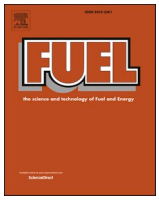
Fuel 351 (2023) 128922

Contents lists available at ScienceDirect

Fuel

journal homepage: www.elsevier.com/locate/fuel



# Bioprocess strategies to augment biohydrogen production from algae

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ARTICLE INFO

*Keywords:*
Algae
Biohydrogen
Strategies
Advancements
Genetic engineering
Future scopes

ABSTRACT

Drastic climatic changes and health hazards, in the recent years, due to combustion of fossil fuels has elicited the need to find sustainable and economical fuel alternatives. Hydrogen gas is a versatile energy carrier that can be used for transportation, electricity generation, and industrial processes. It does not produce greenhouse gases or other pollutants when used, making it a promising alternative to fossil fuels. Among the feedstocks used for hydrogen production, algae are found to be a potential source due to their high growth rate, ability to grow in diverse environments, and ability to produce hydrogen via the process of photosynthesis. However, current hydrogen production rates using algae are low, and strategies to improve these rates are needed. This review is aimed to investigate and evaluate different strategies to enhance biohydrogen production using algae. In addition, the prospects of using macro and microalgae as feedstocks have been elucidated. This review also provides brief insights on the current technologies available for algal biohydrogen production and their bottlenecks. The recent advancements, research gaps and future scopes of this research have been highlighted. Overall, the discussion of the various strategies can provide valuable information for the development of cost-effective and sustainable hydrogen production methods.

## 1. Introduction

Biohydrogen is a renewable source of hydrogen gas that is generated by microorganisms through the fermentation of organic matter [37]. As a fuel source, biohydrogen has various potential advantages. It is a clean-burning fuel that emits only water when burned; thus, it does not contribute to greenhouse gas emissions or air pollution. It is also a very efficient fuel that can be used to power cars, buses, and airplanes, among other vehicles [99]. In addition, several feedstocks, including agricultural and forestry residues, municipal solid waste, and algae, can be used to make biohydrogen [75,36]. The microorganisms commonly used for agricultural residues degradation are *Clostridium* spp., which are anaerobic bacteria that can break down the complex organic molecules in the feedstocks and produce hydrogen as a byproduct [43]. Municipal solid waste (MSW) is another feedstock that can be used for biohydrogen production. The microorganisms commonly used for this purpose are mixed cultures of bacteria and archaea, which can break down the organic matter in the MSW and produce hydrogen as a byproduct. Pretreatment facilitates the disintegration of biomass and hence increases the biohydrogen production [6]. This renders it a potentially sustainable and cost-effective alternative to fossil fuels. Industry and academia have both expressed an interest in exploring the viability of biohydrogen production at industrial scales. Ongoing research aims to eliminate the process's technological bottlenecks to make it economically viable at an industrial scale.

The key aspects of biohydrogen production are decided by a set of processes starting from feedstock selection and preparation, hydrogen production mechanism, hydrogen separation, hydrogen purifications and end at hydrogen storage [19]. The first step in biohydrogen production is selecting an appropriate feedstock, such as agricultural or forestry wastes, municipal solid waste or algae. The feedstock must next be ground or shredded into small bits prior to fermentation. Next, the feedstocks are fermented to produce hydrogen gas. Microorganisms, such as bacteria or yeast, break down the organic matter in the feedstock and produce hydrogen as a by-product in a process known as dark fermentation [79]. Apart from dark fermentation, biohydrogen from

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https://doi.org/10.1016/j.fuel.2023.128922
Received 5 February 2023; Received in revised form 14 May 2023; Accepted 5 June 2023
Available online 15 June 2023
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organic matter/feedstock can be produced through photo-fermentation, bio-photolysis, and thermal gasification [70,72]. Each process has its own advantages and disadvantages; however, the choice of feedstock eliminates certain limitations and allows more flexibility in production. For instance, in the case of dark fermentation, choosing a feedstock having a lesser lignin component can eliminate the requirement of pre-treatment [48]. Similarly, bio-photolysis depends on enzymes such as hydrogenase and nitrogenase, which are available in only certain types of biomasses, such as algae and microbes [72].

Algae contain high carbohydrate content required for biohydrogen production [9]. Other advantages of algae include faster growth, shorter growth cycles, adaptation to extreme conditions, utilization of inorganic carbon ($CO_2$), environment-friendly (doesn't require pesticides and herbicides), and it doesn't require arable land for cultivation [3,8,46]. This diverse range of features enables algal biomass to be a suitable candidate for biohydrogen production. With the appropriate selection of strain and pre-treatment algal biomass, the biohydrogen production potential could be increased significantly.

Though biohydrogen production from algae provides several advantages over other feedstocks, several challenges need to be addressed to make it a competitive candidate for the hydrogen-producing industries. Certain aspects of algal biohydrogen production such as developing high-yield algal strains, optimizing growth conditions, and developing efficient harvesting techniques, are a few of them. This review is aimed to 1) highlight the potential of hydrogen production from algal biomass, 2) briefly discuss the production process, 3) explore the current and upcoming strategies for enhancing the hydrogen production from algal biomasses, and 4) identify the research gaps associated with the process and recommendations for overcoming the challenges. This review intends to summarize the current opportunities and challenges of hydrogen production from algal biomass, understanding which will be helpful in the advancement of algal biohydrogen industries.

## 2. Biohydrogen production from algae

Feedstocks are abundant and diverse resource, ranging from agricultural and forestry waste to microorganisms and algae. The use of biomass for hydrogen production offers a promising alternative to conventional methods, which rely heavily on fossil fuels and emit significant greenhouse gases. However, there are a lot of factors that have be considered between the various generation of biomasses to produce biohydrogen in a more sustainable, cost-effective, and environmentally friendly way. This section discusses the challenges associated with the first- and second-generation biomasses and how algae can be a potential and reliable feedstock for biohydrogen production.

### 2.1. Challenges with different generations of biomass

The biomasses have been classified into three categories, which are: (i) first-generation biomass, (ii) second-generation biomasses, and (iii) third-generation biomasses. Crops intended for food and animal feed are often placed under the first generation. The crops with a relatively higher proportion of starch/sugar, such as potato, corn, wheat, barley, and oil-producing crops such as sunflower, palm, and soybeans, are typical examples of first-generation biomasses [91,14]. Owing to their high sugar and starch fraction, these biomasses could be readily fermentable by anaerobic microorganisms and produce a relatively higher biohydrogen yield. Despite their higher hydrogen production ability, the main obstacle in adopting their use in hydrogen production is the food vs fuel dilemma. Their application in energy production requires a considerable amount of water, arable land, and fertilizers, making their production economically unappealing [100]. Further, their use for fuel production would create a potential food shortage and is unnecessarily burdensome to food security across the globe.

The lignocellulosic biomasses represent the second category. These are comprised of agricultural and municipal wastes, crop, and forest residues. Second-generation biomasses are generally a by-product of various industries and natural ecosystems and hence are quite abundant and economically cheap, moreover, they don't interfere with food production [91]. However, certain limitations exist in their biochemical composition, which impedes their economic feasibility for hydrogen production. These biomasses are constituted of lignin, cellulose, and hemicellulose [88], which are complex polymeric carbohydrates that are difficult to ferment by the anaerobic microbes. Hence, pretreatment strategies should be incorporated when lignocellulosic biomasses are used. Bhatia et al. [10] discussed the recent development in pretreatment technologies for breaking these complex polymers into fermentable form (monomers) specific pre-treatment procedures are required, which makes the process cost intensive. The processes also produce by products such as furfuran aldehydes, acids and phenolics that can inhibit the growth of algae and other microbes. Hence, the application of second-generation biomass for hydrogen production is not economically feasible with the currently adopted pre-treatment techniques [40].

### 2.2. Potential of algal biomass

Algae are classified under third-generation biomasses. Owing to their high carbohydrate content, algae are being used extensively for producing hydrogen. They subdue the challenges possessed by the first and second generations of biomasses [7]. Unlike the first generation, they could be cultivated in higher quantities and do not require arable land, fertilizers, and generally, be produced in a limited period. The algae do not have lignin content so the cost-intensive pre-treatment steps can be omitted. Algae could be classified depending on different parameters [17]. The number of cells could be unicellular (i.e., blue-green algae) or multicellular (i.e., green, brown, and red algae) as well as based on their morphology and size, they can be grouped as microalgae and macroalgae. Generally, this is the standard and widely followed classification; however, algal classification can be further extended as per their dependency on carbon sources. Algae can utilize $CO_2$ (autotrophic), organic carbon (heterotrophic), or both for their growth depending on the conditions (mixotrophic). Algae stores carbon as lipid, starch, and cellulose inside them. The primary source of carbohydrates is starch and cellulose, which can be consumed as a carbon source (by the microbes) during the fermentation [15].

#### 2.2.1. Macroalgae

The macroalgae enhance the biodiversity of the marine ecosystem by contributing to the prevention of pollution and eutrophication. Depending on the presence of pigments, macroalgae could be classified into three major algal categories; green (Chlorophyta), brown (Phaeophyceae) and red (Rhodophyta) [58]. The hydrogen production ability of macroalgae is mainly due to carbohydrates; the proportion of constitutive components varies between the species. For instance, brown, green, and red algae have a carbohydrate proportion of 30–50, 30–60, and 25–60% of dry weight, respectively [51]. The constituents in the carbohydrates vary based on the biomass types. Kim et al. [44] have reviewed the potential of macroalgae through fermentative pathways for hydrogen production and concluded that selective sugar recovery is important for macroalgal biohydrogen production. Different species contain carbohydrates such as agar, floridean starch, carrageenan and glucans, mannitol, sorbitol, alginate, laminarin, sucrose, and sulphated polysaccharides [20,90].

It is crucial to note that the dissimilarity observed in the chemical composition among the different groups could be due to the place of origin and harvested season [39]. For instance, a higher profile of carbohydrates is observed in the summer and autumn compared to other seasons [21]. However, certain exceptions, i.e., *Acanthophora muscoides* (red algae) and *Dictoyota ciliolate* (Brown algae), show a higher carbohydrate percentage in winter. The carbohydrate percentage in algae increases with the day temperature [93]. So, as explained briefly, the carbohydrate portion of lignin-free algae is pivotal for hydrogen

production. The polysaccharide present in the algae needs to undergo hydrolysis to produce fermentable monomeric sugar such as glucose and galactose. Several researchers have identified that species with a higher carbohydrate intake (particularly D-galactose, anhydrogalactose, cellular mannunoric, and gulunoric acid blocks) have a higher potential to produce hydrogen [51]. Certain red algae species such as *Gelidium, Gracilaria*, and *Euchema* have a higher proportion of fermentable sugar such as glucose and galactose; hence these are attractive options for hydrogen production [92].

#### 2.2.2. Microalgae

Microalgae are autotrophic microorganisms that capture carbon dioxide from the atmosphere and convert them to biomasses via the photosynthesis process. Microalgae do not involve complex metabolic processes and lack heterotrophic tissue. This enables them to harness the energy and convert it more efficiently to biofuels, making an excellent option for biofuel production [65,62]. Microalgal energy content is immensely appealing; its carbohydrate, lipid, and protein have a net energy content of 17, 34, and 24 KJ $g^{-1}$, respectively [42]. As the prime focus of this manuscript is on hydrogen production, the discussion is limited to carbohydrates. Carbohydrates are the starting products of photosynthesis and execute a crucial role in microalgae's structural and functional development. Numerous species with different biochemical compositions are categorized under the microalgae group, but the diversified classes of microalgae produce distinct polysaccharides. Chrysolaminarin is one of the common starches (with a linear polymer of β(1,3) and β(1,6) linked glucose units) found in pyrenoids of chloroplasts in major microalgal species, serve as fermentable sugar substrate to the anaerobic microbes that subsequently produces hydrogen [17].

The selection of species from the diversified microalgal community is imperative for biohydrogen production as it is essential, viable, and sustenance to algal biofuel industries. High carbohydrate content, photosynthetic efficiency, growth rate, resistance to environmental stress, and better productivity are essential characteristics that must be considered while selecting a suitable strain [26].

## 3. Current technologies for biohydrogen production from algae and its bottlenecks

### 3.1. Dark fermentation

In dark fermentation, the algae broken down before being combined with water to create a slurry. The slurry is then inoculated with microorganisms, such as yeast or bacteria, and fermented in a closed container. The microbes produce hydrogen gas as a by-product of decomposing algae. Microbes assimilate carbohydrates such as glucose, galactose, and mannose present in the algae and convert them to alcohol and carboxylic fatty acids, producing hydrogen as a by-product. The monomeric sugars are converted to phosphoenolpyruvate and pyruvate through glycolysis. Later, the redox of ferredoxin releases electrons for production of hydrogen from protons [78,70]. The hydrogen produced by the microbes helps vent out excess electrons produced during alcohol and fatty acid production. Because of its simplicity and higher yield, dark fermentation is one of the most used techniques for hydrogen production. The overall mechanism of the biohydrogen formation through fermentation process is given in Fig. 1. The choice of substrate and microbial inoculum plays a crucial role in controlling the yield of dark fermentations; however, operational factors such as reaction temperature, pH, and organic loading rates also influence the hydrogen yield [11]. Moreover, the bioreactor type and configuration also impact the fermentation process. The other products of dark fermentation, such as acetic acid, lactic acid, and butyric acid, can either be utilized as the substrate for other fermentation processes or they can be purified and commercialized [51]. The effect of experimental conditions and the impact of pre-treatment on fermentative biohydrogen production of microalgae and macroalgae have been summarised in Tables 1 and 2, respectively.

### 3.2. Photo-fermentation

In photo-fermentation, microbes produce hydrogen gas as a by-product of decomposing algae in the presence of light. The organic wastes are broken down with the help of ATP to produce carbon dioxide

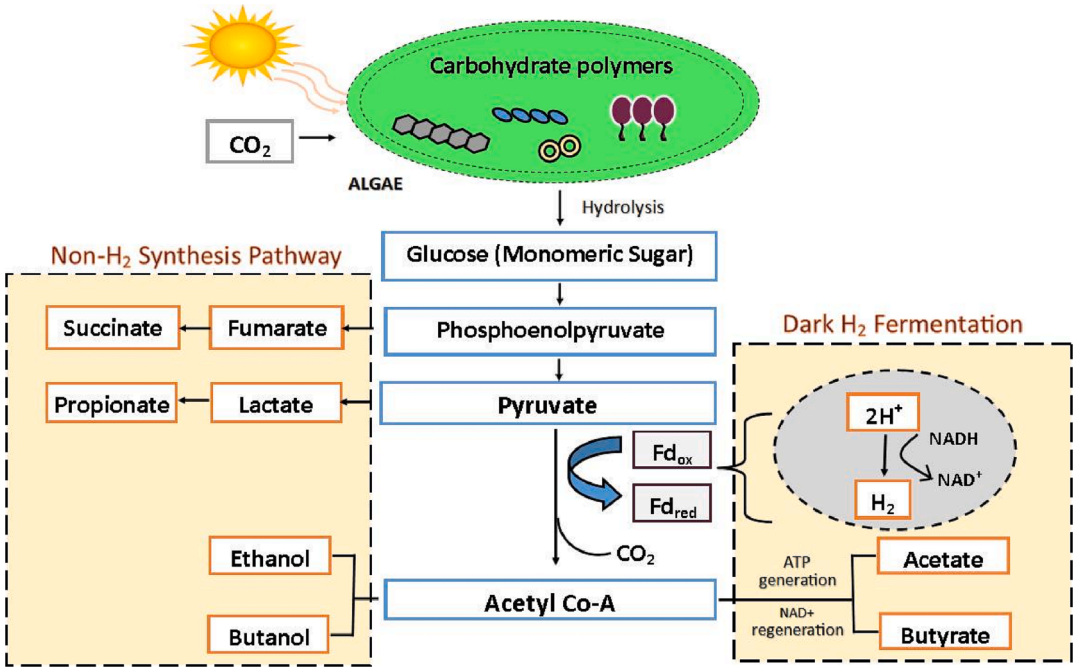


**Fig. 1.** Mechanism of hydrogen production from algae via dark fermentation pathway ($CO_2$: Carbon Dioxide; $H^+$: Hydrogen Ion; $H_2$: Hydrogen; Acetyl CoA: Acetyl Coenzyme A; $Fd_{ox}$: Oxidized Ferredoxin; $Fd_{red}$: Reduced Ferredoxin; ATP: Adenosine Triphosphate; NAD: Nicotinamide Adenine Dinucleotide; NADH: Nicotinamide Adenine Dinucleotide Hydride).

**Table 1**
Hydrogen production from microalgae by fermentative pathways.

| Algal species | Bioreactor used | Algal growth conditions | Carbohydrate (%) | Pre-treatment | Inoculum | Reactor used in biohydrogen reaction | Hydrogen production experimental condition | Mechanism involved | Biohydrogen yield | Hydrogen production rate | References |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Chlorella vulgaris* and *Clostridium* sp. | - (Laboratory cultivated) | BG-11 media | – | $FeCl_3$ for nanoparticle extraction | – | 50 mL serum vials | Nitrogen purged; pH 5.8; 130 rpm agitation speed; 35 °C temperature | Dark fermentation | 192 ± 0.8 mL $H_2$ $g^{-1}$ glucose | 8.54 mL $g^{-1}$ $d^{-1}$ | Yildirim et al., [102] |
| *Chlorella vulgaris* | - (Laboratory cultivated) | 10% landfill leachate | 26.4 | Autoclave with 1% $H_2SO_4$ | – | 150 mL glass fermentor | Nitrogen purged; 35 °C temperature | Dark fermentation | 50.91 mL $g^{-1}$ VS | 6.33 mL $g^{-1}$ VS $h^{-1}$ | Feng et al., [29] |
| Microalgae consortia (*Scenedesmus* and *Chlorella*) | Plastic bag as photo reactor | – | – | Electrolysis<br>Autoclave<br>Ultrasonication<br>No treatment | Clostridia species | 100 mL serum bottle | Strict anaerobic condition; 120 rpm agitation speed; 37 ± 0.1 °C temperature | Dark fermentation | –<br>~24 mL $g^{-1}$<br>~34 mL $g^{-1}$<br>9.5 ± 0.0 mL $g^{-1}$ | – | Kumar et al., [108] |
| Microalgae consortia | Plastic bag as photo-reactor | – | – | – | Mesophilic inoculum | 100 mL serum bottle | Strict anaerobic condition; pH 5.5; 150 rpm agitation speed; 35 °C temperature | Dark fermentation | < 2 mL | – | Kumar et al., [109] |
| | | | | | Thermophilic inoculum | | Strict anaerobic condition; pH 5.5; 150 rpm agitation speed; 55 °C temperature | | – | 90 mL $L^{-1}$ $d^{-1}$ | |
| Taihu blue algae | - (Harvested from lake) | - (Open lake) | – | Thermal<br>Acid<br>Alkaline<br>No treatment | Phototrophic bacteria from municipal sludge | 500 mL glass bottle | pH 7.5 ± 0.2; 150 rpm agitation speed; 35 °C temperature | Dark fermentation | 113 ± 5 mL $H_2$ $g^{-1}$ VS<br>~45 mL $H_2$ $g^{-1}$ VS<br>94 ± 4 mL $H_2$ $g^{-1}$ VS<br>28 ± 2 mL $H_2$ $g^{-1}$ VS | – | Cai et al., [103] |
| *Microcystis wesenbergii* and *Microcystis aeruginosaare* | - (Harvested from lake) | - (Open lake) | – | Hydrothermal heating with a dilute acid<br>Steam heating with dilute acid<br>No treatment | *Clostridium butyricum* from anaerobic sludge | 417 mL glass reactors | pH 6.0 ± 0.1; 150 rpm agitation speed; 35 °C temperature; 72 h in a water bath | Dark fermentation | 24.96 mL $g^{-1}$ TVS<br>18.63 mL $g^{-1}$ TVS<br>1 mL $g^{-1}$ TVS | – | Cheng et al., [104] |
| Microcystis (*Microcystis wesenbergii* and *Microcystis aeruginosaare*) | - (Cultivated in lake) | – | 11.64 | Hydrothermal treatment with different concentrations of $H_2SO_4$ (1 %)<br>2 % | Activated sludge containing hydrogen producing microbes | 417 mL glass reactors | pH 6.0 ± 0.1; 150 rpm agitation speed; 35.0 ± 1.0 °C temperature | Dark fermentation | 1.92 ± 0.11 mL $g^{-1}$ TVS<br>14.53 ± 0.62 mL $g^{-1}$ TVS | 0.16 ± 0.02 mL $g^{-1}$ TVS $h^{-1}$<br>0.90 ± 0.06 mL $g^{-1}$ TVS $h^{-1}$ | Cheng et al., [105] |

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**Table 1** (*continued*)

| Algal species | Bioreactor used | Algal growth conditions | Carbohydrate (%) | Pre-treatment | Inoculum | Reactor used in biohydrogen reaction | Hydrogen production experimental condition | Mechanism involved | Biohydrogen yield | Hydrogen production rate | References |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 4 % | | | | | 37.37 ± 1.25 mL $g^{-1}$ TVS | 2.01 ± 0.08 mL $g^{-1}$ TVS $h^{-1}$ | |
| | | | | 8 % | | | | | 31.44 ± 0.22 mL $g^{-1}$ TVS | 1.86 ± 0.04 mL $g^{-1}$ TVS $h^{-1}$ | |
| | | | | No treatment | | | | | 0.26 ± 0.04 mL $g^{-1}$ TVS | 0.02 ± 0.01 mL $g^{-1}$ TVS $h^{-1}$ | |
| Mixed microalgal cultivation | Vertical transparent plastic bag | Different concentrations of swine manure as culture medium (2.5 g $L^{-1}$) | 1128 ± 42 mg $L^{-1}$ | Ultrasonication and enzymatic treatment | Heat-treated anaerobic digestive sludge | 150 mL fermentation bottle | Strict anaerobic condition; pH 7.0; 150 rpm agitation speed 35 ± 0.1 °C temperature | Dark fermentation | 55.96 mL $g^{-1}$ VS | 3.4 mL $g^{-1}$ VS $h^{-1}$ | Kumar et al., [108] |
| | | 5 g $L^{-1}$ | 1140 ± 6 mg $L^{-1}$ | | | | | | 58.53 mL $g^{-1}$ VS | 4.18 mL $g^{-1}$ VS $h^{-1}$ | |
| | | 7.5 g $L^{-1}$ | 1006 ± 6 mg $L^{-1}$ | | | | | | 56.51 mL $g^{-1}$ VS | 4.46 mL $g^{-1}$ VS $h^{-1}$ | |
| | | 10 g $L^{-1}$ | 1090 ± 10 mg $L^{-1}$ | | | | | | 51.2 mL $g^{-1}$ VS | 3.76 mL $g^{-1}$ VS $h^{-1}$ | |
| *Chlorella* sp. | – | – | 29.2 | Scarification and fermentation (SSF) | Anaerobic granules collected from anaerobic digester | 600 mL serum bottles | pH 6.6; 35 ± 2 °C temperature; Varied biomass concentration (20 g $L^{-1}$) | Dark fermentation | 170 mL $g^{-1}$ VS | 1.6 mL g-$VS^{-1}h^{-1}$ | Giang et al., [106] |
| | | | | Pre-hydrolysis with SSF | | | 10 g $L^{-1}$ | | 132 ± 4 mL $g^{-1}$ VS | – | |
| | | | | | | | 20 g $L^{-1}$ | | 172 ± 11 mL $g^{-1}$ VS | 2.4 mL $g^{-1}$ VS $h^{-1}$ | |
| | | | | | | | 30 g $L^{-1}$ | | 108 ± 6 mL $g^{-1}$ VS | – | |
| | | | | | | | 40 g $L^{-1}$ | | 87 ± 8 mL $g^{-1}$ VS | – | |
| | | | | | | | 50 g $L^{-1}$ | | 68 ± 3 mL $g^{-1}$ VS | – | |
| *Spirogyra* sp. | Glass column bubble photo bioreactor, polyethylene plastic sleeve, open raceway pond (scaled up) | – | 27 | Homogenisation and bead beating | *C. butyricum* DSM 10702 | 120 mL serum bottle | 150 rpm agitation speed; 37 °C temperature; 144 h incubation | Dark fermentation | 47.4 ± 4.3 mL $H_2$ $g^{-1}$ dry wt. | – | Pinto et al., [71] |
| | | | | Acid hydrolysis | | | | | 146.3 ± 12.5 mL $H_2$ $g^{-1}$ dry wt. | | |
| | | | | No treatment | | | | | 54.3 ± 9.7 mL $H_2$ $g^{-1}$ dry wt. | | |
| *A. platensis* | 250 mL triangular flasks | Air bubbling; 15% $CO_2$ | 25 | Dilute acid and steam heating | *Clostridium butyricum* | 300 mL glass bottles | pH 6; 35.0 ± 1.0 °C temperature; 3 d incubation | Dark fermentation | 42 mL $g^{-1}$ VS | 4.4 mL $g^{-1}$ VS $h^{-1}$ | Ding et al., [107] |
| | | 15% $CO_2$; 0.5 M NaCl stressed | 53 | | | | | | 96 mL $g^{-1}$ VS | 5.4 mL $g^{-1}$ VS $h^{-1}$ | |
| | | 15% $CO_2$ | 25 | | *Rhodopseudomonas palustris* | | pH 7; 30.0 ± 0.5 °C temperature; 4 | Photo fermentation | 184 mL $g^{-1}$ VS | 5.3 mL $g^{-1}$ VS $h^{-1}$ | |

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**Table 1** (*continued*)

| Algal species | Bioreactor used | Algal growth conditions | Carbohydrate (%) | Pre-treatment | Inoculum | Reactor used in biohydrogen reaction | Hydrogen production experimental condition | Mechanism involved | Biohydrogen yield | Hydrogen production rate | References |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 15% CO2; 0.5 M NaCl stressed | 53 | | | | d illuminated incubation | after dark fermentation | 333 mL $g^{-1}$ VS | 7.8 mL $g^{-1}$ VS $h^{-1}$ | |
| Mixed microalgae consortia (*Scenedesmus* followed by *Chlorella* sp.) | Plastic bags | 8000–9000 lx light intensity; 12:12 h photoperiod; 23 ± 1 °C temperature | 6.5 ± 1.5 | Electrolysis pre-treatment<br>Autoclave<br><br>Ultra-sonication<br><br>No treatment | Endospore forming Clostridia species | 100 mL serum vials | 120 rpm agitation speed; 37 ± 0.1 °C temperature | Dark fermentation | 37.7 ± 0.4 mL $g^{-1}$ VS<br>29.5 ± 0.8 mL $g^{-1}$ VS<br>36.8 ± 0.7 mL $g^{-1}$ VS<br>9.5 ± 0.0 mL $g^{-1}$ VS | 236 ± 14 mL $L^{-1}$ $d^{-1}$<br>128 ± 11 mL $L^{-1}$ $d^{-1}$<br>160 ± 7 mL $L^{-1}$ $d^{-1}$<br>64 ± 5 mL $L^{-1}$ $d^{-1}$ | Kumar et al., [110] |
| *Gelidium amansii* | – | - (Procured) | 63.7 | – | Spore-forming hydrogen producing bacteria (granular digester sludge with varied inoculum concentration (v/v) 3 %<br>6 %<br><br>9 %<br><br>12 %<br><br>15 % | 150 mL vial | pH 7.0; 150 rpm agitation speed; 35 ± 0.1 °C temperature | Dark fermentation | 43 mL $H_2$ $g^{-1}$ dry wt.<br>45 mL $H_2$ $g^{-1}$ dry wt.<br>39 mL $H_2$ $g^{-1}$ dry wt.<br>37 mL $H_2$ $g^{-1}$ dry wt.<br>38 mL $H_2$ $g^{-1}$ dry wt. | 476 mL $L^{-1}$ $d^{-1}$<br>520 mL $L^{-1}$ $d^{-1}$<br>454 mL $L^{-1}$ $d^{-1}$<br>457 mL $L^{-1}$ $d^{-1}$<br>420 mL $L^{-1}$ $d^{-1}$ | Sivagurunathan et al., [85] |
| *Lyngbya limnetica* | Conical flask | – | – | $Fe_3O_4$ nanoparticles | *Clostridium butyricum* | Glass bottle | pH 7.5; 40 °C temperature | Dark fermentation | 937 mL $L^{-1}$ | – | Srivastava et al., [89] |
| *Chlamydomomas reinhardtii* | – | Tris-acetate-phosphate (TAP) media; 24 °C temperature; 60–90 μmol photons $m^{-2}$ $s^{-1}$ PPFD | – | – | *Pseudomonas putida* 12,264<br>*Escherichia coli*<br>*Rhizobiumm etli* | 155 mL hermetically sealed bioreactor | 200 rpm agitation speed; 25 °C temperature; 50 PPFD illumination | Photo fermentation | 40.8 mL $L^{-1}$<br>35.1 mL $L^{-1}$<br>16.1 mL $L^{-1}$ | – | Fakhimi et al., [28] |

(VS: Volatile Solid; TVS: Total Volatile Solid; d: days; PPFD: Photosynthetic Photon Flux Density).

**Table 2**
Hydrogen production from macroalgae by fermentative pathways.

| Algal species | Bioreactor used | Carbohydrate (%) | Pre-treatment | Inoculum | Reactor used in biohydrogen reaction | Hydrogen production Experimental condition | Mechanism involved | Biohydrogen yield | Hydrogen production rate | References |
|---|---|---|---|---|---|---|---|---|---|---|
| *Laminaria japonica* | - (Purchased in dry form) | – | Gamma irradiation<br>Control<br>10 kGy<br>20 kGy<br>30 kGy | Anaerobic sludge from wastewater treatment plant | 150 mL Erlenmeyer flask | Anaerobic condition; pH 7.0; 150 rpm agitation speed; 36 °C temperature | Dark fermentation | 11.67 mL $g^{-1}$ TS<br>14.17 mL $g^{-1}$ TS<br>mL $g^{-1}$ TS<br>8.33 mL $g^{-1}$ TS | – | Chen et al., [18] |
| *Ulva reticulate* | - (Harvested from Jedda, Saudi Arabia) | 200 mg $L^{-1}$ | Control<br>Disperser<br>Combined acid disperser | Methanogenic bacteria | 1 L bottles | Anaerobic condition; pH 5; 72 h incubation with agitation at 37 °C temperature | Dark fermentation | 11 mL $g^{-1}$ COD<br>40.1 mL $g^{-1}$ COD<br>60.5 mL $g^{-1}$ COD | – | Dung et al., [24] |
| *Laminaria japonica* | – | – | Microwave 100 °C<br>120 °C<br>140 °C<br>160 °C<br>180 °C<br>No treatment | Ionized irradiated digested sludge | 150 mL conical flask | Anaerobic condition; pH 7.0; 150 rpm agitation speed; 36 °C temperature | Dark fermentation | 18 mL<br>25.92 mL<br>30.01 mL<br>38.08 mL<br>30.08 mL<br>19.93 mL | – | Yin et al., [117] |
| *Ulva reticulate* | - (Harvested from coastal region) | 38.2 | Microwave disintegration<br>Microwave disintegration and $H_2O_2$<br>Microwave disintegration and alkaline treatment<br>No treatment | Digested sludge from an anaerobic digester | 300 mL serum bottle | 130 rpm agitation speed; 37 °C temperature incubation | Dark fermentation | 72.5 mL $g^{-1}$ COD<br>77.6 mL $g^{-1}$ COD<br>87.5 mL $g^{-1}$ COD<br>10.3 mL $g^{-1}$ COD | – | Kumar et al., [109] |
| *Ulva* sp. | – | 50.34 | –<br>Acid hydrolysis | *Clostridium butyricum* CGS5<br>*Clostridium butyricum* CGS5<br>*Clostridium pasteurianum* CH4 | Serum bottle | pH 7; 200 rpm agitation speed; 37 °C temperature<br>Varied substrate concentration<br>4 g RS $L^{-1}$<br>8 g RS $L^{-1}$<br>12 g RS $L^{-1}$<br>16 g RS $L^{-1}$<br>pH 5<br>pH 5.5<br>pH 6<br>Control experiment | Dark fermentation | 0.2 mol $H_2$ $mole^{-1}$ RS<br>1.01 mol $H_2$ $mole^{-1}$ RS<br>0.14 mol $H_2$ $mole^{-1}$ RS<br>0.4 mol $H_2$ $mole^{-1}$ RS<br>0.65 mol $H_2$ $mole^{-1}$ RS<br>1.0 mol $H_2$ $mole^{-1}$ RS<br>0.7 mol $H_2$ $mole^{-1}$ RS<br>0.7 mol $H_2$ $mole^{-1}$ RS<br>1.39 mol $H_2$ $mole^{-1}$ RS<br>0.9 mol $H_2$ $mole^{-1}$ RS<br>0.9 mol $H_2$ $mole^{-1}$ RS | 1.74 mL $L^{-1}h^{-1}$<br>73.2 mL $L^{-1}h^{-1}$<br>5.41 mL $L^{-1}h^{-1}$<br>25 mL $L^{-1}h^{-1}$<br>45 mL $L^{-1}h^{-1}$<br>75.3 mL $L^{-1}h^{-1}$<br>45 mL $L^{-1}h^{-1}$<br>80 mL $L^{-1}h^{-1}$<br>208.3 mL $L^{-1}h^{-1}$<br>140 mL $L^{-1}h^{-1}$<br>75 mL $L^{-1}h^{-1}$ | Margareta et al., [61] |

(*continued on next page*)

**Table 2** (*continued*)

| Algal species | Bioreactor used | Carbohydrate (%) | Pre-treatment | Inoculum | Reactor used in biohydrogen reaction | Hydrogen production Experimental condition | Mechanism involved | Biohydrogen yield | Hydrogen production rate | References |
|---|---|---|---|---|---|---|---|---|---|---|
| *L. japonica* | – | – | No treatment | Anaerobic sludge | 150 mL erlenmeyer flasks | Anaerobic condition; pH:7; 120 rpm agitation speed; 36 °C temperature | Dark fermentation | 15.09 mL $g^{-1}$ TS | 1.82 mL $h^{-1}$ | Yin et al., [118] |
| | | | Microwave | | | | | 18.12 mL $g^{-1}$ TS | 1.98 mL $h^{-1}$ | |
| | | | Microwave + 0.5 % $H_2SO_4$ | | | | | 22.16 mL $g^{-1}$ TS | 2.62 mL $h^{-1}$ | |
| | | | Microwave + 1.0 % $H_2SO_4$ | | | | | 28 mL $g^{-1}$ TS | 2.81 mL $h^{-1}$ | |
| | | | Microwave + 2.0 % $H_2SO_4$ | | | | | 14.99 mL $g^{-1}$ TS | 1.78 mL $h^{-1}$ | |
| *K. alvarezzi* | – | – | Enzymatic treatment | *C. beijerinckii* Br21 | 50 mL vials | – | Fermentation | 23.8 mmol of $H_2$ $g^{-1}$ | – | Rodrigues et al.,[119] |
| *Gelidium amansii* | - (Collected from littoral zone) | 84.2 | – | Heat treated inoculum | 500 mL serum bottle | Anaerobic condition; pH 7 | Dark fermentation | 54.6 mL $g^{-1}$ TS | 0.33 L $L^{-1}$ $d^{-1}$ | Nguyen et al., [116] |
| | | | | | 300 mL glass bottle was used as a single chamber MEC | | MEC | 203.2 mL $g^{-1}$ TS | 0.34 L $L^{-1}$ $d^{-1}$ | |
| | | | | | | | Dark fermentation, MEC | 403.5 mL $g^{-1}$ TS | 0.51 L $L^{-1}$ $d^{-1}$ | |
| | | | | | | | Simultaneous dark fermentation and MEC | 438.7 mL $g^{-1}$ TS | 0.64 L $L^{-1}$ $d^{-1}$ | |
| *Laminaria digitata* | – | – | No treatment | – | AMPTSII systems | – | Dark fermentation | 35 mL $g^{-1}$ VS | – | Ding et al., [107] |
| | | | Hydrothermal | Liquid digestate of an industrial digester treating food waste | | 60 rpm agitation speed; 35 °C temperature | | 45 mL $g^{-1}$ VS | | |
| | | | Hydrothermal and acid | | | | | 57 mL $g^{-1}$ VS | | |
| | | | Enzymolysis | | | | | 42 mL $g^{-1}$ VS | | |
| | | | Hydrothermal and enzymolysis | | | | | 55 mL $g^{-1}$ VS | | |
| | | | Hydrothermal, acid and enzymolysis | | | | | 58 mL $g^{-1}$ VS | | |
| *Ulva reticulate* | - (Collected from coastal area) | 37.23 | No treatment | Anaerobic digester (*Clostridium cellulosi*, *Clostridium tyrobutyricum* and *Eubacteria*) | 300 mL serum bottles | pH 5.5; 130 rpm agitation speed; 37 °C temperature | Dark fermentation | 12 mL $H_2$ $g^{-1}$ COD | – | Kumar et al., [52] |
| | | | Microwave | | | | | 72.5 mL $H_2$ $g^{-1}$ COD | | |
| | | | $H_2O_2$ and microwave | | | | | 81.5 mL $H_2$ $g^{-1}$ COD | | |
| | | | $H_2O_2$, microwave and acid | | | | | 92.5 mL $H_2$ $g^{-1}$ COD | | |
| *Chaetomorpha antennina* | - (Collected from coastal area) | 24 | No treatment | Anaerobic digester (spore forming acidogenic hydrogen producing microbes) | 300 mL serum bottles | 130 rpm agitation speed; 37 °C temperature incubation | Dark Fermentation | 19 mL $H_2$ $g^{-1}$ COD | – | Kumar et al., [120] |
| | | | Microwave disintegration | | | | | 63 mL $H_2$ $g^{-1}$ COD | | |
| | | | Surfactant aided microwave disintegration | | | | | 74.5 mL $H_2$ $g^{-1}$ COD | | |

(RS: Reduced Sugar; TS: Total solid; MEC: Microbial electrolysis Cell; VS: Volatile Solid; COD: Chemical Oxygen Demand).

and hydrogen. Microorganisms are cultivated in an appropriate growth medium, such as seawater or wastewater, utilizing sunlight or artificial light as the energy source [94]. To enhance microbe growth and hydrogen production, the growth parameters, such as pH, temperature, and nutrition levels, are meticulously managed. The microbes involved in this process can switch between photo-fermentation and photosynthesis depending on the experimental conditions. It was discovered that the presence of carbon-rich substrate promotes photosynthesis, and nitrogen-deficient conditions are ideal for photo fermentation. Hydrogen gas is produced at low concentrations and is often combined with oxygen and carbon dioxide [70,38].

### 3.3. Bio-photolysis

Bio-photolysis is the utilization of microorganisms, such as algae or cyanobacteria, to generate hydrogen gas via photosynthesis. The process by which the reductants produced after splitting water are directed towards hydrogen production rather than the Calvin cycle distinguishes bio-photolysis from photo-fermentation. At the thylakoid membrane of algae and cyanobacteria, where the light-absorbing pigments are grouped into two functional arrays, photosystems (PS) I and II, photochemical oxidation occurs. When the system absorbs excess energy, certain microbes transfer electrons to hydrogenase and convert $H^+$ ions into hydrogen gas [5]. Based on the steps involved in hydrogen production, bio-photolysis can be classified as direct or indirect photolysis. In cases of direct photolysis, PS II absorbs light and decomposes water molecules, leading to the generation of hydrogen gas (Fig. 2). In indirect photolysis, light energy is utilized to generate biomass through chemical reactions, and then these biomasses are digested by hydrogenase and nitrogenase enzymes to produce hydrogen in the presence of water and light (Fig. 2) [70]. The effect of experimental conditions and the impact of different stresses on photolytic biohydrogen production have been summarised in Table 3. The drawbacks of the existing biohydrogen production techniques from algae are shown in Table 4.

## 4. Strategies to improve algal biohydrogen production

### 4.1. Pre-treatments

To ensure the feasibility of alga biohydrogen production, access to the carbohydrate region of the fermentable sugar present in the complex biomass is crucial. Microalgae and macroalgae do not comprise lignin and hence do not require pre-treatments like other conventional biomass. However, DF's efficiency depends on several parameters like the substrate composition, pH, temperature, inoculum type, and hydraulic retention time (HRT). In certain situations, only half of the volatile solids will be available for consumption in DF, which may not satisfy the theoretical stoichiometric of 670 mL $H_2$ $g^{-1}$ VS. To overcome this constraint, several cell lysis techniques have been devised. These techniques implemented after harvesting the algae could be categorized into four categories: physical/mechanical, chemical, biological, physicochemical, and thermal [13,64]. Physical or mechanical methods involve direct cell disruption or degeneration of the targeted biomasses. This involves mechanical processes such as ultrasonication, bead beating, electroporation, high-pressure homogenization, extrusion, and pyrolysis. The cells could also be disrupted chemically by treating them with acids, alkalis, and other surfactants [83]. Some reported chemical treatment methods are ozonolysis, acid/alkali treatment, organosol process, and ionic liquid-based treatment [61,71]. For instance, acid-catalyzed treatment of defatted algal biomass results in more solubilized reduced sugar, producing higher hydrogen [49]. Chemical treatment is less energy-intensive than physical treatment since it does not require much heat or electricity. Algae have long aliphatic unsaturated hydroxy fatty acid monomers termed algaenans. These non-hydrolyzable biopolymers consist of liner polyester chains and are exceedingly resistive. Hence, algal biomasses are treated with enzymes such as alcalase, cellulose, lysozyme, papain, pectinase, neutrase, and snaillase. These enzymes can degrade the cell wall and allow the separation of lipids. Physicochemical treatment involves biomass exposure to steam/ammonia/$CO_2$, wet oxidation, or microwave irradiation [51]. The last category, thermal treatment includes use of high temperature, autoclaving, freeze drying/thawing and steam explosion [64].

A combination of the above methods is generally implemented to

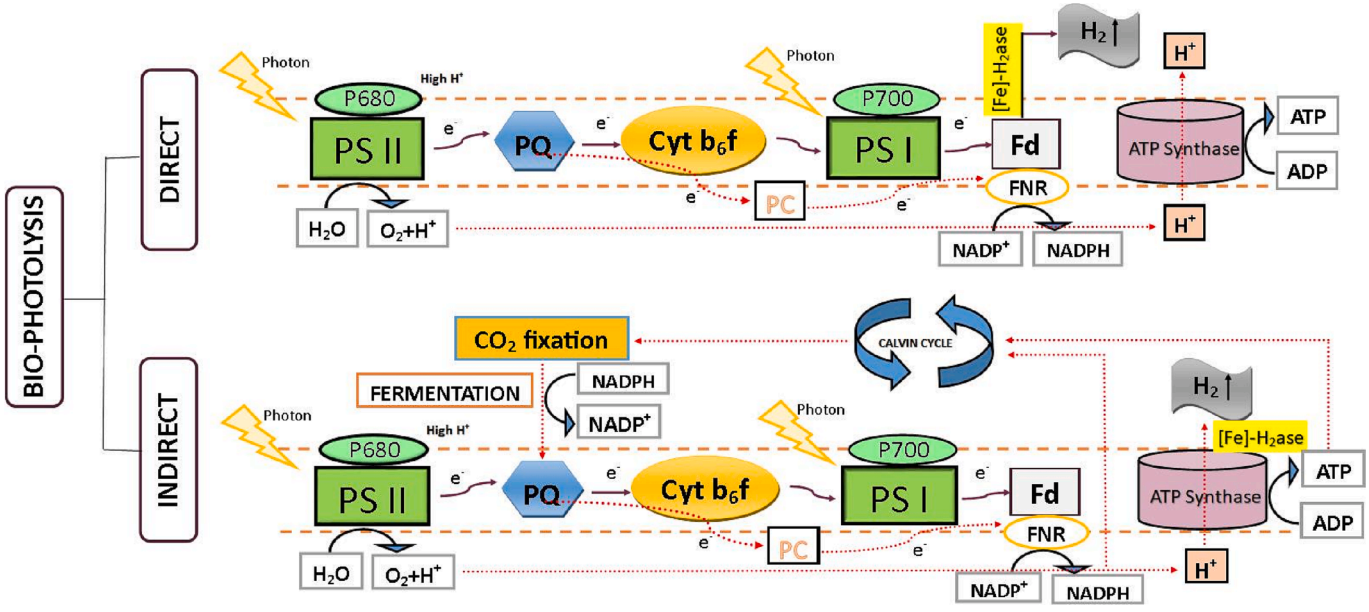


**Fig. 2.** Mechanism of hydrogen production from algae via bio-photolysis pathway (PSI: PhotoSystem I; PSII: PhotoSystem II; $H_2O$: Water; $O_2$: Oxygen; PQ: Plastoquinone; PC: Plastocyanin; Cyt $b_6f$: Cytochrome $b_6f$ complex; Fd: Ferredoxin; FNR: Ferredoxin-NADP(H) Reductase; [Fe]-$H_2$ase: Iron-Hydrogenase; ATP: Adenosine Triphosphate; ADP: Adenosine Di-Phosphate; NAD: Nicotinamide Adenine Dinucleotide; NADH: Nicotinamide Adenine Dinucleotide Hydride; NADPH: Nicotinamide Adenine Dinucleotide Phosphate Hydride).

**Table 3**
Hydrogen production from algae by bio-photolysis pathways.

| Algal species | Bioreactor used | Algal growth condition | Additional growth conditions | Biohydrogen yield | Hydrogen production rate | References |
|---|---|---|---|---|---|---|
| *Spirulina platensis* | Photobioreactor with hydrogen production, storage and algae recovery | – | Varied sulphur concentration | 66.32 mL $g^{-1}$ VS | – | Nazarpour et al., [66] |
| *Anabaena* sp. (UTEX 1448) | Duran glass bottles as photobioreactor | Growth medium: BG-11; 1.05 kg $m^{-3}$ of glucose (hydrogen production stage)<br>pH: 9.2<br>Temperature: 24 °C<br>Light intensity: 4440 lx | Nitrogen deprivation | 6.277 ± 0.41 mmol $L^{-1}$ | 42.55 ± 2.14 μmol $L^{-1}$ $h^{-1}$ | Vargas et al., [113] |
| | | pH: 10.2<br>Temperature: 32 °C<br>Light intensity: 2220 lx | | 9.73 ± 0.41 mmol $L^{-1}$ | 67.07 ± 2.14 μmol $L^{-1}$ $h^{-1}$ | |
| *Anabaena* sp. (UTEX 1448) | BOD incubator with photoperiod and temperature control | Growth medium: 2.1 g $L^{-1}$ of glucose pH: 10.2<br>Temperature: 32 ± 0.1 °C<br>Light intensity: 2200 lx | Ammonium deprivation (Exponential phase) | 13.36 ± 1.83 mmol $H_2$ mg Chl $a^{-1}$ | – | Vargas et al., [115] |
| | | | Ammonium addition (Exponential phase) | 11.19 ± 1.13 mmol $H_2$ mg Chl $a^{-1}$ | | |
| | | | Ammonium deprivation (Stationary phase) | 2.90 ± 0.67 mmol $H_2$ mg Chl $a^{-1}$ | | |
| | | | Ammonium addition (Stationary phase) | 1.25 ± 0.33 mmol $H_2$ mg Chl $a^{-1}$ | | |
| *Chlorella pyrenoidosa* | – | Growth medium: TAP<br>pH: 7.0<br>Temperature: 28 °C<br>Light intensity: 90 ± 10 μE $m^{-2}$ $s^{-1}$ | Sulphur deprivation without glucose | 65.5 mL $L^{-1}$ | – | Liu et al., [111] |
| | | | Sulphur deprivation with glucose (0.7 g $L^{-1}$) | 121.1 mL $L^{-1}$ | | |
| *Chlamydomonas reinhardtii* (CC425) | 500 mL duran glass bottles as photobioreactors | Growth medium: Axenic TAP<br>pH: 7.2<br>Temperature: 24 °C<br>Light intensity: 60 μmol photons $m^{-2}$ $s^{-1}$ | – | 61.10 ± 7.16 mL $L^{-1}$ of culture | 17.02 ± 3.83 μmol $L^{-1}h^{-1}$ | Vargas et al., [114] |
| *Chlamydomonas moewusii* (SAG 24.91) | | Photoperiod: 12:12 h | | 20.67 ± 3.10 mL $^{-1}$ of culture | 5.12 ± 0.37 μmol $L^{-1}h^{-1}$ | |
| *Scenedesmus obliquus* | Hermitically sealed bottles (diameter 5 cm, height 9.5 cm) | Growth medium: Mixotrophic culture medium (5 g $L^{-1}$ glucose)<br>Temperature: 30 °C<br>Light intensity: 100 μE $m^{-2}$ $s^{-1}$ | 50 % potassium<br>25 % potassium | 1 mL<br>1.1 mL | – | Papazi et al., [112] |
| | | | 10 % potassium<br>1 % potassium<br>No potassium<br>Control | 1.2 mL<br>1.4 mL<br>1.9 mL<br>0.9 mL | | |
| *Chlamydomonas reinhardtii* (CC124) | 15 mL gas-tight vials | Growth medium: HSM<br>pH:<br>Temperature: 25 °C<br>Light intensity: 60 μE $m^{-2}$ $s^{-1}$<br>Agitation speed: 120 rpm | Autotrophic medium | No emission | No emission | Khosravitabar & Hippler, [41] |
| | | Growth medium: HSM | Autotrophic medium and oxysorb | 10.7 ± 2.1 μmol mg $chl^{-1}$ | 0.11 ± 0.003 μmol mg $chl^{-1}h^{-1}$ (max. rate) | |
| | | Growth medium: TAP | Photoheterotrophic medium | 25.6 ± 4.3 μmol mg $chl^{-1}$ | 0.93 ± 0.01 μmol mg $chl^{-1}h^{-1}$ | |
| | | | Photoheterotrophic medium and oxysorb | 139.6 ± 3.2 μmol mg $chl^{-1}$ | 2.05 ± 0.08 μmol mg $chl^{-1}h^{-1}$ | |
| | | Growth medium: HSM | Autotrophic medium | No emission | No emission | |
| | | | Autotrophic medium and oxysorb | 23.3 ± 3.2 μmol mg $chl^{-1}$ | 0.89 ± 0.02 μmol mg $chl^{-1}h^{-1}$ | |
| | | Growth medium: TAP | Photoheterotrophic medium | 250.0 ± 18.6 μmol mg $chl^{-1}$ | 3.35 ± 0.24 μmol mg $chl^{-1}h^{-1}$ | |
| | | | Photoheterotrophic medium and oxysorb | 460.0 ± 16.6 μmol mg $chl^{-1}$ | 4.1 ± 0.21 μmol mg $chl^{-1}h^{-1}$ | |
| *Chlamydomonas reinhardtii* FACHB-265 | 250 mL glass flask | Growth medium: TAP<br>Temperature: 30 °C<br>Light intensity: 50 μmol $m^{-2}$ $s^{-1}$<br>Photoperiod: 12:12 h | – | 120.0 mL $L^{-1}$ μmol mg $chl^{-1}$ | – | Ban et al., [5] |

(BOD: Biological Oxygen Demand; HSM: High Salt Minimal; TAP: Tris-acetate-phosphate).

**Table 4**
Bottlenecks of current technologies available for biohydrogen production from algae (Adapted from [12,54,70] and [50].

| Biohydrogen production techniques | Bottlenecks |
|---|---|
| Dark fermentation | • Low yield and hydrolysis efficiency<br>• Requires pre-treatment<br>• Low substrate conversion efficiency<br>• Production of $H_2$-$CO_2$ gas mixture<br>• Thermodynamic restrictions with increase in $H_2$ yield<br>• Reactor design |
| Photo-fermentation | • Low hydrolysis efficiency<br>• Requires nitrogen limited conditions<br>• Requires external light source<br>• Low light transformation efficiency<br>• Process limitation due to light/dark cycle utilization<br>• Reactor design<br>• Contamination and $H_2$ consumption by other organisms |
| Direct bio-photolysis | • Requires high energy<br>• Oxygen sensitivity<br>• Demand for designing and cost of photobioreactor<br>• Low light utilization efficiency<br>• Complex uncontrollable pathways<br>• Electron competition |
| Indirect bio-photolysis | • Long process<br>• Low light transformation efficiency<br>• Poor hydrogen production<br>• Low rate of hydrogen production<br>• Oxygen generation by PSII |

maximize the fermentation process's efficiency. One such widely used process is anaerobic solid-state fermentation (ASSF). It poses many advantages, including low energy consumption, reactor size, wastewater generation, and the hydrolyzing enzymes secreted extracellularly during the process, enhancing substrate breakdown for nutrient release. Lunprom et al. [59] used ASSF and DF by *Chlorella* sp. The recorded yield of biohydrogen was 16.2 mL $g^{-1}$ volatile solid, of which the contribution from ASSF was 11.6 mL $g^{-1}$ volatile solid. $H_2$ production was increased by 412% when *Athirospira maxima* were exposed to boiling, bead milling and ultrasonic combined with enzymatic pre-treatments. Sambusiti et al. [78] conducted a study, in which an 87% increase in $H_2$ was achieved by pre-treating microalgae *Chlorella pyrenoidosa* with steam and microwave combined with dilute acid. Moreover, algal biomasses pre-treated with microwave and hydrogen peroxide under an alkaline condition yield higher hydrogen than those treated under an acidic environment. Because under alkaline condition, microwave treatment induces higher decomposition of hydrogen peroxide, resulting in more $OH^-$ radical production. The latter promotes solubilization of COD and generate higher hydrogen [51]. Other pre-treatment methods often reported in the literature are pH shock, heat shock, loading shock, fluctuating redox potential, aeration, and a combination of these methods. Heat shock and pH control can also eradicate methanogens (considered prime hydrogen-consuming microorganisms). Acid-alkali treatments ($pH < 6.3$ or $pH > 7.8$) are also efficient alternates for subsiding methanogens [49].

Although the treatment of biomass enhances algae's biohydrogen production ability, it also faces certain constraints like the formation of inhibitors during the process [35]. The treatments could induce glucose degradation via side-chain reactions and form a toxic compound called hydroxymethylfurfural (5-HMF), which can impair cellular growth and respiration [35]. The generation of inhibitors lessens the hydrogen production efficiency and poses a threat to the fermenter. One of the critical steps in the pre-treatment of algae is the detoxification of treated algae. Several promising methods have been devised for cost-effective detoxification. Applying excess calcium hydroxide and subsequent adsorption using charcoal is one of the most used techniques. Besides, other used procedures for detoxification are chemical addition (addition of alkalis/reducing agents), enzymatic and microbial treatments, heating and vaporization, solid–liquid and liquid–liquid extraction [86]. This would ensure effective and sustainable biohydrogen production.

### 4.2. Nutrient supplementation/limitation

Sulphur deprivation is the most common nutrient limitation technique used for enhancing biohydrogen production. Deprivation of sulphur in the culture media is a method of optimizing $H_2$ synthesis via bio-photolysis. It can be classified into an initial growth phase followed by a non-growth phase. The microalgae are first cultivated under suitable conditions and transferred to sulphur deficient media for $H_2$ production. Deprivation of sulphur in the nutrient growth medium can reversibly inhibit the photosynthetic activity of algae and maintain low $O_2$ partial pressure. In such cases, the protein biosynthesis is hindered, forbidding the algae to attain the necessary turnover of the D1/32-kD reaction of PS II centre protein. This can also be mentioned as psbA chloroplast gene product of the algal thylakoid membrane. Hence, PS II D1 polypeptide activity declines, reducing the photosynthesis to respiration ratio, increasing algae's respiration rate. Thus, a reduction in the $O_2$ concentration prevails, further reduced by mitochondrial respiration. Due to this imbalance in the ratio, the anaerobic condition will stay in the sealed, light-dependent system. This circumstance in the chloroplast will provoke the [Fe]-hydrogenase pathway to photosynthetically produce $H_2$ by exploiting 60–90% of electrons from splitting of water and the remaining 20–30% from carbohydrate catabolism via fermentation. This shows that both direct and indirect bio-photolysis are utilized in sulphur-deprived conditions. Still, repetitive switching between sulphur depletes and replete conditions are quite difficult to overcome by using immobilized algal cells for continuous hydrogen generation [32,82]. Another possibility for indirect bio-photolysis under sulphur deprivation is to circumvent the effect of [Fe]-hydrogenase to $O_2$ by temporal partitioning of the photo-production of $O_2$ and $H_2$. The consumption of internal protein and starch by algae is very high, and such catabolic reactions endure indirect $H_2$ production. During the course, the electrons generated flow between PS I and II through the plastoquinone pool. Daylight assimilation and electron transfer to PS I increase the electrons' redox potential to levels equivalent to [Fe]-hydrogenase and ferredoxin. Sulphur deprivation restricts ~ 75% of the PS II activity by suppressing D1 proteins synthesis essential for PS II repair. Such partial activity is vital for $H_2$ production via photolysis. However, a significant reduction would affect the overall efficiency of $H_2$ production [4,74].

When a modified TAP (Tris–Acetate–Phosphate) growth medium was used as the organic substrate for biohydrogen production in the presence of microalgae and bacterial species, improvement in the yield, biomass growth and acetic acid consumption was observed [12]. Rashid et al. [73] found that addition of glucose to sulphur-deprived cultures of *Chlorella vulgaris* can improve the biohydrogen production by 18 times under partial light conditions.

### 4.3. Bioreactor designs

To commercialize biohydrogen produced using algae, construction of photobioreactors is indispensable. The design of a bioreactor for biohydrogen production is a critical attribute as the process efficiency is dependent on various factors such as light illumination, pH, temperature, mixing of nutrients and gas, depth, and interfacial biofilm formation. These parameters must be maintained at an optimum condition to improve the overall performance of the reactor and vary based on the algal strain used. For example, excessive illumination can cause photoinhibition and the higher temperature caused can in turn disrupt the metabolism and growth regulation of algae [1]. Hence, a cost-effective light material with minimum heat generation is recommended. To make the reactor more cost-effective, it can be made light penetrative to utilize sunlight. There are various kinds of photobioreactors designed for

this application such as stirred tank, flat panel, vertical (bubble/airlift) and horizontal tubular column, tubular coiled and immobilization [3,87]. Stirred tanks are simple, easy to handle reactors that have agitation and sensor systems to maintain the operational parameters inside. Erlenmeyer flasks, roux-type and cylindrical bottles were used as stirred tank reactors. Though the flat panel photobioreactors are only being used for small-scale applications, it is more economical and has high photosynthetic activity due to effectively regulated systems controlling the temperature, pH, and optical density for hydrogen production [87]. Vertical column bioreactors are constructed with transparent glass body with an outer covering of water sheets to control temperature and prevent excess light penetration. Similarly, horizontal tubular columns are also transparent, and the algal suspension is passed through the tubes using airlift pumps [3,67]. Tubular coiler reactors are like horizontal tubular columns with an added advantage of higher surface to volume ratio. It is reported that the light conversion efficiency can be further increased with conic helical shaped coils [87]. Immobilization of algal cells in the form of alginate beads or films can also offer higher surface to volume ratio and continued hydrogen production for prolonged time due to higher cell density and short light path [87]. The opportunities and drawbacks of various photobioreactors used for algal biohydrogen production is showed in Table 5.

**Table 5**
Opportunities and obstacles of various photobioreactors designed for biohydrogen production from algae.

| Photobioreactor design | Opportunities | Obstacles |
|---|---|---|
| Continuous stirred tank reactor (CSTR) | • High mass transfer (nutrients) due to efficient mixing<br>• Substrate-microbe contact<br>• Operates under different hydraulic retention time (HRT) and substrate conditions<br>• Cheap and easy to handle | • Design and reactor configuration<br>• Short HRT causes washout of cells<br>• High energy consumption<br>• Low surface to volume ratio |
| Flat panel | • Avoids backpressure of biohydrogen<br>• High surface to volume ratio<br>• Better light capture angle<br>• Low shear stress | • Agitation via aeration causes dilution of hydrogen |
| Vertical column (bubble/airlift) | • Efficient gas exchange due to mixing<br>• Low cost and energy consumption<br>• Low shear stress | • Low surface to volume ratio<br>• Low light capture efficiency<br>• Inconvenient reactor designs |
| Horizontal tubular column | • High surface to volume ratio<br>• High biomass productivity | • Low gas exchange<br>• Accumulation of biomass<br>• High energy consumption |
| Fixed bed reactor | • Maintains optimum conditions | • Non-uniform flow circulation<br>• Low mass transfer<br>• Low substrate conversion efficiency<br>• Heterogenous activity of microorganisms |
| Membrane bioreactor | • High biomass yield<br>• High substrate conversion efficiency | • Membrane fouling<br>• Low shelf life of membranes<br>• High operation cost |
| Tubular coiled reactor | • High surface to volume ratio<br>• High light conversion efficiency | • High shear stress<br>• Low gas exchange<br>• High power consumption |
| Immobilization reactor | • Utilization in CSTR can avoid cell washout even at short HRTs<br>• Very high surface to volume ratio<br>• No shear stress | |

Though several designs have been explored, there are certain characteristics that should be considered before construction of photobioreactors suitable for algal cultivation and hydrogen production. For example, agitation is essential for gas exchange for algal growth; however, it might dilute the hydrogen produced in the system. Similarly, the reactors should have provision for insertion of sensor to better control the parameters; however, it can pose the risk of leakages. Also, the reactor materials that encounter the hydrogen gas should have impenetrable properties. The design should also allow sufficient headspace for the culture as well as to avoid hydrogen tightness. The reactor should contain cost-effective measures for controlling multiple parameters such as cooling jackets for temperature, atmospheric $CO_2$ supply for algal cultivation and mechanical agitation instead of external supply. In addition, the materials used for construction should be durable, heat resistant, corrosion resistant, autoclavable, leakage proof, cheap, easily available and should not have inhibiting effect on algae.

### 4.4. Integrated hybrid systems

The integrated system will assist in overcoming the drawback of a process while aiding the other. This advancement will further increase the productivity and yield of biohydrogen production. Dark and photofermentation processes can be combined for the cooperative activity of photosynthetic and non-photosynthetic bacteria under light and dark conditions, respectively. The organic co-products such as acids of acetic, lactic, and butyric formed during DF can increase the system's acidity, lower the enzymes' activity, and yield $H_2$. These organic substances could serve as substrates for photoautotrophic microbes in the presence of light to convert carbon compounds to $H_2$ and $CO_2$. These hybrid systems can yield 12 mol $H_2$ $mol^{-1}$ hexose in theory. It was stated that the biohydrogen potential increased from 96.6 to 337 mL $g^{-1}$ of total solids when this combined process was used. However, only the rich VFAs in the effluent were found to be converted into biohydrogen. Photo-fermentation can serve as a suitable post-treatment to DF because purple sulphur bacteria consume a wide range of organic acids as substrates and result in high production yields. Generally, the processes are combined in a single reactor to prevent organic acid accumulation by photo-fermentation [76,16].

Nevertheless, the integrated process can be more effective when running in two different reactors as the process requires other optimum conditions (i.e., DF occurs at 37–50 °C, whereas photo-fermentation requires 30 °C. However, the DF effluent must go through a cooling stage before undergoing photo-fermentation, especially in thermophilic DF. Another bottleneck of the system can be the high concentration of organic acids generated in DF, which could inhibit organisms used in photo-fermentation. To address this, few researchers have utilized the effluents of dark fermentation to cultivate algae because algae can assimilate the organic residues (acetate and butyrate) from the effluent, this will not only reduce the acid content but also generate more algal biomass for biohydrogen production [76]. Other parameters that should be considered in such arrangements are selecting microbial strains, DF/photo-fermentation biomass ratio, substrate concentration and light regimes such as source and intensity [13,53].

The COD-rich effluent from DF can also be utilized as a substrate for microbial fuel cells (MFCs) and microbial electrolysis cells (MECs). MFCs have exoelectrogens that can oxidize the organic matter at the anode terminal and reduce protons at the cathode terminal to generate electricity. In MECs, an external voltage supply is used to mobilize electrons across terminals to produce $H_2$ by proton reduction at the cathode. These integrated systems also achieve a high biohydrogen production rate and yield [69,95].

### 4.5. Use of metals and nanomaterials

Lack of substrate accessibility, reduced efficiency of bioconversion reaction and low enzyme production are the major bottlenecks for biohydrogen production in terms of sustainability and economic feasibility. Several organic and inorganic nanomaterials have been found to address these issues and significantly improve the yield of $H_2$ and substrate biotransformation, owing to their advantages of the precise structural and physicochemical characteristics including surface to volume ratio, large surface area, ability to maintain thermal and pH stability of enzymes and high electro-conductivity. Inorganic nanomaterials of several metals like iron, nickel, silver, copper, gold, and titanium have been studied so far, among which iron and nickel are established to be the most efficient ones as they act as co-factor for the active site of $H_2$-ase and $N_2$-ase enzymes. For instance, when $Fe_3O_4$ nanoparticles were used during the co-fermentation of algal biomass (*Lyngbya limnetica*) and glucose, the cumulative hydrogen production was observed to increase by 37.14%[89]. Besides, they serve as oxygen scavengers, eliminating the undesirable oxygen produced during fermentation, thus reducing the redox potential. This will further create a suitable anaerobic condition for hydrogenase activity [13,65]. Though titanium had been reported to have high catalytic performance, low-toxicity and cost, the fact that it triggers reverse reaction of photocatalytic mechanism leading to the production of oxygen limits its use [81]. Carbon nanoparticles are the widely used organic nanomaterials which are employed in various forms such as nanotubes, powdered activated and nano activated carbon. However, Mohan et al. [63] recorded that activated carbon alone wasn't effective (1.57 mol kg $COD^{-1}$ $day^{-1}$) in comparison to biocompatible silica (7.02 mol kg $COD^{-1}$ $day^{-1}$). Yet, when used in combination with mesoporous platinum and titanium dioxide, a highest of 7490 μmol $h^{-1}$ g $photocatalyst^{-1}$ hydrogen production was achieved [33].

Ban et al. [5] applied $Ca^{2+}$ to *Chlamydomonas reinhardtii* culture to enhance its photolytic $H_2$ production. Calcium also enhanced the strain's chlorophyll and protein content and decreased Reactive Oxygen Species (ROS) levels, protecting the PS II activity accountable for direct bio-photolysis. Also, the starch synthesis is improved, contributing to the indirect bio-photolysis of $H_2$ generation. The mitochondrial respiration rate was enhanced, leading to a decrease in the headspace $O_2$. It advances the biohydrogen production using algae growing in the culture. Though these results show the efficacy of using nanomaterials, optimization of its dosage to increase its bioavailability and minimize feedback inhibition, toxicity towards the microorganisms, and save disposal is important. This will promote its usage in continuous systems for commercial applications.

### 4.6. Gene manipulation

Alteration at the genome level of the strains employed can benefit biohydrogen production by improving the process's yield and kinetics. The genes involved in the mechanism of biohydrogen production can be engineered metabolically to overcome the drawbacks of varied performance of the same organism with a change in substrate, low resilience of the strain for adjustments in the operational conditions, and contamination of the inoculum [60].

Generally, direct bio-photolysis is reported to contribute more to the overall $H_2$ production. Because, in indirect bio-photolysis, the electrons are supplied through an external substrate making it inaccessible for consumption. This obstacle can be overcome by modifying the transporter proteins for enhancing substrate transfer inside the cell. HUP1, a hexose symporter gene from *Chlorella kessleri,* was expressed heterologously in the stm6 cells of *Chlorella reinhardtii* that lack glucose transporters. This insertion enabled the import of glucose, and the transformed *C. reinhardtii* stm6Glc4 synthesized five-times more $H_2$ than the wild type [23]. In another study, a random gene insertion was performed in *C. reinhardtii* to correct the irregularity in the respiratory metabolism and the inability to execute cyclic electron transport around the PS I. The insertion aided in retaining starch for a relatively longer duration under an anaerobic environment due to inhibition of energy intake by mitochondrial respiration [47].

The sensitivity of [FeFe]-hydrogenases to $O_2$ is a significant issue in biologically catalyzing $H_2$ synthesis. Genetic engineering can be an alternative solution to prevent inhibition by involving genetic engineering to identify and isolate organisms with hydrogenases tolerant to $O_2$. Yang et al. [101] genetically engineered the hydrogenase enzyme of green alga *Chlorella* sp. by modifying the amino acid sequences A105I, V265W, G113I, or V273I around the gas tunnel to avert the oxygen molecules from entering the active centre. This strategy increased the hydrogen production up to 30-folds. Ghirardi [30] attempted a random mutagenesis method to find $O_2$-tolerant hydrogenases in *C. reinhardtii* and thus, the mutagenized cells were exposed to two selection pressures: hydrogen metabolizing capability and cell's ability to liberate biohydrogen when exposed to $O_2$. Also, the cells were screened with a chemochromic sensor to detect $H_2$ activity. Such selections were reported to increase the $O_2$ tolerance of cells for sustained $H_2$ release. However, more studies on this aspect must be done to identify $O_2$-tolerant [FeFe]-hydrogenases from different algal species. PsbO is the gene responsible for oxygen generation in the PSII system. The knocking out of this gene yielded nine times more hydrogen due to induction of the hydrogenase enzyme in *Chlorella* sp. DT strain [57,54]. Overexpression of certain genes can also lead to enhanced production of hydrogen. In a study, the codon optimization genes in *lba* and *hemA* of *C. reinhardtii* were overexpressed to achieve a 20% increase in hydrogen by means of direct photolysis. This genetic engineering approach also led to a 6.8-fold increase in the *hemH-lba* protein expression (Wu et al., 2011) [54].

Another challenge is to bypass the electron competition by hydrogenase enzyme A with ferredoxin-NADP + -reductase (FNR) (primary receptor) in biophotolysis. Eilenberg et al. [25] fused the electron donor ferredoxin with hydrogenase in an *in vivo* model microalga *C. reinhardtii.* When the fusion gene was expressed in the system, the enzyme was able to directly intercept with the photosynthetic electrons for effective hydrogen generation. Similarly, mutation of Moc 1 gene in *C. reinhardtii* improved transfer of electrons to hydrogenase through a respiratory electron transport chain and simultaneously the hydrogen generation [2].

Algae can be engineered to upsurge the light conversion efficiency for growth, biohydrogen synthesis and avoid photo-inhibition. In a study, conversion efficiency up to 10–13% was attained using genetically modified microbial culture. However, improving the conversion of natural solar light should also be considered from a commercialization point of view. Mutant algal strains with less chlorophyll content were cultivated, which augmented light distribution into deeper biomass layers for pilot scale applications [22].

### 4.7. Algal-Bacterial interaction

The algal-bacterial interaction is ubiquitous in microbial ecology. In addition to the exchange of $O_2$ and $CO_2$, the interdependence between algae and bacteria has enhanced the accumulation of starch and maintained protein and chlorophyll content, which are the key factors to improving $H_2$ yield [31]. The co-existence has proven effective in $H_2$ synthesis by achieving anaerobic conditions. Researchers have found that bacteria assist algae by exhibiting a co-metabolism pattern. While algae supply $O_2$ and organics for bacterial growth, the latter shows an ability to reduce algal hydrogenase's $O_2$ sensitivity. For instance, when *Pseudomonas* sp. was co-cultivated with green algae *Chlorella* sp., the bacterial growth rapidly consumes the dissolved oxygen and the oxygen present in the headspace, resulting in an aerobic environment (essential for the proper working of algal hydrogenases) [5]. Similarly, *Pseudomonas* sp. enhanced the photolysis-mediated hydrogen production from *C. reinhardtii* and *Scenedesmus* sp. [5]. Similarly, the co-cultivation of *Chlamydomonas reinhardtii* with *Pseudomonas putida* was beneficial in

maintaining the acetic acid concentration, which is essential for bio-hydrogen production, but their higher concentration inhibited bacterial growth [28]. Chen et al. [17] used thermophilic bacteria *T. eurythermalis* A501 to utilize algal residue *Dunaliella primolecta* for hydrogen production and obtained a yield of 192.35 mL $g^{-1}$ VS without any pre-treatment. However, metals like calcium harmed the algal-bacterial co-cultivation system as it affected the $O_2$ consumption, resulting in relatively high $O_2$ content in the headspace. Xu et al. [98] co-cultured *C. reinhardtii* cc124 and bacteria *A. chroococcum* to achieve a 16-fold increase in hydrogen yield (255 μmol mg $Chl^{-1}$) than the control.

Co-cultures of bacteria had been reported to influence the yield, rate, and duration to increase hydrogen production from algae compared to monocultures [54]. A study investigated the effect of bacterial co-cultures with Chlamydomonas for hydrogen production at low light intensities. The results showed increased hydrogen release of 46, 24, and 32% for *P. stutzeri, E. coli,* and *P. putida,* respectively with decreased growth of Chlamydomonas. This was because of the higher respiration rate at low light intensities [27]. Fakhimi et al. [28] also demonstrated the role of sugar supplementation in the medium to improve hydrogen generation from algae in the presence of bacterial co-cultures. When *C. reinhardtii* was co-cultured with *E. coli*, *P. putida* and *Rhizobium etli* in a sugar-containing medium, hydrogen production of 35.1, 40.8 and 16.1 mL $H_2$ $L^{-1}$ was attained. The enhanced performance was attributed to the consumption of bacteria-synthesized acetic acid by microalgae and simultaneous production of hydrogen from bacteria and microalgae.

### 4.8. Co-digestion of microalgal biomass with other raw materials

Several microalgal biomasses contain high proportion of protein than carbohydrates, which alters the optimum C/N ratio, making them less favorable for biohydrogen production. Co-digestion is a process in which microalgal biomass is mixed with other organic materials, such as food waste or agricultural waste, to adjust C/N ratio of the feedstocks to produce biohydrogen. This can also be used as a strategy to dilute the toxic compounds generated by one substrate and enhance the organism's synergistic effect. This process occurs in a bioreactor, where microorganisms break down the organic materials and release hydrogen gas as a by-product. Co-digestion has been found to be an effective way to increase the efficiency and yield of hydrogen production, as well as to reduce the cost of the process. Co-digestion of microalgal biomass with other raw materials for bio hydrogen production can be done using a variety of different feedstocks. Some examples include microalgal biomass mixed with food waste containing high organic content; agricultural waste such as straw or corn stover, to address issues related to land use and food security; sewage sludge, a by-product of wastewater treatment, which can be a good source of nutrients for microorganisms; and livestock manure [77,97]. Xu et al. [97] co-digested *Microcystis wesenbergii* and *Microcystis aeruginosaare* with food waste to enhance the C/N ratio to 22.65 and hydrogen yield (31.42 mL $H_2$ g-$VS^{-1}$) by 12.13 times compared to dry algae. Similarly, Sivagurunathan et al. [85] co-digested *Gelidium amansii*, a carbohydrate-rich macroalgae, with protein-rich microalgae, *Chlorella* sp. and *Scenedesmus* sp., to achieve better C/N ratio compared to mixed microalgae.

### 4.9. Sparging inert gas

$O_2$ inhibition of [FeFe]-hydrogenase during direct bio-photolysis is a significant bottleneck for $H_2$ production. $O_2$ molecules can bind to the iron atom of the cluster and prevent the attachment of protons necessary for $H_2$ liberation. Another prospect of overcoming this limitation is sparging inert gas for spatially or temporally removing $O_2$ as and when it is formed for [FeFe]-hydrogenase enzyme to remain active throughout the $H_2$ metabolism. Inert gases can lower the partial pressure of $O_2$ and maintain it below 0.1% for long-term hydrogenase activity. However, a huge volume of gas might be essential for stream dilution, which is impractical in case of economic feasibility, cost, and energy aspects [53]. Also, containing the large volume of gas needed in the process might add to the cost making this strategy unsustainable [84].

### 4.10. PS II inhibitors

Inhibitors of PS II, namely DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea), can impede the electron flow between PS II and the plastoquinone pool. In simple, it prevents the linear flow between PS II and PS I. This action might generate $H_2$ and $CO_2$ in the stoichiometric ratio of 2:1. DCMU blocks the generation of $O_2$ by PS II, thereby decreasing the headspace $O_2$ [5]. The following anaerobic environment upon illumination will induce [Fe]-hydrogenase enzyme ensuing considerable rates of $H_2$ generation when DCMU is involved [53].

### 4.11. $O_2$ scavengers

$O_2$ scavengers are a strategy used to improve biohydrogen production using microalgae by reducing the amount of oxygen present in the culture, which can inhibit the activity of hydrogen-producing enzymes [26,68]. Khosravitabar & Hipplera [41] used oxysorb, as a chemical means to scavenge oxygen from the sulphur containing culture of *Chlamydomonas reinhardtii* by inducing anaerobiosis. Sodium ascorbate present in oxysorb acts as a reducing agent to convert oxygen to water, which is catalyzed by a small amount of cupric sulfate. Several other examples of $O_2$ scavengers which include the following.

- The use of anaerobic bacteria, such as Clostridium or Desulfovibrio, can consume oxygen and maintain anaerobic conditions in the culture.
- Sodium sulfite can be added to the culture as an oxygen scavenger. It reacts with oxygen to form sulfur dioxide, which can be removed from the culture by bubbling through the solution [68].
- A sealed culture system can be used to maintain anaerobic conditions by preventing the exchange of gases with the atmosphere.
- A low agitation rate can reduce the amount of oxygen that is dissolved in the culture.
- A low aeration rate can reduce the amount of oxygen that is supplied to the culture.
- Photobioreactor with low light intensity: By reducing the light intensity, photosynthesis is slowed down, and hence the amount of oxygen produced by photosynthesis is reduced.

### 4.12. Dark-light cycles

Dark-light cycles have been shown to be an effective strategy for improving biohydrogen production using microalgae. The dark phase allows for the accumulation of intracellular storage compounds, such as starch, which can then be converted to hydrogen during the light phase. Additionally, the dark phase also allows for the maintenance of a low dissolved oxygen concentration, which can prevent the oxidation of hydrogen. Kosourov et al. [45] found that a 14: 10 h light/dark cycle under sulphur deprived condition can lead to earlier anaerobiosis in the system and thereby hydrogen production. Rashid et al. [73] recorded the highest hydrogen production of 530 ± 5 mL $L^{-1}$ when immobilized, sulphur-deprived cultures of *Chlorella vulgaris* were exposed to 48 h of darkness after 24 h of light.

## 5. Advanced strategies in biohydrogen production from algae

Apart from the above-discussed strategies for biohydrogen production from algae, few other researchers attempted novel advanced strategies to enhance the biohydrogen production from algae, which are summarised below:

- Novel hybrid electrochemical approaches using microbial electrolysis cells (MECs) are developing. They are established based on

microbial fuel cells (MFCs) where the organic matter is oxidized by electroactive biofilms to produce green and sustainable energy. In short, MFC is the bioelectrochemical process and MEC is the bio-hydrogen production reactor combining MFC and electrolysis [80]. A variety of organic substances can be used for this process and the microorganisms utilize them to produce electrons and protons at the anode which gets transferred to the cathode via the external circuit and reaction medium, respectively [34,54]. These electrons and protons come together to form hydrogen at the cathode. As the hydrogen is produced extracellularly, it is considered unconventional and termed as electro-bio-hydrogenation [54]. MECs can also be integrated with other techniques, especially dark fermentation, to further improve the yield and by-product utilization. MECs and MFCs are considered the cleanest, environment-friendly, and most efficient hydrogen production techniques.

- Algal-based photo-fermentative biohydrogen production from the effluents of dark fermentation is a promising advancement. In addition, the $CO_2$ generated during dark fermentation reaction could be utilized by the algae for growth. Also, the algae could be able to produce more hydrogen in the presence of bacteria as they were able to maintain a low dissolved oxygen level in the system.
- The potential of microRNA (miRNA) in regulating biohydrogen levels in microalgae has been reported by Anwar et al. [3]. The miRNAs were differentially expressed under sulphur deprived conditions in *C. reinhardtii* which directly regulated the photo-production of biohydrogen. They were targeted towards genes responsible for photosynthesis, which created an anaerobic environment and stimulated hydrogenases activity. Endogenous miRNAs (cre-miR1166.1, cremiR1150.3 and cre-miR1158) were upregulated after heat induction in response to sulphur deprivation leading to enhanced biohydrogen production compared to the wild type. Similarly, artificial miRNAs were developed to target oxygen evolving enhancer (OEE2) gene through a heat inducible expression vector. The transgenic *C. reinhardtii* showed reduced expression of OEE2 produced under sulphur deprived conditions, resulting in higher hydrogen. Such sustained hydrogen production was also reported to be possible in *C. reinhardtii* using long-chain non-coding RNA (LncRNA).
- Light induced regulation of artificial miRNA was developed by Wang et al. [96], where a blue light was used to establish optogenetic control in *C. reinhardtii* to achieve higher hydrogen yield. The transformed algae were initially cultivated in red light to improve biomass growth and later shift to blue light to stimulate miRNA transcription and suppression of targeted genes.
- Incorporation of nanotechnology in bioreactor designing to enhance the overall illumination capacity. Liao et al. [56] developed a biofilm photobioreactor coated with $GeO_2$-$SiO_2$-chitosan medium to improve its refractive index and light conversion efficiency without heat generation. This increased the hydrogen production by 1.56-fold than the uncoated reactor. Later, lanthanum hexaboride ($LaB_6$) was further included with the mixture by Li et al. [55] to produce a photothermal nanobiomaterial to absorb (380–510 and 660–780 nm) and transmit (590 nm) light and improve the biofilm development and hydrogen production up to 2.92 mmol $h^{-1}$ $m^{-2}$.
- Atmospheric and room-temperature plasma (ARTP) is a mutation tool developed as a part of whole-cell mutagenesis technology. It uses helium radio-frequency glow discharge plasma to generate even scattered particles under atmospheric pressure and mutate the strands of DNA with irreversible gene repair efficiency [54,4]. This technique has several advantages over conventional mutagenesis methods like chemical or ultraviolet radiation such as operator's safety, high activity, high mutation efficiency, and user-friendly. Ban et al. [4] adopted this technology to reduce the size of chlorophyll and increase the hydrogen production efficiency of *C. reinhardtii* mutant up to 2.7–3.1 times (356.5–405.2 mL $L^{-1}$) when cultivated in co-cultures.
- Thermochemical approaches such as thermal and supercritical water gasification for biohydrogen production from algae is considered economical and a promising alternative as wet biomass can be directly utilized instead of cost-intensive drying [52].

## 6. Research gaps

Though the number of publications on biohydrogen production are increasing in recent times, the gaps and inadequacies still exist, that prevents its widespread acceptability. Out of the exhaustive literature survey conducted, the research gaps identified were as follows:

- Selection of microalgal strains suitable for optimum biohydrogen production in conventional and especially the novel hybrid systems. For example, identifying strains that can tolerate the organic acids produced as by-products during dark fermentation or even utilize them to growth and produce not only hydrogen but also other value-added products is imperative. Also, the shift in diversity of organisms compared to the inoculum should be pondered to positively exploit them.
- Understanding the complex and dynamic mechanisms governing the hydrogen generation processes for better genetic and metabolic engineering approaches.
- C:N:P balance in the medium and contamination of bioreactor with methane and oxygen are critical concerns that should be focussed on and some of the state-of-the-art bioinformatic tools like polyomics and statistical design of experiments could help.
- The microalgae-bacteria symbiotic interaction has shown to be a promising strategy to improve biohydrogen production by promoting starch retention, protein, and chlorophyll formation. However, there still exists a gap due to limited investigation on the effect of bacteria consortia on microalgae in large-scale biohydrogen systems.
- In addition to optimization of process influencing factor, focusing on configuration/designing of biohydrogen reactors can offer higher photosynthetic efficiency.
- Though dark fermentation technique using algae has been implemented at a large-scale for biohydrogen production, there is so far no photoheterotrophic algal reactors installed. Efforts to design such reactors can help make the process and product more economical.
- Considering that the existing life cycle assessments on biohydrogen production from algae are solely interpretations of laboratory results, further studies on life cycle, technoeconomic assessments and environmental sustainability can promote scale-up and commercialization.

## 7. Conclusion and future scopes

Carbon neutrality, energy-effective and sustainability are the three main attributes that popularized biohydrogen production from algae. Some of the conclusions of the review are discussed below.

- Technical challenges for large-scale implementation of algae-derived biohydrogen should be overcome. For examples, the firmness of the algal cells affects its hydrolysis efficiency via dark fermentation and photo-fermentation. In addition, challenges like substrate conversion, mass storage of feedstock, biomass pre-treatment cost and overall production cost hinders commercialization and should be further worked upon.
- Studies have indicated that the integration of wastewater and microalgae in a fermentation-bioelectrochemical system containing bacteria (photosynthetic and non-photosynthetic) can increase the yield and efficiency of biohydrogen production. Such use of industrial wastewater for bioenergy production can not only make the technology economically feasible but also serve as a wastewater management approach.

- The main cost bearing factors of biohydrogen production from algae are construction of photobioreactors. This can be tackled by recycling the metabolic products produced by algae in the photobioreactors, which can reduce the expense of nutrient addition and 80–85% of the overall cost.
- Researchers should focus on increasing the photosynthetic performance of microalgae or hydrogenase enzyme level or activity in microalgae.
- Measures to overcome the oxygen sensitivity of hydrogenase is necessary. Use of genetic engineering can help structurally modify the enzyme to make them more tolerant to oxygen.

## CRediT authorship contribution statement

**Krishnamoorthy Nageshwari:** Conceptualization, Data curation, Investigation, Writing – original draft. **Abhijeet Pathy:** Conceptualization, Formal analysis, Writing – original draft. **Arivalagan Pugazhendhi:** Investigation, Supervision, Writing – review & editing. **Paramasivan Balasubramanian:** Conceptualization, Investigation, Supervision, Writing – review & editing.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

No data was used for the research described in the article.

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