD(P) (FNR) [32].

Hydrogen evolution in green algae is carried out by two light-dependent and one light-independent fermentative pathway by unidirectional [Fe] or [FeFe] hydrogenases. In all the three pathways, ferredoxin acts as key electron donor to hydrogenases enzyme. In the first pathway, splitting of water molecule at PSII acts as a source of electrons. The second pathway is PSII independent where the electrons come from the catabolism of starch and lipids reserves. As a result, NADPH molecule is produced which is further oxidized to NPQR to generate electrons. The third route however occurs during the dark fermentation process. During the dark anoxic conditions, algae tend to degrade its starch reserves to generate fermentative end product such as hydrogen along with acetate, formate and ethanol [33, 34]. Nevertheless, the efficiency of biofuel production by the fermentative pathway is significantly affected by the availability of intracellular carbohydrate and lipid reserves. The presence of algaenan and other complex carbohydrates present in the cell wall of the algae restricts the availability of intracellular biomolecule to various hydrolytic enzymes; as a result, the overall process compromises in terms of biofuel output. Consequently it is imperative to have an efficient pretreatment process to disrupt algal cell wall and increase the overall biofuel yield. Different algal biomass pretreatment methods are discussed in the following section [35, 36].

3 Pretreatment of algal biomass for hydrogen production

For sufficient biohydrogen production, high amount of carbohydrate must be present. As the biomass undergoes different stages of extraction, pretreatment is done to preserve the carbohydrate [1]. The pretreatment thus provides improved yield of biohydrogen. This step disfigures the cell and exposes the carbohydrates in its cell wall [37]. Effect of pretreatment methods on algal biomass for bio-hydrogen production is shown in Table 1. Sambusiti et al. [1] mentioned three categories of pretreatment (Fig. 2):

3.1 Physical pretreatment

3.1.1 Bead milling

Bead milling method is generally used for microalgae, mainly cyanobacteria, where beads of ceramic/glass/quartz/steel are used to disrupt algal cell. The wet biomass is agitated along with these beads, this physical agitation causes bumping of the biomass, as a result, causing cell wall distortion. This outcome is due to the kinetic energy of the beads and this leads to heat up of the apparatus thus requires cooling after agitation that makes large-scale operations cost intensive [51]. For cyanobacterial cells, the sugar yield via this treatment is less as compared to other methods; however, carbohydrate and protein yield are found to be satisfactory [1]. Various parameters such as agitation speed, design of the chamber, biomass concentration, size and density of the beads and exposure time affect the optimization of bead milling-assisted cell disruption [52]. Postma

Table 1 Effect of pretreatment methods on algal biomass for bio-hydrogen production

Algal feedstock	Pretreatment conditions	Hydrogen yield	Comparison with untreated biomass	References
Chlorella vulgaris	Crude enzymes (35 °C/52 h)	43 ml H_2/g biomass	1.42 times higher yields	[38]
Scenedesmus obliquus	Autoclaving (121 °C/15 min, 1.4 bar)/*Enterobacter aerogenes*	40.9 mL H_2/g biomass	7.7 mL H_2/g biomass	[39]
Spirogyra sp.	Homogenization, bead beating, thermal-acid treatment (H_2SO_4-1 N/g biomass, 1 h, 121 °C)/*Clostridium butyricum*	146.3 mL H_2/g biomass	54.3 mL H_2/g biomass	[40]
Chlorella sp.	Heat-treated anaerobic sludge	8.29 mL H_2/g VS	-	[41]
Microcystis wesenbergii	Hydrothermal heating and acid: 135 °C, 15 min, 2% H_2SO_4/heat-treated anaerobic sludge	24.96 mL H_2/g TVS	0.30 mL H_2/g TVS	[42]
Microcystis aeruginosaare	Steam heating in an autoclave and acid: 135 °C, 15 min, 2% H_2SO_4/heat-treated anaerobic sludge	18.63 mL H_2/g TVS	0.30 mL H_2/g TVS	[42]
Scenedesmus obtusiusculus	Acid-thermal treatment: 160 °C, 3 h, 3% HCl/granular sludge	48.08 mL-H_2/g-VS	28.53 mL-H_2/g-VS	[43]
Spirogyra sp.	Acid treatment: 1 N H_2SO_4–60 min/2 N H_2SO_4–30 min at 121 °C/*Clostridium butyricum*	10.4 L H_2/L alga hydrolyzate	-	[44]
Nannochloropsis oceanica	Acid: H_2SO_4 0–2.0%; Microwave: 80–180 °C, 5–25 min/anaerobic sludge	2-39 mL H_2/g VS	0	[45]
Ulva reticulate	Alkaline condition (pH 7–12), microwave and H_2O_2 combination	87.5 mL H_2/g COD	10.3 mL H_2/g COD	[46]
Dunaliella primolecta	Lipid-extracted biomass/*Thermococcus eurythermalis* A501	192.35 mL H_2/g VS	36.84 mL H_2/g VS	[47]
Dunaliella tertiolecta	Lipid-extracted biomass/*Thermococcus eurythermalis* A501	183.02 mL H_2/g VS	25.34 mL H_2/g VS	[47]
Chlorella sp.	Light intensity 5000Lx, temperature 30 °C, pH − 7.0, protease: 15%, 25 g/L substrate conc.	43.62 mL H_2/g VS	1.31 mL H_2/g VS	[48]
Laminaria digitata	Hydrothermal pretreatment (140 °C for 20 min), pH − 4.5 and glucoamylase	55.2 mL H_2/g VS	35.7 mL H_2/g VS	[49]
Chlorella vulgaris	Thermal treatment: 100 °C, 60 min/digested sludge	190.90 mL H_2/g VS	13.3 mL H_2/g VS	[50]

Where *VS* volatile solids, *TVS* total volatile solids, *COD* chemical oxygen demand

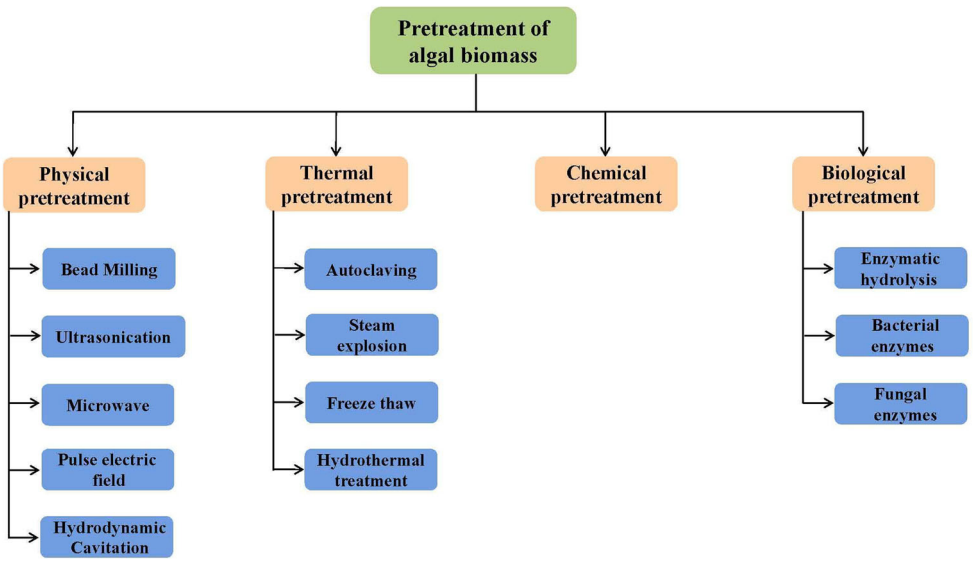

Fig. 2 Classification of different pretreatment of algal biomass

et al. [53] reported lower energy consumption and improved cell disruption efficiency in various algal species with smaller beads measuring 0.3–0.4 mm in size. In a study performed with wet biomass of alga *Arthrospira maxima*, an increase of 412% of hydrogen production was achieved using bead milling assisted with thermal, ultrasonic and enzymatic pretreatment method [54].

3.1.2 Ultrasonication

When ultrasonic waves travel, they create areas of compression and rarefaction and this develops micro bubbles that cause cavitation inside the liquid. These bubbles rupture, releasing amounts of energy and causes change in the immediate environment of the cell; as a result, imbalance is created in the temperature and pressure [55]. This release of energy supports the cell wall distortion, and the energy applied through ultrasonic wave's ranges from 10 kHz to 20 MHz. The degree of disruption also depends upon other factors which include size and shape of the cell, age of the culture and cell suspension density [51]. Ultrasonication at higher frequencies, however, causes generation of the free radicals resulting in the oxidative injure to the various cellular components [56]. One of the drawbacks of using this process is the generation of excess heat while cell disruption, consequently additional cooling setup is required, thus increasing overall cost of the process. Study performed by Kurokawa et al. [57] on three algae (*Chaetoceros gracilis*, *Chaetoceros calcitrans* and *Nannochloropsis* sp.) resulted the requirement of different optimal intensities for each algae. Another study resulted combination of higher (3.2 MHz, 40 W) and lower (20 kHz, 100 W) frequencies for efficient cell disruption of algae *Scenedesmus dimorphus* and *Nannochloropsis oculata* [58]. Apart from the power and frequency, exposure time of ultrasonication also plays significant role in efficient cell disruption. Microalgae consortia of *Scenedesmus* sp. and *Chlorella* sp. upon treatment at 10 W frequency for 30 min resulted in hydrogen yield, production and productivity of 36.8 mL/g, 28.7 ml and 160 mL/L/day^{-1} respectively [59].

3.1.3 Microwave

These electromagnetic waves (ranges 0.3–300 GHz) interplay with dielectric and polar molecules in the liquid medium which increases heat in the immediate surroundings and thus increasing the pressure, which eventually leads to disruption of the cell. Microwaves heat up the water in the cell and lead to cell wall distortion. This technique is highly efficient as its disruption rate is higher in a short period of time [51]. The macromolecules of the cell are polarized through microwave followed by solubilization of the cell components and modification in the protein conformation. This method is beneficial for lipid extraction from microalgal biomass as it slightly dehydrates the cell suspension making it viscous in nature [55]. Microwave-treated algal biomass of *Nannochloropsis oceanic* showed significant increase in the reducing sugar [45]. In a study conducted with seaweed *Laminaria japonica*, microwave assisted with mild acid pretreatment enhanced hydrogen yield to 28 mL/g in comparison to 15 mL/g total solids in control [22]. Cheng et al. [60] reported improved hydrogen yield of 283.4 mL/g in cyanobacteria *Microcystis* sp. upon microwave-assisted mild acid treatment; the yield was recorded via the combination of light and dark fermentation. Though, the microwave process is highly effective, but the large-scale application requires higher maintenance cost. In addition heat-sensitive products cannot be extracted by this process.

3.1.4 Pulsed electric field

In this method electric current is passed across the cell culture with a high voltage of 100–300 kVcm^{-1} resulting in permeations in the cell wall, thus easing extraction of pigments and antioxidants. An important initial step before applying electric current is deionization (absence of changed ions) of cell culture medium as it reduces the degree of cell disruption. Numerous other factors like size and shape of the cell, position in the electric field, intensity of the voltage, number of pulses and duration of voltage applied also modify the outcome. Extraction of macromolecules is not fruitful in this method due to size difference between the pore and macromolecules. Lipid extraction percentage was high in *Ankistrodesmus falcatus* after pulsed electric treatment. This method provides ease of extraction as there is no production of impurities, as a result, making downstream processing more convenient in comparison to other pretreatment methods [51, 55, 61]. Additionally, during this pretreatment method less toxic solvents are used, which are cost effective and poses less damage to the environment. Pulse electric field in combination with heat treatment (55 °C) of *Chlorella vulgaris* SAG 211-11 strain yielded 23–39% and 3–5% of carbohydrate and protein respectively [62]. One of the constraints with this method is that it does not facilitate the release of macromolecules such as proteins from the cell. In a study conducted by Lam et al. [63] on *Chlorella vulgaris* and *Neochloris oleoabundans*, only 13% protein content was achieved by pulse electric field (150 kWh kg_{DW}^{-1} cells) as compared to 45–50% by bead milling method.

3.1.5 Hydrodynamic cavitation

This process is similar to that of ultrasonic treatment in which formation of micro bubbles and pressure difference takes place, the only difference is that, in this method, the cell suspension is passed through a crevice hence creating microbubbles, which, while coming back under ambient pressure, bursts, releasing

fierce waves and increasing pressure and temperature inside the cell. This ultimately ruptures cell wall. This method of pretreatment is considered better than ultrasonic pretreatment as it is energy efficient and can be used for industrial production [51]. Due to its ease of access, this pretreatment method was used for the removal of algal blooms [64]. However, for a particular algal species a specific setup is required in terms of an orifice, applied pressure and application time. In this regime, the favourable factors for green alga *Chlorella pyrenoidosa* NCIM 2738 were orifice as a cavitation device, 5 bar pressure, 0.45% w/v solid loading and 180 min processing time [65].

3.2 Thermal pretreatment

3.2.1 Autoclaving

This is the most common method for routine sterilization and rupturing of the microbial cells. It sterilizes through steam at 121 °C temperature and pressure of 15 psi. This similar principle is applied on microalgal cells. Such treatments prove effective when done in coordination with an acid or an alkali. The outcomes for distortion are not the same in each experiment, as it also relies on several other factors like time required for lysing, volume of the cells and strength of the acid/alkali used. Batista et al. [66] performed an experiment with *Scenedesmus obliquus* at normal autoclaving conditions (121 °C and 15 min time), the autoclaved biomass was subjected to dark fermentation with *Clostridium butyricum*, and as a result, 113.1 ml of hydrogen gas per gram volatile solids from 50 g/L dried algal biomass was achieved. In another study conducted with green alga *Nannochloropsis oculata*, lipid yield was higher in the biomass pretreated with autoclaving in comparison with other pretreatment methods [67]. Though the process is common and no expertise is needed, but the large-scale operations are not feasible due to cost-intensive nature.

3.2.2 Steam explosion

This method of biomass pretreatment employs steam for cell disruption. The cell suspension is treated with steam at 180–240 °C of temperature and pressure of 1–3.45 MPa for a brief time followed by instantaneous depressurization to normal conditions. This leads to bursting of the cell, resulting in the release of cellular contents for further extraction [51]. Lorente et al. [68] conducted a study with green alga *Nannochloropsis gaditana*, out of various biomass pretreatment methods he employed, highest lipid yield of 18.2% was obtained with steam explosion at 150 °C temperature. In *Nannochloropsis gaditana* Lubián strain CCMP1775, out of 18.8% of total carbohydrate content, 12.9% carbohydrate was recovered by using steam explosion pretreatment method [69]. Instead of its ease of operation, the process is not used at industrial scale due to its cost-intensive nature to maintain high temperature and pressure.

3.2.3 Freeze drying/freeze thaw

In this method of pretreatment, biomass is subjected to freezing in a lyophilizer leading to formation of ice crystals. These crystals rupture the cell wall as the volume increases by ice crystal formation. Thawing of biomass at room temperature releases the intracellular contents via the ruptured cell wall. Repeated cycles are required in this process to cause successful cell disruption [51, 55]. Freeze thaw pretreatment method is one of the simplest methods to perform that do not require much expertise, but still very few studies have reported this microalgal pretreatment method for biohydrogen production. This is probably due to its high energy input requirement. Wang et al. [70] reported 1.5–2.5 folds increase in hydrogen production in freeze thaw pretreated activated sludge.

3.2.4 High-temperature thermal pretreatment/hydrothermal treatment

This method causes cell wall disruption by thermal denaturation in neutral, acidic or alkaline medium at temperatures ranging from 80 to 160 °C for a short span of time. For acidic and alkaline treatments, strong acids and alkalis are used for example sulphuric acid and hydrochloric acid and sodium hydroxide respectively. This treatment also decreases N content and enhances carbon content in the oil obtained as demonstrated in *Nannochloropsis occulata* [55]. Optimization of temperature and treatment time according to the microalgal strain is required. This method is practiced at large scale due to its ease and no strict requirement for skilled personnel [51]. Stanislaus et al. [50] carried out hydrothermal pretreatment of microalga *Chlorella vulgaris* at 100 °C for 60 min, upon fermenting the pretreated biomass with digester sludge 190.9 ml H_2/g volatile solids was achieved. During hydrothermal pretreatment pressure applied also plays vital role in terms of the overall output. Hydrothermal pretreatment of filamentous cyanobacteria *Arthrospira platensis* at 108 °C for 30 min at 0.5 atm pressure increased the hydrogen output by 60% [71]. In another study, acid (0.75% v/v H_2SO_4)-assisted hydrothermal pretreatment (160 °C for 30 min) of *Chlorella* sp. yielded 1079 ml-H_2/L [72]. One of the demerits of this pretreatment process is that the heat-sensitive compounds such as antioxidants, pigments and proteins cannot be extracted via this process.

3.3 Chemical pretreatment

Employing acid/alkali for microalgal cell rupturing is called chemical pretreatment. Generally strong acid and alkali like sulphuric acid and sodium hydroxide are used. This treatment

is concomitantly used with hydrothermal pretreatment. This method is very unique as acid usage disrupts cell wall and bases saponify its lipids. The working conditions of acid require high temperature. Alkali treatment is known to affect the particle size. Factors on which the efficiency of the treatment depends include the type of strain, duration of the treatment, synchronization with other treatment and recovery time. The harsh conditions of the reaction in due course of time degrade the container due to high pH and temperature, denaturation of protein followed by constant release of pigments from the cell suspension. The release of noxious chemicals is also a big question as its disposal poses a great threat. Due to the degradation of pentose sugars in harsh conditions, there is immediate formation of fermentation inhibitors like furfural and 5-hydroxyl methyl furfural. Some other inhibitors like acetate and propionate also reported in minute amounts. This causes low extraction of carbohydrate; hence, addition of inhibitor-resistant fermentative strains would prove beneficial [51]. In this regime, a genetically engineered furfural-resistant strain of *Clostridium beijerinckii* NCIMB 8052 is used for hydrogen fermentation, and as a result, the fermentation process remains unaltered even in the presence of 4–6 g/L of furfural [73].

3.4 Biological pretreatment

3.4.1 Enzymatic hydrolysis

In this method a cocktail of enzymes is used to treat the microalgal cell. This treatment works under specific conditions without leaving behind any impurities and under comparatively sensitive conditions. Microalgal cell wall is generally composed of cellulose, hemicelluloses, pectin and algaenan. Algaenan and sporopollenin are highly resistant to enzymes; hence, they undergo a pretreatment before enzymatic treatment [51]. While the cell wall component is not the same and fluctuate depending on factors such as the growth conditions, nutrient availability and algal strain [55], enzymes like cellulases, amylases, cellobiohydrolase, hemicellulase, lysozymes, pectinases, chitinases, proteases and lipases are used according to the strain to be acted upon [51]. To disintegrate the glycosidic bonds of the glucose and maltose, enzymes like glucanases and glucosidases are used [55]. Though they are efficient in treating algal biomass, enzymatic pretreatment still has few demerits, such as high cost associated with the enzymes and selective and specific nature. The enzymes are very specific to their substrate therefore detailed knowledge of the structural components of cell wall is important. Instead of using a specific enzyme, a cocktail or mixture of enzyme results in better hydrolysis of biomass. In a study conducted with *Nannochloropsis* sp., a combination of cellulose and mannanase was used for pretreatment; as a result, the lipid recovery was enhanced from 40.8 to 73% [74]. In another study, the biomass of green alga *Chlorella vulgaris* SAG 211-12 was treated with the mixture of macerating enzyme from *Rhizobium* sp. and cellulase form *Trichoderma viridae*, and as a result, the increase of 7 fold in hydrogen production was observed [75].

3.4.2 Pretreatment by bacterial enzymes

Due to fast growth rate and metabolic activity of bacteria, the bacterial-assisted biomass pretreatment offers advantages over fungal-assisted pretreatment. Enzymatic pretreatment has a limitation, as the activity of enzymes is lost over a period of time. On the other hand, hydrolytic bacteria, under favourable growth conditions, continue to produce hydrolytic enzymes over a long period of time, thereby making the pretreatment process more cost effective [76]. Hydrolytic bacteria such as *Thermomonospora* sp., *Clostridium* sp., *Streptomyces* sp., *Cellulomonas* sp. and *Bacillus* sp. can also be used for the pretreatment of lignocellulosic biomass, and besides this, few hydrolytic bacteria shows significant increase in methane yield under thermophilic conditions of biogas slurry [77]. Cheng et al. [78] used hydrolytic bacteria for the pretreatment of algal biomass due to their algicidal activity. In a previous study, *Bacillus thuringiensis* ITRI-G1 strain treated biomass of *Chlorella vulgaris* has shown increase yield of 44.3% in lipid content [79]. In another study, different percentages of pure bacterial culture of *Bacillus licheniformis* were used for the anaerobic digestion (37 °C for 60 h) of *Chlorella* sp.; as a result, volatile fatty acids and methane production of anaerobically digested biomass were increased by 17.3–44.2% and 9.2–22.7% respectively [80].

3.4.3 Pretreatment by fungal enzymes

There has been a lot of reports on the pretreatment of algal biomass using purified and cocktail of enzymes; several attempts have also been made for the pretreatment of algal biomass using bacterial enzymes. Though the fungi have exceptional ability to secrete various enzymes such as cellulose and xylanase, very few studies of pretreatment of algal biomass through fungal crude enzymes have been reported. One such study was performed on two fungal strains of *Aspergillus lentulus* and *Rhizopus oryzae* for the production of crude enzymes. It was reported that crude enzyme of *Aspergillus lentulus* resulted in ~100% cell death of *Chroococcus* sp. upon 48 h of incubation; in addition to this, total sugar and COD of the biomass were also solubilized up to 44% and 46% respectively by the action of crude enzyme [81]. In another study, pretreated (using crude enzymes from *Aspergillus lentulus*) biomass of cyanobacteria *Chroococcus* sp. was subject to anaerobic digestion, as a result, the methane production was enhanced to 324.38 mL CH_4 g^{-1} VS_{fed} as compared to 254.73 mL CH_4 g^{-1} VS_{fed} from untreated algal biomass [82]. Advantage of using fungal crude enzymes is that they

are cheap in comparison to other commercial enzymes and use if these enzymes at industrial scale significantly reduce the overall cost of biomass pretreatment.

4 Impact of pretreatment

Pretreatment is an important criterion for hydrogen production as it can make or break the result. A suitable pretreatment for particular operation is extremely vital to enhance the production of simplified carbohydrates which ultimately leads to hydrogen production. As carbohydrate is the main source of hydrogen production, it is present mainly in the cell wall of the algal biomass and thus its disintegration is vital to access the polysaccharide. For a particular type of microalgae, there is requirement of specific temperature and pH to obtain desired results. Sambusiti et al. [1] mentioned microwave treatment aided with acid treatment on *Arthrospira platensis* to uncover its carbohydrate content for the action of glucoamylase enzyme. This glucoamylase enzyme assists in hydrolyzing α-1, 4 and α-1, 6 glycosidic linkage in carbohydrates hence breaking the complex carbohydrates into simpler ones. Wet algal biomass of *A. platensis*, however, shown lower yield of carbohydrates by microwave then enzymatic hydrolysis pretreatment. *Chlorella pyrenoidosa* biomass was treated with steam heating and diluted acid and microwave-assisted heating with diluted acid to enhance its hydrogen yield as compared to heating treatments alone (like steam heating, microwave heating and ultrasonification) [54]. For lipid-extracted *Scenedesmus* sp. biomass, thermal (100° or 120 °C) and diluted alkali-assisted thermal treatment proved efficient for protein and carbohydrate hydrolysis. Physical method like bead milling was used on cyanobacterial cells but was less effective than ultrasonic treatment. For macroalgae, usual methods used are just similar, i.e. diluted acid and thermal treatment. In *L. japonica*, cellulose and hemicelluloses are the vital components; as a result they yielded glucose and xylose on hydrolysis [22]. Roy et al. [37] treated algal biomass with hydrochloric acid at different gradations and found that HCl de-clumped the cellular clusters and also de-shaped them as carbohydrate was degraded. The debris produced in the treatment was less in comparison to H_2O_2 treatment, as it oxidized the debris and chlorophyll content due to the release of nascent oxygen, and the same result was obtained with physical treatments also. In a recent study, marine macro alga *Ulva reticulate* pretreated with a microwave in combination with H_2O_2 under alkaline conditions showed a COD solubilization of 34% and the highest hydrogen yield of 87.5 mL/g COD was achieved [46].

After the pretreatment of algal biomass, it could be efficiently used for the production of any kind of biofuel. To select the best pretreatment approach, however, the analysis (qualitatively and quantitatively) of pretreated biomass is required. One of the most common approaches of qualitative detection of pretreated biomass is through visual observation via microscopy. In general light microscopy gives basic idea about the pretreatment process; nowadays however, more advanced microscopic techniques such as SEM, TEM and fluorescent microscopy are being used [81]. Use of SEM and TEM requires skilled techniques and the initial cost of installing these facilities is quite high. Fluorescent microscopy, on the other hand, is a cost-effective and user friendly approach that can be used to easily differentiate various pretreatment methods. This approach uses certain fluorescent dye, for example Sytox® Green a nucleic acid stain. This dye is impermeable to living cell, but has a peculiar property of binding with the exposed DNA of the damaged cell, and it has emission and excitation maximum of 523 nm and 504 nm, respectively. Therefore, on the basis of the intense green colour on the microscopy images, one could have an idea about the pretreatment process [83]. Qualitative analysis has some limitations in the sense that it gives a general idea and differentiates comparatively; however for more precise indications, quantitative methods are used. One such approach for differentiation among different pretreatment methods is the cell counting technique. In this method live and dead cells are counted after the biomass pretreatment with the help of fluorescent microscope equipped with automated cell counter [81]. The percentage of cell death is determined by the equation:

$$\text{Cell death } (\%) = (X_0 – X_t / X_t) \times 100 \quad (1)$$

where, X_0 and X_t are live cells mL^{-1} at time 0 and t during the pretreatment process, respectively. In another method, the pretreatment of biomass could be measured indirectly on the basis of sugar concentration, solubilization of biomass and soluble chemical oxygen demand (sCOD) with respect to pretreatment time by using the following equation [84]:

$$S_D = (COD_S – COD_{S0}) / (COD_T – COD_{S0}) \times 100 \quad (2)$$

where, S_D is degree of solubilization (%), COD_{S0} is the soluble COD in the raw algal biomass, COD_T the total COD of the algal biomass and COD_S is the soluble COD after pretreatment [35]. Merits and demerits of pretreatment methods are shown in Table 2.

5 Enzymes involved in hydrogen production

There are certain key enzymes present in microalgae and cyanobacteria that are known to produce biohydrogen. These are as follows.

5.1 Hydrogenase

This metalloprotein is present in chloroplast of microalgae (eukaryote) and in the cytoplasm of cyanobacteria

Table 2 Merits and demerits of different pretreatment processes [28, 30]

S.no	Method	Merits	Demerits
1.	Bead milling	• Suitable for wet biomass • Higher degree of disruption	• High energy demand • Heating during process
2.	Ultrasonication	• Higher degree of disruption • No supplementary requirement • Less time consuming	• Oxidative damage at high frequency • Heat generation during process • Scattering of energy with increased distance
3.	Microwave	• Efficient biomass solubilization • Less time consuming • Advances digestibility of starch • Eases lipid extraction	• Expensive maintenance • Heat generation • Requirement of additional treatments for desired results
4.	Pulsed electric field	• Low energy requirement • No extreme conditions given to the cell • No production of impurities • Less noxious solvent usage • Comparatively cheaper	• High cell mortality • Requirement of de-ionization • Heat generation
5.	Hydrodynamic cavitation	• Less energy requirement • Applicability for large-scale production	• Sophisticated equipment requirement • Complex optimization • Heat generation
6.	Autoclaving	• Short working time • Less maintenance costs • High degree of cell wall disruption • Easy to operate	• More energy requirement • Inappropriate for heat labile components
7.	Freeze drying/freeze thaw	• No further treatment requirement • Appropriate for sensitive compounds • No homogenization required	• High maintenance • Complex machinery • Less efficient cell wall disruption
8.	Hydrothermal treatment	• Hydrolyses starch • Reduces N content in bio-oil	• Protein and sensitive compound denaturation • Requires acid/alkali • High energy requirement
9.	Acid/alkali	• Effective disruption of cell wall • Easy availability of raw materials	• Extreme pH • Susceptible to corrosion • Formation of inhibitors • Unavailability of safe disposal
10.	Enzymatic treatment	• No secondary pollution • No requirement for sophisticated equipment • Less energy requirement	• Maintenance of mild, accurate conditions • Expensive in nature • Requirement of extensive study before employing

(prokaryote). Hydrogenase leads to the formation of hydrogen (H_2) from proton (H^+) with no ATP consumption (Fig. 3) [9]. Production of H_2 via hydrogenase is shown in the following reaction.

$$2H_2O + \text{light} \rightarrow 2H_2 + O_2 \quad (3)$$

There are three classes of hydrogenase, viz. [FeFe] hydrogenase, [NiFe] hydrogenase and [Fe] hydrogenase [19, 85]. [Fe] hydrogenase is 38 kDa homodimeric protein occurs in some of the methanogenic archaea. It carries out the conversion of CO_2 and H_2 into methane through a complex chemical reaction. The substrate for the catalytic reaction of [Fe] hydrogenase differs from rest of the two hydrogenase enzymes; it has a peculiar H cluster inside the active centre and recently there are several reports of isolating [Fe] hydrogenases. The *hyd A* gene has been isolated from *Chlorella fusca*, *Scenedesmus obliquus* and *Chlamydomonas reihardtii* [86, 87].

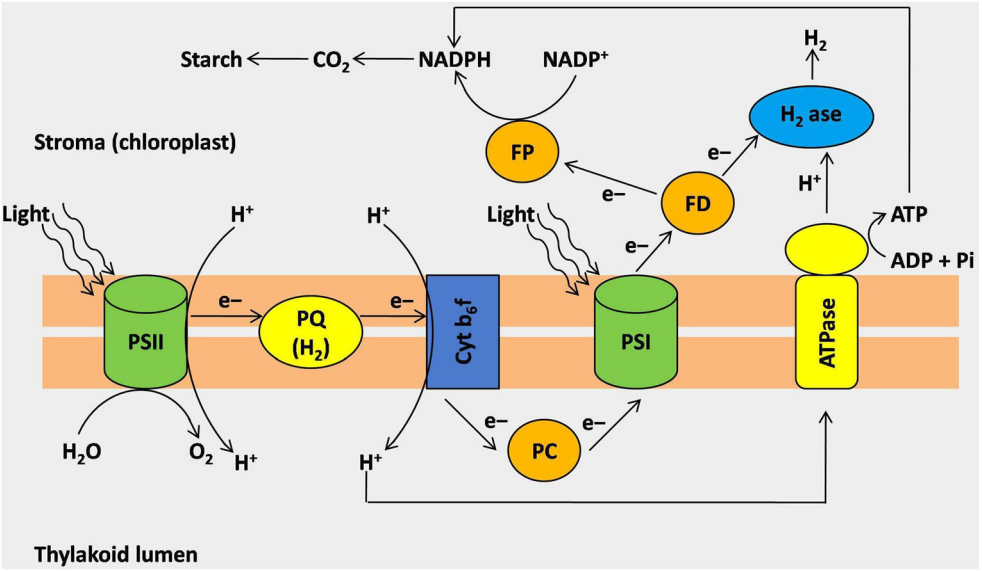

Fig. 3 Schematic illustration showing H2 production by algae. PSII, photosystem II; PSI, photosystem I; PQ, plastoquinones; PQ (H2), reduced plastoquinones; Cyt b6f, cytochrome b6f complex; PC, plastocyanin; ATPase, ATP synthase; FD, ferredoxin; FP, ferredoxin-NADP reductase; H2ase, hydrogenase

[FeFe] hydrogenase is produced by gene *hydA*, and it is responsible for enzyme formation. This enzyme has a peculiar core; hence, it has improved efficiency as it is 100 times more productive than other classes of hydrogenase. The core is extremely sensitive to oxygen and thus makes it tough to liberate H_2 under oxygenic conditions; the core is also the site for electron transfer from ferredoxin. The molecular weight of this hydrogenase is 45–50 kDa. The catalytic site/H-cluster consists of FeFe bond with sulphur bridge, 4Fe-4S residue, CN and CO ligands joined to each Fe atom at the centre [19]. Absence of sulphur in the environment aids hydrogen production as its presence stimulates suppresser protein for hydrogen production. High light intensity and optimum pH are also stimulators for hydrogenase [9]. One peculiar thing about this [FeFe] hydrogenase is that it can work bidirectionally as it can either produce or consume hydrogen, and this switch depends on the environmental conditions [21].

NiFe hydrogenase is the fundamental enzyme in cyanobacteria for hydrogen production and also found in archaea and anaerobic bacteria. It may present in cyanobacteria in both the forms, i.e. uptake and bidirectional hydrogenase enzyme. It consists of two units; the larger unit acts as an active site having [NiFe] bond; on the other hand, Fe-S cluster is present in the smaller unit. Reduction of H^+ to hydrogen is carried out by the transfer of electron from smaller unit to the active site of the enzyme. In the case of NiFe hydrogenase, the sources of electron could be both flavodoxins and ferredoxins [19, 88, 89].

5.2 Uptake hydrogenase

As the name suggests, this enzyme consumes hydrogen. It is encoded by gene hupSL and works efficiently in nitrogen-fixing cyanobacteria. It is found in all nitrogen-fixing bacteria except some Synechococccus strain and Chroococcidiopsis isolates. It is found in the thylakoid membrane of heterocyst of unicellular and filamentous cyanobacteria. This enzyme shows resistance to oxygen, and cells with active uptake hydrogenase enzyme show less amount of net H2 in the cell; however, mutant cells deficient of uptake hydrogenase had thrice the amount of hydrogen than the wild ones [9, 90].

$$H_2 \rightarrow \text{uptake hydrogenase} \rightarrow 2H^+ + 2e^- \quad (4)$$

5.3 Nitrogenase

Cyanobacteria own this enzyme, and this enzyme helps convert nitrogen to ammonia followed by hydrogen (shown in Eq. 5). Nitrogenase is of different types on the basis of the cofactor, viz. molybdenum nitrogenase, vanadium nitrogenase and iron nitrogenase. Thoroughly studied nitrogenase enzyme out of these three is molybdenum nitrogenase [16]. It is composed of two units, dinitrogenase (MoFe protein or protein I) and dinitrogenase reductase (Fe protein or protein II). Dinitrogenase is a homotetramer consisting of α_2 β_2 encoded by *nifD* and *nifK* genes. This enzyme breaks the N_2 bond to form NH_3. Dinitrogenase reductase is a homodimer encoded by *nifH* gene. It delivers electron to dinitrogenase after receiving it from an external electron donor. Nitrogenase works efficiently in nitrogen deficient environment and requires electron from a donor, reductant and 16 molecules of ATP [19]. It is an irreversible reaction [9]. Cyanobacteria use nitrogenase for hydrogen production in heterocystous cells. Heterocysts are thick-walled cells that lack photosystem II (PS II) thus lacking O_2

as there is no photolysis of water [19, 90]. The overall reaction of nitrogenase is shown below:

$$H_2O \rightarrow \text{photosynthesis} \rightarrow [CH_2O] \rightarrow \text{ferredoxin} \rightarrow \text{nitrogenase} \rightarrow H_2 \quad (5)$$

Cyanobacteria occur as both nitrogen-fixing and non-nitrogen-fixing bacteria and thus their mechanism for hydrogen production is quite different and as follows [9]:

In presence of nitrogen:

$$N_2 + 8H^+ + 8e^- + 16\ ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16P_i \quad (6)$$

In absence of nitrogen:

$$8H^+ + 8e^- + 16\ ATP \rightarrow 4H_2 + 16\ ADP + 16\ P_i \quad (7)$$

6 Mechanism of hydrogen production

Currently large-scale production of hydrogen is carried out by methods like electrolysis of water and coal gasification which are somewhat destructive and harmful to the environment, so to make production of hydrogen energy efficient, certain methods are considered, i.e. using microorganisms. Algal biomass is known to produce biohydrogen through different methods. Biohydrogen is produced by algae through two methods that are photosynthesis and fermentation. Biohydrogen production through photosynthesis further branches to two different methods known as (i) direct biophotolysis and (ii) indirect biophotolysis and the fermentation process can also occur through two different paths, namely (i) photo fermentation and (ii) dark fermentation [55]. The chlorophycean members of microalgae produce biohydrogen only through direct biophotolysis due to the evolution of oxygen, whereas cyanobacteria can undergo both direct and indirect biophotolysis due to the cell differentiation they possess. As cyanobacteria have specialized cells called heterocysts, there is no oxygen evolution and thus undergo indirect biophotolysis, whereas the vegetative cells carry out the direct biophotolysis [19, 85].

6.1 Direct biophotolysis

Microalgae have all the machinery to carry out hydrogen formation in the presence of light. They have photosystem I, photosystem II, ferredoxin, chlorophyll a, hydrogenase and nitrogenase. Direct biophotolysis involves photochemical oxidation of water and subsequent hydrogen production with the assistance of [Fe] hydrogenase [2, 91]. PSII situated in the thylakoid membrane receives the electrons from the dissipation of water which are ultimately received by Fe hydrogenase through ferredoxin intermediate, resulting in the formation of hydrogen. During cyclic photophosphorylation these electrons reduce plastoquinone (PQ) to plastoquinol (PQH_2) which further reduce $NADP^+$ to NADPH. ATP synthase enzyme uses the protons to create a gradient for the ATP formation. Thus, there is production of oxygen and hydrogen simultaneously. The hydrogen gas produced by direct photolysis is 98% pure [91].

6.2 Indirect biophotolysis

The process of indirect photolysis is an anaerobic hydrogen production due to sulphur stress in green algae. Sulphur deprivation hinders protein formation which ultimately reduces photochemical activity of D1 protein of PSII, hence, obstructed oxygen formation. This creates an imbalance between oxygen production and consumption. The respiration increases as compared to the photosynthesis hence creating anaerobic conditions in the growth medium [91]. It is a two-step process: (i) CO_2 assimilation in green algae and cyanobacteria with oxygen evolution; (ii) the stored carbohydrates are used for the production of H_2 by reversible hydrogenases [15]. In this method the consumption of starch and protein is more due to anoxygenic environment. Studies conducted on cyanobacterium *Gloeocapsa* reported the pH range of 6.8–8.3 and temperature between 30 and 40 °C for optimal hydrogen production [91].

6.3 Photo fermentation

It is a method which involves generation of hydrogen and carbon dioxide from complex organic acid compounds through ferredoxin and nitrogenase using light as energy source [9]. The endogenous substrates undergo photo fermentation and give rise to electrons from organic compounds. Electrons from this catabolism flow to PQ. Assimilation activity of photosystem I (PS I) brings the redox potential of ferredoxin and [Fe] hydrogenase to an equivalent. Molecular hydrogen is formed as hydrogen ions are the terminal electron acceptor. To yield hydrogen efficiently, it has also been found that the presence of PS II inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) obstructs electron flow from PSII to PQ and this leads to the production of molecular hydrogen and carbon dioxide in stoichiometric ratio of 2:1 [17, 91]. Nitrogenase enzyme requires ATP; it works under unavailability of fixed nitrogen and the substrate should have high C/N ratio [92]. Activity of nitrogenase is deterred by the presence of oxygen and high ammonia concentrations, also increased light intensity showed improved outcomes but high intensities adversely affected light conversion efficiencies [15]. Purple non-sulphur (PNS) bacteria produce hydrogen from organic compounds like acetic acid, glucose, fructose, succinate and malic acid. Aromatic compounds and alcohols, ethanol and propanol are also used. *Rhodobacter* and *Rhodopseudomonas* are some examples of

photosynthetic PNS bacteria. PNS bacteria are less sensitive to oxygen and continue to produce hydrogen through nitrogenase enzyme [9].

6.4 Dark fermentation

It is a multistep pathway which does not require sunlight, water and oxygen. It is performed by anaerobes and some microalgae like green algae. Starch and glycogen present in green algae and cyanobacteria respectively are converted to pyruvate from sugars and ultimately to hydrogen under dark conditions. The pyruvate molecule is converted into acetyl coA via TCA cycle. The pyruvate is acted upon by two enzymes pyruvate formate lyase (PFL) and pyruvate ferridoxin oxido-reductase (PFOR). Per pyruvate molecule gives 1 to 2 mole of hydrogen. This low yield is due to uptake hydrogenase enzyme which consumes some part of hydrogen. The oxidative pentose phosphate pathway (OPP) in microalgae forms 4 mol of hydrogen and 2 mol of acetate from 1 mol of glucose [9, 93]. There are four stages in this process. In the first stage, polymeric compounds like carbohydrates, proteins and fats undergo hydrolysis to form smaller molecules. In the second stage, the fermentative bacteria release enzymes which acidify these smaller compound molecules. Formation of molecules like hydrogen, carbon dioxide, acetate, organic acids is accompanied by acidogenesis. The third stage leads to formation of acetate and hydrogen through acetogenesis of compounds formed in previous stage. Here homoacetogenic bacteria convert available hydrogen and carbon dioxide into acetate. However, the hydrogen formed is an intermediate of acidogenesis and acetogenesis and is further utilized by methanogens. Finally methanogenic bacteria form methane by reducing carbon dioxide and decarboxylating acetate [17]. Advantages and disadvantages of various hydrogen production methods are discussed in Table 3.

Table 3 Advantages and disadvantages of various hydrogen production methods [2, 22, 25, 39]

Direct photolysis	Indirect photolysis	Photo fermentation	Dark fermentation
Advantage			
a) Easy availability of raw materials, i.e. water and sunlight b) Efficient conversion of solar light c) Does not require carbon sources	a) Separate evolution of hydrogen and oxygen b) Yields high amounts of hydrogen c) By-products also yield	a) Uses industrial waste for organic compounds b) Reduces pollution c) Uses organic acid of dark fermentation d) Uses wider wavelength of light e) Can use wastewater also	a) Variety of carbon sources can be used b) No light required c) By products are also useful like butyric acid, lactic acid and acetic acid d) No oxygen produced e) Works under non-sterile conditions
Disadvantage			
a) Sensitivity of enzyme hydrogenase to oxygen b) Requirement of high--intensity light c) Reduced hydrogen production d) Generated mixture of H_2-O_2 is explosive e) Expensive in nature	a) Large-scale requirement of adenosine triphosphate (ATP) b) Needs continuous light supply c) Photosynthetic conversion efficiency is inefficient	a) Oxidative inhibition of nitrogenase b) High ammonia concentration also inhibit the enzyme c) Incompetence to use high--intensity light d) High cost of reactors	a) Requirement of nitrogen less conditions b) Low yield c) Substrate wastage due to incomplete utilization d) Expensive pretreatment e) Low-cost reactor

7 Influence of different factors on microalgal hydrogen production

In the biofuel industry, whether it is associated with biodiesel production or biohydrogen production, one of the main cost-intensive processes is the cultivation of algae. At industrial scale, the higher cultivation cost could be compensated with high product recovery. Better understanding of the growth pattern/nutrient requirements of the algae is crucial to overcome such problems. Certain factors are discussed below that play a crucial role in hydrogen production by algae.

7.1 Light intensity

Light intensity is one of the most important factors that significantly affect the microalgal hydrogen production. Contrary to the algae grown in natural habitat, artificially grown algae in lab condition require optimal illumination, so that its overall productivity will not be affected. Microalgae have an ability to produce hydrogen via the process of photosynthetically mediated water oxidation. Both microalgae and cyanobacteria have a potential to fix sunlight into high energy H_2 molecules to the maximum theoretical competence of around 13% [94]. However, the major challenge for this process is the oxygen (O_2) sensitivity of hydrogenases enzyme (responsible for H_2 production) as both O_2 and H_2 are coproduced by algae in the same volume [95]. For the algae to produce higher amount of H_2, O_2 from the cell has to be continuously removed. Under aerobic conditions, light-driven activation of PSII mediates the photolysis of H_2O molecule, resulting in the generation of oxygen, hydrogen and electron. Finally the electron and hydrogen

are utilized by $NADP^+$ to yield NADPH, the reduced form [96]. However, during anaerobic conditions, instead of $NADP^+$, the electrons and protons are taken up the hydrogenases enzyme resulting in the formation of H_2 molecule.

To generate higher quantities of H_2, a unique approach was employed in which the culture of green algae is suddenly switched from dark to light condition. During dark condition, anaerobic environment is maintained due to the inactivation of PSII, as a result expression of hydrogenases enzyme takes place. As soon as the algal culture is shifted to light condition, a sudden spike in the H_2 production is observed due to the utilization of electrons and protons (produced by PSII) by hydrogenases enzyme. H_2 production by this strategy lasts only for a short span of time until the activation of Calvin Benson cycle and the deactivation of hydrogenases enzyme by increasing concentration of O_2 by PSII [97, 98]. This approach, however, could be applied repeatedly to attain higher H_2 production.

7.2 Spectral selectivity

Hoshino et al. [97] conducted an experiment with green alga *Chlorella reinhardtii* for H_2 production under the influence of different light intensities. The basis of their study lies on the fact that both PSI and PSII reaction centers require different wavelengths of light to carry out their chemical reactions. In the case of *C. reinhardtii*, the peak of action spectra of PSI and PSII lies approximately at 680 nm and 670 nm respectively. Keeping this in mind, if a selective light intensity of 680 nm with the brief intermittent exposures of 670 nm will be provided to the alga, it will lead to the constitutive expression of PSI, whereas the activity of PSII will be suppressed to the greater extent. As a result, oxygen generation by PSII downregulates several folds, and at the same time mitochondrial respiration continues to occur resulting in an anaerobic condition inside the cell. All these events favour the expression of hydrogenases enzyme in an anaerobic condition and production of higher amounts of H_2. It has been reported that under the influence of spectral selectivity the rate of photosynthetic O_2 production decreases as much as 40–50%; on the other hand, the rate of photo H_2 production increases around 80% [97, 99, 100].

7.3 Nitrogen deprivation

Nitrogen deprivation is one of the well-known strategies to induce lipids and starch accumulation in green algae. Nitrogen limitation for H_2 production in green algae, however, did not get much attention due to its very low yield. In green algae, H_2 production under nitrogen stress is mainly due to the fermentation of starch and lipids since, apart from PSII, cytochrome b6f complex is also degraded due to nitrogen stress resulting in the hindrance in electron transfer to hydrogenases enzyme [101, 102].

As discussed earlier, nitrogenase is the enzyme that is responsible for H_2 production in blue green algae (cyanobacteria). The activity of nitrogenase enzyme in heterocyst-bearing cyanobacteria is, however, inhibited by the presence of nitrogen source in the growth medium. Shah et al. [103] reported that presence of any nitrogen source in the growth medium such as inorganic nitrogen forms as well as amino acids inhibits the activity of nitrogenase enzyme. In non-heterocyst-bearing cyanobacteria such as *Synechocystis* sp., H_2 production is carried out by the presence of [NiFe] hydrogenases (also called as Hox- hydrogenases). The expression of *hox* genes occurred under nitrogen depleted conditions in the growth medium [104].

7.4 Effect of pH

pH of the culture medium is one more essential factor that significantly affects the H_2 production of algae. It has been reported that the activity of hydrogen producing enzyme is inhibited by both acidic and extremely alkaline conditions [105]. On the basis of the earlier reports, it has been concluded that, depending upon the type of substrates used, different algal species require different optimum pH conditions and most of the algal species works well near neutral pH conditions for efficient H_2 production [106]. A study on green alga *Tetraspora* sp. CU2551 was performed to study the effect of pH on the flow of H^+ ions to hydrogenases enzyme. As a result, maximum H_2 production (24% higher than control) was observed at pH 6.5 [107]. In another study conducted with sulphur-deprived cultures of *Chlorella reinhardtii*, the maximum H_2 production was observed at pH 7.7 [108]. Most of the earlier reports reveals that the H_2 production was much compromised at acidic than that of alkaline pH conditions, and it may be due to the fact that, during acidic conditions, algal cells lose their ability to maintain their optimum pH conditions resulting in lower levels of ATP generation [109].

8 Genetic modifications to enhance biohydrogen production

Very small part of the light energy is used for H_2 production, cell survival and the maintenance of the algal cell. Larger portion (60–80%) of light energy is wasted in the form of heat [110]. Therefore, there is a scope to enhance the photosynthetic efficiency of the cell via the genetic manipulation of the photosynthetic regulation. LHCI (light harvesting complex) and LHCII of PSI and PSII respectively play an important role in photosynthesis. In the presence of higher light intensities, the expression of LHC in wild-type strains is downregulated, resulting in the death of the cell. However, it has been reported that the light harvesting potential was significantly improved in genetically modified cells with reduced chlorophyll antenna

size. This strategy helps algal cells to sustain high light intensities without compromising photosynthetic efficiency [111, 112].

H_2 production in algae can also be enhanced via engineering hydrogenase enzyme. Molecular weight of hydrogenase is 48 kDa [113]; the active site of the enzyme is called H-cluster which produces hydrogen gas. Supply of e−, H^+ and O_2 to the active site of the enzyme is carried out by numerous microchannels present on the apo-enzyme. The activation of the enzyme is carried out via the various sequences of gene expression followed by assembly of proteins commonly called as maturation process [114–116]. As we have discussed earlier, the biggest challenge with this enzyme is its O_2 sensitivity. Still there are certain ways by which the efficiency of hydrogenase enzyme could be improved. One such way is to decrease the O_2 sensitivity of *HydA* gene which plays a vital role in the maturation process of the enzyme. This gene works in a reversible manner, i.e. activated in anaerobic conditions and inactivated in aerobic conditions [117, 118]. Engineering *HydA* gene for O_2 tolerance will definitely improve the efficacy of hydrogenase enzyme; contrary to this, modification of microchannels (that carries O_2 to the active site of the hydrogenase enzyme) can also be done. Improving catalytic production of H_2 from H^+ ions is yet another approach to enhance the catalytic activity of hydrogenase enzyme; this can also be achieved by redirecting the flow of e− from PSI towards the enzymes active site [112, 119, 120].

9 Discussion

Hydrogen is one of the cleanest forms of energy that can be used to fulfill our future energy demands. Enormous efforts are being made to improve biohydrogen production from microorganisms. Algae-derived biohydrogen production is a promising approach; perhaps at the same time it has some technical challenges such as pretreatment of biomass, substrate conversion efficiency, scale up operations, large-scale storage and overall production cost. Algae are very rich in terms of their diverse varieties, but contrary to this, at present there are very few algal species that are being explored for biohydrogen potential; therefore, it is needed to explore more number of species that may have improved potential of hydrogen production. Moreover, a comprehensive knowledge of algal cell wall structure is needed to develop effective enzyme cocktail for degradation of cell wall. Few studies in recent years focused on the development of cell-wall less mutants of algae, and these studies will definitely add up new dimensions to algal biohydrogen production. In a recent study, cell-wall less mutant of green microalga *Chlamydomonas reinhardtii* cc-400 has been developed; mutant strain has shown 70% release of intracellular protein upon pretreatment with pulse electric field with very low energy input [121]. In a study performed with *Nannochloropsis gaditana* strain 127 (SAG 2.99), cell wall thinning up to 50% (27.8 to 12.6 nm) has been achieved via incubating microalgal slurry at 38 °C in dark conditions for 24 h [122]. Biomass pretreatment step is imperative for biohydrogen production; therefore, carbohydrate-rich lipid-extracted microalgal biomass might have a better potential for biohydrogen feedstock. This synergistic approach could lower the overall cost of biofuel production.

Dual approach of waste water treatment in conjugation with biohydrogen production has been discussed since long, but the practical application of this dual approach is still lacking. Wastewater streams are inhabited by variety of microalgal species; the application of microalgal consortia (mixed culture) for biohydrogen production could be a better option in terms of its stability, wide range of substrate utilization, operational ease and cost effectiveness. Apart from this, the effect of various nutrient availability could also be studied; as in previous studies it has been widely reported that the modulation of nutrients in growth medium can significantly alter the metabolism and hydrogen production potential of microalgae. The effect of macro as well as micronutrients can be used to unfold mystery of complex regulatory network involved in hydrogen production.

Metabolic engineering, particularly targeted genome engineering of microalgae, is yet another approach that can be used for major breakthrough in algal biohydrogen production. This technique can help in exploring various metabolic pathways that may improve the substrate utilization and help in diverting carbon flux towards the hydrogen producing pathways. Random and targeted mutagenesis techniques are being used in this regime, as a result of which more stable and oxygen-tolerant hydrogenase enzymes are being screened.

10 Conclusion

Certain methods for production of biohydrogen have been employed like photo fermentation, dark fermentation and biophotolysis. For dark fermentation low yields and accumulation of by-products are the reasons for encumbrance to industrial production. Perhaps bio-photolysis faces impediment due to oxygen sensitivity of enzyme and incompetent substrate conversion. The drawbacks include the expensive prices of carbon source and long time required for hydrogen production. Pretreatment of the biomass is a crucial method to determine the outcomes of fermentation and efficient hydrogen production. Improvement in cell immobilization techniques for desired results and simultaneously using different treatments on algae can help in comparing the results with that of the conventional biofuel producing methods. Thorough research on management of by-products and any usefulness they provide can

prove to be a breakthrough in hydrogen production. An in-depth study regarding algal cell wall compositions and their growth in different conditions can help to a larger extent to improve hydrogen production through microalgae. Genetic engineering of high producing algal strains according to desired outcomes can prove to be a quantum leap in this avenue. So with the efforts of researchers further technological advancements can change the course of the biofuel industries reducing dependence on the fossil fuels and stress to the Earth's natural resources.

Acknowledgements S.K.M. is thankful to UGC-DSKPDF, Delhi, India for PostDoctoral research grant. S.K.M. is also thankful to the vice-chancellor, Panjab University Chandigarh, for providing necessary laboratory facilities and support. Author N.S. thankfully acknowledges Department of Chemical Engineering and Technology, IIT (BHU) Varanasi for providing PostDoctoral Fellowship. Author N.S. also acknowledges the Department of Chemical Engineering and Technology, IIT (BHU) Varanasi for providing the experimental facilities.

Funding This work was supported by [UGC-DSKPDF], grant numbers [F.4-2/2006 (BSR)/BL/16-17/0518].

Declarations

Competing interests The authors declare no competing interests.

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