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Reviewing biohydrogen production from microalgal cells through fundamental mechanisms, enzymes and factors that engendering new challenges and prospects

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ABSTRACT

Hydrogen is the most promising alternative energy recently that is renewable and clean due to its sustainability, great potential as energy source and efficiency during the conversion. In fact, microalgal biohydrogen has attracted significant attentions as a clean and sustainable fuel to achieve the carbon neutrality and sustainability in environment. In this review, the mechanisms of biological methods and electrochemical methods, and the enzymes involved in the biohydrogen generation from microalgae have been inclusively and rudimentary detailed. The review also summarizes the benefits and drawbacks of each technique, and compares the biohydrogen yields from each method based on the results reported by the recent studies. Furthermore, the key factors that influence the biohydrogen yields including pH, light intensity, cell density, temperature and substrate introduced have been addressed as well in this review. Moreover, the review also highlights the primary shortcomings encountered which are oxygen sensitivity of enzymes and low biohydrogen yield. Lastly, the resolutions to enhance the biohydrogen production together with the future prospects for sustainable biohydrogen production from microalgae are opined systematically.

1. Introduction

Energy consumption has drastically expanded due to the urbanization, industrialization, and rising population, and this phenomenon has significantly increased the utilization of fossil fuels [1]. As a non-renewable energy source, fossil fuels will be depleted eventually

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and unable to fulfil the future generation demands. Besides, the burning of fossil fuels has released a considerable amount of carbon dioxide into the atmosphere which causing air pollution and global warming. Hence, a renewable energy source that is environmentally friendly and able to capture carbon dioxide has to be explored to satisfy the increasing demands for energy.

Nowadays, biofuels have received more attentions to replace fossil fuels for some reasons including its ability to minimize the emission of greenhouse gases (GHG). Biofuels are produced from biomass, and they are recognized as carbon neutral because the quantity of carbon dioxide emitted and captured are virtually equal. Biofuels can be categorized into three groups which are the first generation (food crops), second generation (lignocellulosic materials), and third generation (microalgae biomass). However, the first- and second-generation biofuel feedstock have some downsides including they need arable land to grow, their growth dependent on environment factors and their biofuels production requires a lot of energy [2]. On the ther hand, the biofuel produced by microalgae has some benefits such as microalgae feedstock are able to grow under harsh conditions and on non-arable land. Some parts of the world have employed biofuels to partialy replace the utilization of fossil fuels. For example, Brazil uses sugarcane as a biofuel source, while Europe as well as some regions in Asia use palm oil. In the case of microalgae as a biofuel source, China, Brazil, the United States, and Japan are the leading countries in this sector [3,4].

Hydrogen has been touted to be the most promising alternative energy that is renewable and clean recently due to its sustainability, great potential as energy source and efficiency during the conversion. According to Li et al. [4], 1 kg of hydrogen has the energy value of around 120 MJ which exceeding the majority of conventional fuels. Furthermore, hydrogen is a fuel that is free of carbon as it only produces water as the byproduct after combustion process [5]. Based on Sharma and Arya [6], hydrogen is most frequently used in the petroleum and fertiliser industries, at about 50 % and 37 %, respectively. Hydrogen is typically generated via hydrocarbon reforming or non-reforming ways such as electrolysis and gasification. Nevertheless, these methods have some drawbacks such as involving the extensive usage of fossil fuels, high electricity consumption as well as poor thermal efficiency [7].

The biological methods are another option to produce hydrogen. Although biological techniques for hydrogen production have a slower rate of productivity than physicochemical modes, they are more environmental beneficial in the synthesis of hydrogen. The majority of biological processes occur at room temperatures and pressure, and they require lesser energy than thermochemical hydrogen production processes. Biohydrogen is the term used to describe hydrogen generated through a biological process. Of late, the biohydrogen production from microalgae has received a lot of attentions. Microalgae provide a number of benefits over other biological sources, including a rapid growth rate, minimal energy requirements, and they do not require expensive pretreatment procedures because of the absence of lignin [8]. In addition, microalgae are able to utilize carbon dioxide to produce new microalgae biomass, namely, 1.83 kg of carbon dioxide are required to produce a 1 kg of biomass in dry weight basis [9]. Microalgae can produce biohydrogen via two types of biological methods which are fermentation and biophotolysis processes. Each of them has two subcategories which are direct-biophotolysis or indirect-biophotolysis and dark-fermentation or photo-fermentation. However, it has been emphasized that the biophotolysis hydrogen production is inhibited by the oxygen sensitive enzymes, while the fermentation process is not feasible because of organic substances employed are not entirely transformed into carbon dioxide and hydrogen [10]. Besides, the biological hydrogen can also be generated from microalgae via electrochemical methods which are microbial photoelectrochemical cell (PEC) and microbial electrolysis cell. They are commonly chosen over typical biological, chemical, or biochemical processes because of their extensive application potentials.

In short, microalgae biological hydrogen has attracted significant attentions as a clean and sustainable fuel to achieve the carbon neutrality and sustainability in nature. The review article emphasizes on the recent information related to generation of biohydrogen from microalgae, technologies to produce biohydrogen from microalgae, biohydrogen synthesizing enzymes in biological pathways, key parameters for biohydrogen generation from microalgae and difficulties encountered as well as ways forward to overcome the challenges.

2. Microalgal cells

Microalgae are the earliest photosynthetic life forms on Earth and the main producers in every ecosystem. They are microscopic aquatic microorganisms and visually invisible. Microalgae do not have vegetative parts of plant such as roots, stems and leaves. Their main photosynthetic pigment is chlorophyll, and they use solar energy, water, and carbon dioxide to yield biomass and oxygen [11]. Microalgae are prokaryotic and eukaryotic microbes that can undergo photosynthesis process and they often exist in freshwater and marine environments. They are divided into prokaryotic microalgae (Cyanobacteria), eukaryotic microalgae (green microalgae Chlorophyta), red microalgae (Rhodophyta), and diatoms (Bacillariophyta) [12]. Microalgae can grow rapidly and survive under stress environment due to their simple structure.

Besides, microalgae can be grouped into three types based on the types of carbon sources they take up which are autotrophic, heterotrophic and mixotrophic microalgae [13]. The inorganic elements such as carbon dioxide, salts and sunlight are required for the autotropic microalgae to grow. Since the heterotrophic microalgae are non-photosynthetic type, they need an alternative source of organic substances as source of energy. The mixotrophic microalgae are able to undergo photosynthesis process and obtain extra organic components. In order to survive and obtain external organic nutrients, mixotrophic microalgae must undergo photosynthesis process where the adenosine triphosphate (ATP) and oxygen are produced by converting the sunlight and carbon dioxide. The energy is subsequently employed in the respiration to supply energy for growth.

Microalgae have a dry weight carbon content of about 50 %. Generally, most of the carbon content in microalgae is derived from carbon dioxide [11]. According to Wang and Yin [11], the amount of carbon dioxide that can be fixed by 100 g of microalgae biomass is about 183 g. Microalgae are mainly composed of carbohydrate (5 % − 60 %), lipids (8 % − 30 %), protein (40 % − 60 %) and nuclei acid (5 % − 10 %). The components in microalgae are affected by species, environments of cultivations and characteristics of biochemicals [14]. The composition of microalgae can be used to produce biofuels such as hydrogen. The general components of several microalgal species are shown in Table 1.

Microalgae are currently referred as a third-generation biofuel feedstock that will not compete with foods and terrestrial crops. They are able to generate a significantly higher amount of oil against other types of biofuel feedstock. In addition, the lipid productivities of microalgae are 15–300 times greater than that of conventional oil crops [17]. The notable benefits of microalgae as the feedstock include high rate of grow, not reliant on arable land to grow, strong environmental adaptability, and able to tolerate high carbon dioxide level [11,18,19].

Table 1
General cells' compositions of various microalgal species.

Microalagal species	Carbohydrate (%)	Lipid (%)	Protein (%)	Reference
Chlamydomonas reinhardtii	17	21	48	[15]
Chlorella vulgaris	12–17	14–22	51–58	[15]
Scenedesmus obliquus	10–17	12–14	48–56	[15;16]
Spirulina maxima	13–16	6–7	60–71	[15]
Synechococcus sp.	15	11	63	[15]

In this regard, the potential employment of microalgae to generate hydrogen fuel has been intensively investigated, besides the lipid to biodiesel, considering its advantages that are sustainable intrinsically.

3. Mechanisms of biohydrogen production from microalgae

Microalgae can produce hydrogen via bio-photolysis or fermentative process. The fermentative processes can be further separated into light-dependent and light-independent modes. The dark fermentation is a process that does not dependent on light, while the bio-photolysis or light fermentation process is classified as the light-dependent process. In bio-photolysis process, hydrogen and oxygen are generated by microalgae from solar energy and water. It can be also separated into direct and indirect bio-photolysis. Besides, biohydrogen can be as well produced from microalgae via electrochemical methods which are microbial fuel cell (MFC), microbial photoelectrochemical cell (PEC) and microbial electrolysis cell (MEC). They are frequently preferred to typical chemical, biological, and biochemical processes because of their extensive application potentials.

3.1. Direct bio-photolysis

Bio-photolysis process is similar to photosynthesis. According to Anwar et al. [14], green microalgae are the only organisms that can directly undergo biophotolysis to generate biohydrogen by splitting water under anaerobic enviroment. Photosystem II (PSII) is responsible for the water splitting action in the presence of light [9]. Direct bio-photolysis is a two-step reaction, and the processes are illustrated in Fig. 1. Firstly, water molecules are converted into proton, electron, and oxygen by photosystem II (PSII). Then, hydrogen is produced with the aid of hydrogenase enzymes [20,21]. The reactions are represented by the equations below:

$$Photosynthesis\ reaction :: 2H_2O + Sunlight \rightarrow 4H^+ + 4e^- + O_2 \quad (1)$$

$$Hydrogen\ production\ reaction : 4H^+ + 4e^- \rightarrow 2H_2 \quad (2)$$

During the direct bio-photolysis process, the electrons are created from the oxidation of water through the PSII. The electrons are activated by photons obtained from sunlight via PSII before they are transferred through electron transport chain. The transport of electrons is facilitated by ferredoxin (Fd) and photosystem I (PSI). Plastoquinol generated through electron transfer reduces $NADP^+$ to NADPH. To produce oxygen and hydrogen, the electrons are replaced by oxidizing water and the PSII utilizes these electrons obtained from water for photosynthesis. In order to produce ATP using ATP synthase, a proton gradient is formed by the protons (hydrogen ions) produced from the water-oxidation reaction. The protons are served as terminal electron acceptors during photosynthesis in the microalgae chloroplast. Hence, this process generates both hydrogen and oxygen gases at the same time [21,23].

Indeed, direct bio-photolysis is a fascinating technique because it employs sunlight to convert water, which is easily accessible, into oxygen and hydrogen molecules. In addition, it is more beneficial as no organic carbon substrates are needed for the growing medium. Direct bio-photolysis relies heavily on the photosynthetic ability of microalgae and does not release any greenhouse gas. Furthermore, the microalgae have the solar conversion rate that is ten times greater than that of terrestrial plants. Nonetheless, the hydrogenase system will inhibit the biohydrogen production when the thereshold amount of oxygen is present because the hydrogenase is particularly sensitive towards the oxygen [7]. Specifically, the [Fe-Fe] hydrogenase is highly sensitive towards oxygen. The existence of low concentrations of oxygen will ruin the [4Fe-4S] cluster that presents in the active site of hydrogenase and make it catalytically inactive [7,9]. Based on Stripp et al. [24], the reactive oxygen species were formed first at the distal Fe atom of the [Fe-Fe] subcluster of the active site. The reactive oxygen species would then destroy the [4Fe-4S] cluster and decrease the enzymatic activity quickly. The production of hydrogen molecules decreased noticeably when such hydrogenase activities were suppressed.

A two-stage photosynthesis process via sulphur deprivation was invented to enable the spatial separation of hydrogen and oxygen productions. Firstly, microalgae are cultured in a sulphur-rich medium which effectively improves cell development and photosynthesis. They perform normal photosynthesis which involves the water oxidation and the evolution of oxygen in such environment. Secondly, the microalgae are moved to a sulphur deprivation medium and has resulted in the inhibition of photosynthesis and oxygen evolution. In sulphur deprivation cultures, the oxygen-evolving photosystem II (PSII) is partially deactivated. The reaction-center D1 proteins, which are sulphur -rich, are basically damaged during water splitting in PSII and require to be replaced. However, the production of D1 protein is inhibited and the PSII repair cycle is halted under sulphur-deprived conditions, resulting in a low level of oxygen generation [25]. The culture creates an anaerobic environment when the rate of photosynthetic oxygen evolution decreased below the rate of cell respiration, which is advantageous for activating the hydrogenase [26]. The production of hydrogen and hydrogenase activity are promoted under sulphur deprivation environment [21]. According to Wang et al. [27], *Chlorella pyrenoidosa* could produce hydrogen without a sulphur-deficient environment, but only at extremely low light intensities (<30 μE/m^2/s) that considerably below the light intensity at which photosynthetic oxygen evolution fulfils

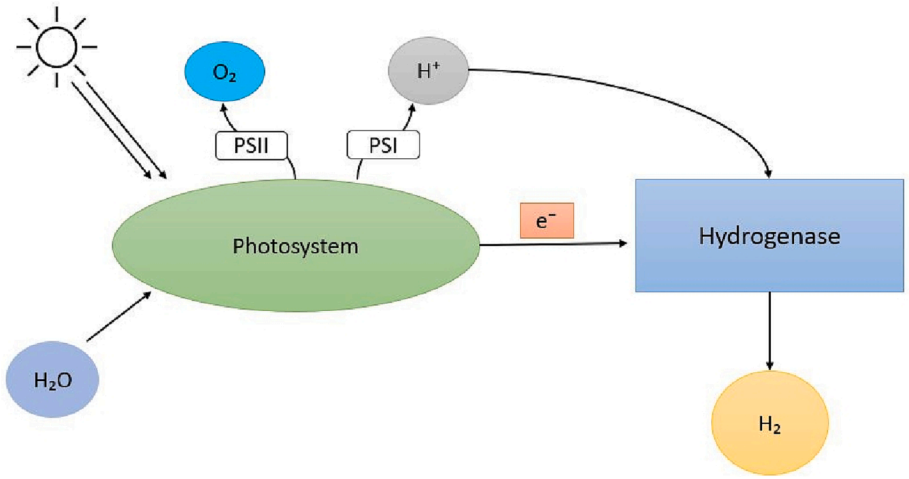

Fig. 1. Schematic diagram of direct bio-photolysis.

respiratory needs. The corresponding production rate of hydrogen at these relatively low light intensities was very poor which was merely 6 μmol/L/h. Vargas et al. [28] examined the production of hydrogen with two Chlamydomonas strains which were *Chlamydomonas reinhardtii* (CC425) and *Chlamydomonas moewusii* (SAG 24.91) under sulphur deprivation environment. The maximum hydrogen productions obtained by *Chlamydomonas reinhardtii* (CC425) and *Chlamydomonas moewusii* (SAG 24.91) were 2.69 mmol/L and 0.78 mmol/L respectively. The hydrogen productivities achieved by *Chlamydomonas reinhardtii* (CC425) and *Chlamydomonas moewusii* (SAG 24.91) were 17.02 μmol/L/h and 5.12 μmol/L/h respectively. Thus, the sulphur-deprived medium can enhance the hydrogen production.

In addition, the bio-photolysis process to produce biohydrogen is inefficient due to the limitation of microalgae growth and low conversion efficiency of photosynthetic mechanism. Hydrogen is generated from proton in bio-photolysis process; hence, the efficiency of photon conversion is vital. Although direct bio-photolysis had a photon conversion of 80 %, the optimum photon conversion efficiency achieved by green microalgae was only about 12.5 % [7]. In fact, the efficiency was quite low which at a rate of 1 % or below [29]. Furthermore, production of biohydrogen is also significantly affected by the efficiency of light absorption of microalgae. Microalgal growth and biomass can be increased by providing maximum light intensity. Microalgae can capture maximum light in a natural condition where the incident light is limited by building large chlorophyll antennas in photosystem. Nevertheless, the efficiency of photosynthetic decreased when the microalgae are exposed to a high light intensity. The photon absorption rate in the top layers of chlorophyll antennae molecules is substantially faster than the rate at which the photons are employed for photosynthesis at high light intensities, which causes the excessive photons to be dissipated via fluorescence or heat [30].

3.2. Indirect bio-photolysis

Indirect bio-photolysis is introduced in order to overcome the challenge faced by the direct bio-photolysis, Microalgae undergo indirect biophotolysis in anoxic environment to generate hydrogen from accumulated glycogen and starch via fermentation or respiration process [9,14]. The indirect bio-photolysis method involves two steps to generate hydrogen. Fig. 2 displays the schematic representation for indirect bio-photolysis. Firstly, microalgae transform solar energy into chemical energy in the form of lipids, carbohydrates, and other organic compounds to increase the amount of endogenous substrate and generate oxygen as a by-product [7]. In the second step, the metabolism utilizes the fixed carbon that presents as carbohydrates to produce a large amount of electrons. The electrons are then transported immediately to the Plastoquinone pool and PSII. The electron transport chain stops working when there is a lack of oxygen. When oxygen is exhausted, an anaerobic environment is created and the oxygen-sensitive hydrogenase enzyme is activated. The free electrons are then transported to PSI, ferrodoxin (Fd), and hydrogenase, which then use them to generate hydrogen [31]. The reactions are represented by the equations below:

$$Aerobic\ reaction: 6H_2O + 6CO_2 + Solar\ energy \rightarrow C_6H_{12}O_6 + 6O_2 \quad (3)$$

$$Anaerobic\ reaction: C_6H_{12}O_6 + 6H_2O + Solar\ energy \rightarrow 6CO_2 + 12H_2 \quad (4)$$

Vargas et al. [32] had studied the generation of hydrogen through indirect biophotolysis with cyanobacteria, *Anabaena* sp. (UTEX 1448), by utilizing glucose as a substrate. The maximum hydrogen production and hydrogen productivity obtained were 9.73 mmol/L^3 and 67.07 μmol/L/h. The advantages of indirect bio-photolysis includes the oxygen sensitivity problem can be solved as the evolutions of hydrogen and oxygen are a separate process. Additionally, it can generate more hydrogen in comparison to direct bio-photolysis [6]. However, indirect biophotolysis still produces relatively limited hydrogen due to hydrogen consumption by the uptake hydrogenase [33].

3.3. Dark fermentation

In contrast to the photosynthetic process, an excessive amount of hydrogen can be generated without the presence of light at ambient pressure during the dark fermentation. The process is classified as light-independent. During this process, the organic substances will be converted into simple substances through a variety of processes. Fig. 3 shows the flow diagram of treating wastewater to produce biohydrogen from dark fermentation. For instance, the carbohydrates are transformed into reducing sugar via hydrolysis. Then, the simple products are acidified by the enzymes secreted by the fermentative microbes. Next, the products undergo acetogenesis process in which they are further converted into biohydrogen and acetate [9,23]. Biohydrogen is generated as an intermediate by-product at the previous process and it is then utilised as electron donor in the methanogenesis process to produce methane. Thus, the biohydrogen produced must be captured before the

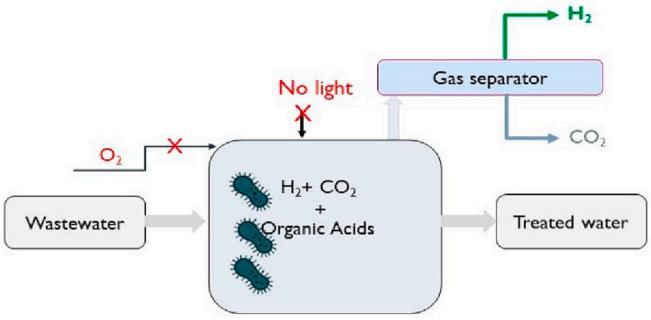

Fig. 3. Flow diagram of dark fermentation process. Reprinted from [34]. Copyright 2021, with permission from MDPI.

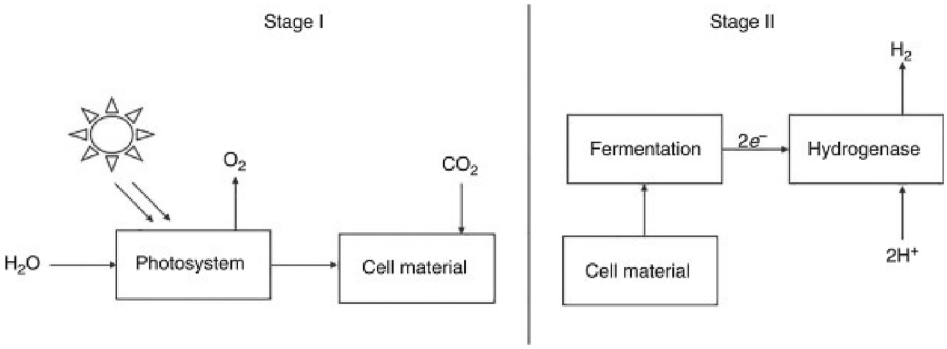

Fig. 2. Schematic diagram of indirect bio-photolysis. Reprinted from [22]. Copyright 2022, with permission from Elsevier.

methanogenesis reaction [21]. The overall dark fermentation process is presented by the equation below:

$$C_6H_{12}O_6 + 6H_2O \rightarrow 6CO_2 + 12H_2 \quad (5)$$

The *Enterobacter aerogenes* had been found could produce 36.08 mL of Hydrogen/g raw material by utilizing corn stover as the substrate through dark fermentation [35]. Besides, Lin et al. [36] discovered the improvement in hydrogen yield in dark fermentation by employing *Enterobacter aerogenes* as fermentative bacteria and glucose as substrate with the addition of furfural in low concentration. Hydrogen yields surprisingly increased to 193.7 mL/g when 5 mM of furfural was added as opposed to 163.5 mL/g (without addition of furfural) during the process. The dark fermentation is appealing as it is environmetally benign and practical. Besides, it utilizes various sources of organic carbon to synthesis hydrogen without the presence of light. Furthermore, there is no issue with oxygen inhibitory. Nevertheless, it is not widely adopted due to the decent yield of hydrogen. In dark fermentative process, the main issue is the incomplete conversion of organic substrates utilized to produce carbon dioxide and hydrogen gases. The organic acids including acetic acid, butyric acid, and lactic acid are as well formed and served as the major end products of the fermentative process in which have resulted in the low yield of hydrogen; besides, hydrogen is also produced as one of the by-products. There is only 15 % of the total energy being transformed into hydrogen, and the remaining 85 % is found being trapped in organic substrates that are usually escaping in the effluent which could be hazardous to the environment. Nevertheless, this shortcoming can be resolved by combining dark fermentation with a microbial fuel cell (MFC), which further generates electricity from the remaining energy of organic substrates produced during fermentation [37]. Furthermore, the carbon dioxide separation is also required throughout the process because a mixture of hydrogen and carbon dioxide gases is generated throughout the fermentative process.

3.4. Photo-fermentation

Photo-fermentation is the fermentative process that converts the organic substances into biohydrogen as well as carbon dioxide when light is present. This mechanism uses sunlight as an energy source rather than glucose. Generally, the purple non-sulphur photosynthetic (PNS) bacteria perform photo-fermentation by utilizing solar energy to transform carbon compounds such as organic acids into biohydrogen and carbon dioxide through nitrogenase enzymes [38,39]. Fig. 4 shows a process flow diagram for photo-fermentation. The organic acids are first oxidised to generate protons, electrons and carbon dioxide. Then, the electrons generated are transported via electron carrier in the membrane and protons are simultaneously pumped across the membranes to create a proton gradient, which subsequently generating energy in the ATP form. The nitrogenase enzyme uses the energy in the ATP form to catalyse the hydrogen gas generation from protons [40]. When molecular nitrogen is present, the purple non-sulfur photosynthetic (PNS) bacteria reduce the molecular nitrogen into ammonia via nitrogenase enzyme. However, only one molecule of hydrogen is generated for every molecule of nitrogen that is fixed into ammonia. Conversely, four molecules of hydrogen are generated under nitrogen-limited or nitrogen deficient condition as nitrogenase enzyme loses its ability to fix nitrogen [41]. The reactions of nitrogense enzymes in the presence or absence of molecular nitrogen are described by Equations (6) and (7). Purple non-sulfur photosynthetic (PNS) bacteria are regarded as the greatest hydrogen producers due to their high yields from the substrates' conversion [38]. The equation (8) below can be used to describe the conversion of acids into hydrogen:

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

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

$$CH_3COOH\ (aceticacid) + H_2O + Sunlight \rightarrow 2CO_2 + 4H_2 \quad (8)$$

The photosynthetic bacterial HAU-M1 had been found producing 141.42 mL of Hydrogen/g raw material by using corn stover as substrate [35]. Besides, Assawamongkholsiri et al. [42] used sugar manufacturing plant wastewater as the carbon source to investigate the production of hydrogen by *Rhodobacter* sp. KKU-PS1 and the maximal hydrogen yield of 2614 mL of Hydrogen/L was achived. García-Sánchez et al. [43] utilized tequila vinasses as a substrate to generate hydrogen via photo-fermentation with Rhodopseudomonas pseudopalustris DSM 123. The maximal hydrogen yield of 2249 mL Hydrogen/L was obataind at a light intensity of 270 W/m^2. One of the benefits of photo-fermentation is the pollutants can be removed from the wastewater as different organic wastes can be utilized and converted into biohydrogen and carbon dioxide. Furthermore, it could be employed during the second stage in order to obtain more hydrogen from dark fermentation of effluents [44]. Although nitrogen deficient or nitrogen limited condition is preferable for biohydrogen production via photo-fermentation, partial nitrogen condition is required. Nitrogen is a crucial component for purple non-sulfur photosynthetic (PNS) bacterial growth by aiding cells to produce compounds that contain nitrogen [45]. Moreover, the industrial effluent needs to be pre-treated as it may be hazardous, causing inhibition [14]. Besides, photo-fermentation requires sufficient ATP input

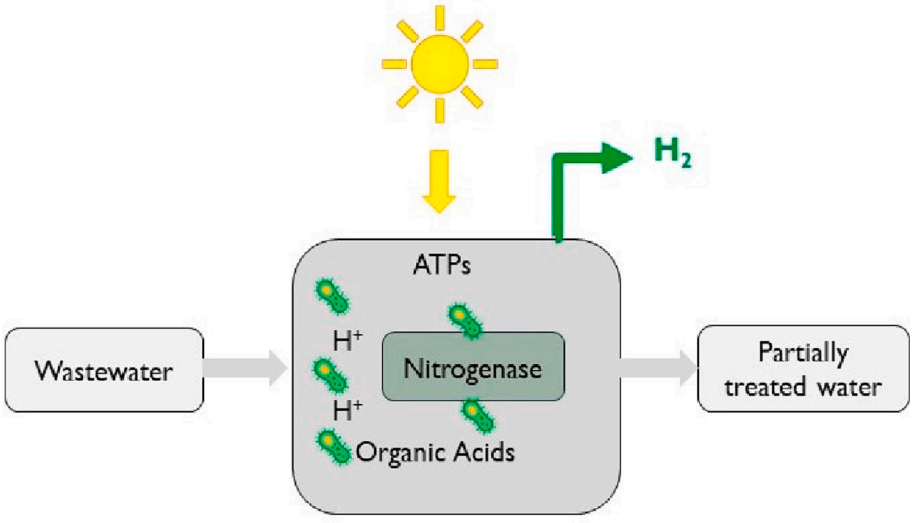

Fig. 4. Flow diagram of photo-fermentation process. Reprinted from [34]. Copyright 2021, with permission from MDPI.

because nitrogenase uses a large amount of energy to produce biohydrogen. Therefore, the efficiency of photo-fermentation is directly influenced by the effectiveness of light conversion [22].

3.5. Microbial fuel cell

The Microbial fuel cells (MFCs) are technologies that oxidise organic and inorganic substrates via employing exoelectrogenic microorganisms as catalysts to produce electric current. The MFCs are also known as microbial photo-electrochemical fuel cell (MPFC). MFCs consist of a cathode and anode that are split by an ion exchange membrane. The microorganisms are found in the anaerobic anode compartment where they produce electrons, protons and carbon dioxide by oxidizing the organic substances [46]. Then, the electrons migrate from anode to cathode over an external circuit to generate electricity, while the protons are delivered to the cathode via the membrane. For the cathode reaction, oxygen is frequently used as an electron acceptor. Under typical operational condition, the electrons will eventually combine with protons and dissolved oxygen to form water. Fig. 5 presents the flow diagram of microbial fuel cell. The half-reactions for MFC under normal conditions are:

$$Anode: n\ CH_2O\ +\ n\ H_2O \rightarrow n\ CO_2 +\ 4n\ e^- +\ 4n\ H^+ \quad (9)$$

$$Cathode: n\ O_2 +\ 4n\ e^- +\ 4n\ H^+ \rightarrow 2n\ H_2O \quad (10)$$

However, the hydrogen production from the protons and electrons generated by microbial metabolism is thermodynamically undesirable. To produce hydrogen in a conventional MFC, a voltage of about 0.23 V or more must be provided to the anodic potential to overcome the thermodynamic barrier. In this phase, hydrogen is generated when protons and electrons from the anodic reaction combine at the cathode. Besides, the oxygen present at the cathode chamber should be eliminated to avoid the leakage of oxygen to the anodic chamber[47]. The half-reactions for MFC with additional voltage supply are as follows:

$$Anode: n\ CH_2O\ +\ n\ H_2O \rightarrow n\ CO_2 +\ 4n\ e^- +\ 4n\ H^+ \quad (11)$$

$$Cathode: 4n\ e^- +\ 4n\ H^+ \rightarrow 2n\ H_2 \quad (12)$$

Exoelectrogenic yeasts are a possible alternative to exoelectrogenic bacteria in MFCs to increase the biocatalytic activity of MFCs. Yeast *Cystobasidium slooffiae* JSUX1 had been reported to produce 67 mW/m^2 of electricity and 23 L/m^3 of hydrogen simultaneously by using xylose as the source of carbon [48]. The advantages of using MFCs to produce biohydrogen include it can use a variety of complicated biomasses as a substrate, direct conversion of the substrate into energy and it uses microorganisms as a biocatalyst. One of the drawbacks of this system is the requirement of additional power supply to drive the hydrogen evolution reaction at cathode. Besides, the operating cost of MFCs is high because the utilization of pricey electrode materials, separator materials and catalyst.

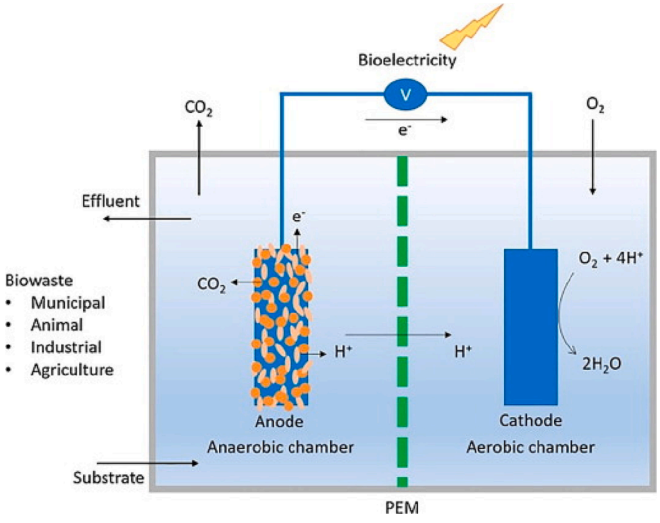

Fig. 5. Flow diagram of microbial fuel cell. Reprinted from [49]. Copyright 2018, with permission from Elsevier.

3.6. Microbial electrolytic cell

The microbial electrolytic cell is a hybrid system that able to produce more hydrogen than biophotolysis and fermentation processes due to the occurrence of photosynthetic and non-photosynthetic microorganisms. Microbial electrolytic cell (MEC) is a modified microbial fuel cell where electric current is required for anodes to promote the bacterial oxidation. The main distinction between MFC and MEC is that the former generates electricity through microbial decomposition, while the latter initiates microbial decomposition using electricity. The microorganisms in MECs serve as electrocatalysts, accelerating the electrochemical reaction [50]. Common MEC structures are divided into membrane-less single chambered and two-chambered versions which are illustrated in Fig. 6. MECs contain two electrodes which are anode and cathode. The electrodes can either be installed together in a single chamber or separately in two different chambers. In the double chambers MEC, a proton exchange membrane is typically utilised to split the two chambers. The active microorganism oxidize the organic substrate to generate protons, electrons, and carbon dioxide in the anode chamber of MEC. The generated electrons are carried to the cathode over the external circuit, while the protons are delivered directly to the cathode through the membrane. Then, the electrons react with protons in the solution to create hydrogen. The equations below show the production of hydrogen from acetate via MEC:

$$Anode: CH_3COOH\ +\ 2H_2O \rightarrow 2CO_2 +\ 8e^- +\ 8H^+ \quad (13)$$

$$Cathode: 8e^- +\ 8H^+ \rightarrow 4H_2 \quad (14)$$

$$Overall: CH_3COOH +\ 2H_2O \rightarrow 4H_2 +\ 2CO_2 \quad (15)$$

Gandu et al. [52] investigated the hydrogen production rates of single-chamber MECs with immobilized *Geobacter sulfurreducens* at anode and non-immobilized *Geobacter sulfurreducens* at anode by utilizing acetate as the substrate. Alginate and a mixture of alginate and chitosan were used to immobilize the bacterial anode. They reported that the MEC with non-immobilized *Geobacter sulfurreducens* at anode obtained the highest hydrogen production rate of 0.58 m^3/m^3/d. In contrast, the MEC with alginate *Geobacter sulfurreducens* at anode and alginate and chitosan *Geobacter sulfurreducens* at anode obatined the hydrogen production rates of 0.43 m^3/m^3/d and 0.39 m^3/m^3/d, respectively. The commercial hydrogen generation with MEC technology faces a number of significant challenges. One of the challenges is the use of high catalytic and high cost catalyst such as platinum on the surface of cathode to boost hydrogen production and decrease the overpotential to activate the hydrogen evolution reaction (HER) [53]. In this context, transition metals are gaining popularity as an efficient candidate of catalysts for cathodic hydrogen evolution reaction due to their availability, cheap, and minimal toxicity to microorganisms. The frequent used carbon-based electrodes showed poor wettability high cost when they are loaded with catalyst. Thus, biocathodes are developed as an ideal and cheaper electrode material. In biocathode, the surface of cathode serves as an electron acceptor for the microbes to catalyse hydrogen evolution reaction by combining produced protons and electrons to generate hydrogen. Finally, additional voltage supply is required to synthesize hydrogen at the cathode because the process is a non-spontaneous reaction [54]. Theoretically, an extra voltage of a minimum 0.114 V must be applied to drive the hydrogen evolution reaction in the MEC system.

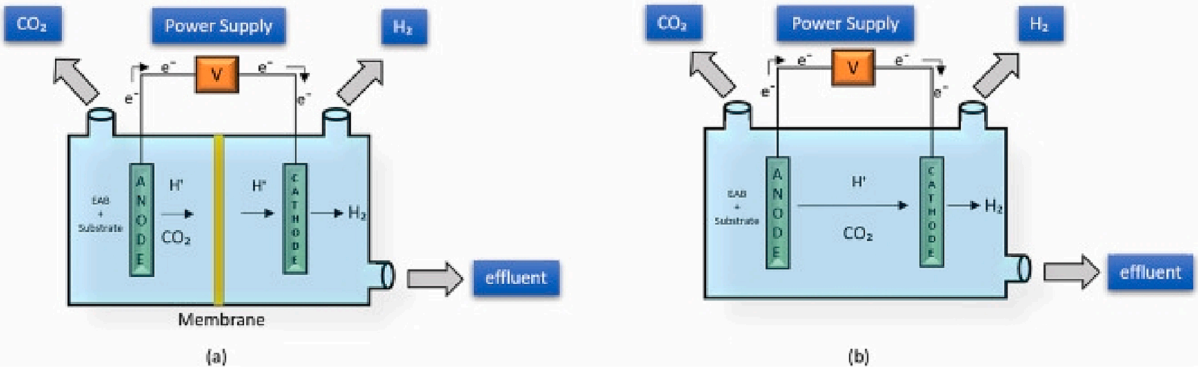

Fig. 6. Microbial electrolytic cells: (a) Two-chamber MEC and (b) Single-chamber MEC. Reprinted from [51]. Copyright 2021, with permission from Elsevier.

3.7. Microbial photo-electrochemical cell

Microbial photo-electrochemical cell (MPEC) is a modern technology that uses synergistic microbial decomposition of organic substances to produce sustainable biohydrogen [53]. Electrochemical active microorganisms such as *Shewanella putrefaciens* and *Aeromonas hydrophila* are employed in the microbial photo-electrochemical cell to produce electrons from the organic components [23]. MPEC is made of semiconductors (electrodes) that are divided by a membrane. MPECs can be classified into two types which are photocathode MPECs and photobioanode MPECs. Fig. 7 and Fig. 8 show the microbial photoelectrochemical cell with photocathode and microbial photoelectrochemical cell with photobio-anode, respectively. In the case of bio-anode MPECs, the active microorganisms produce electrons electrochemically from the organic substrates. Next, the electrons are transferred to the cathode. In photocathode MPECs, electrons and holes are produced at the conduction-band and the valence-band, respectively and separately, when a photon from sunlight is absorbed by the photocathode [55]. Then, the electrons produced at the conduction band are utilised to initiate the reduction of hydrogen process and the holes created at the valance-band are occupied by the electrons generated from the bio-anode. The additional power source is no longer required in MPEC system due to the high conduction-band position of semiconductor [56]. According to Wang et al. [57], titatnium dioxide (TiO_2) was an efficient photocatalyst for hydrogen production because of its high photocatalytic performance, stability, cheap, and non-toxicity. When TiO_2 was utilised as a photocathode under UV irradiation, a yield of 3.5 μmol/h of hydrogen was produced in 200 mL chamber without applying external voltage [53].

For photo-bioanode MPECs, solar energy and bioenergy are incorporated at the same electrode. A photo-bioanode is a two-sided electrode with a semiconductor side that is exposed to light, and a back side that is

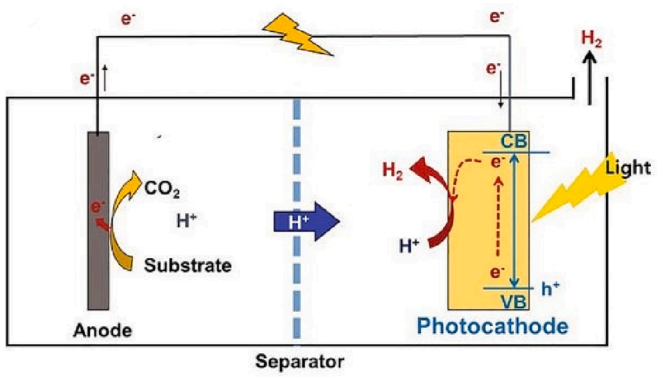

Fig. 7. Microbial photoelectrochemical cell with photocathode. Reprinted from [53]. Copyright 2021, with permission from Elsevier.

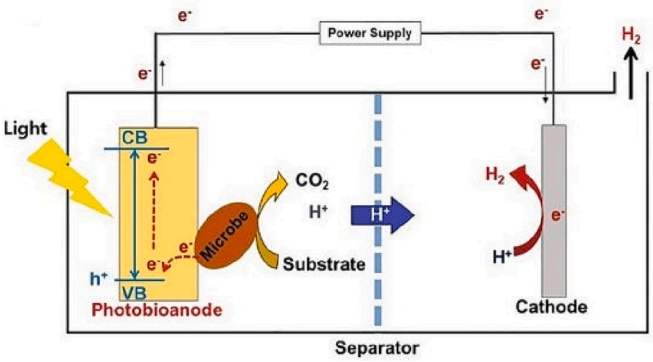

Fig. 8. Microbial photoelectrochemical cell with photo-bioanode. Reprinted from [53]. Copyright 2021, with permission from Elsevier.

in contact with electrogenic microorganisms [58]. Generally, the semiconductor side absorbs light to produce electron-hole pairs, while the conversion of organic substrates into electricity by the microbial through bio-catalysis happens at the back side. Then, the created electrons are delivered to the cathode via an external circuit to initiate the cathode reduction reaction. Pophali et al. [59] proposed to use the metal-free CeO_2-rGO based carbon film as a photo-bioanode in the MPEC and hydrogen was generated at a rate of 5 $m^3/m^3/d$ was obtained. There are currently very few researches using photo-bioanodes to produce energy or hydrogen. Thus, more researches on photo-bioanode in producing hydrogen and electricity should be explored.

Lu et al. [60] developed a novel MPEC that installed with a Fe_2O_3 photobio-anode and a black-silicon (b-Si) photocathode to improve the electricity and hydrogen generation from wastewater. The two photoelectrodes generated photoelectrons and holes with increased absorption of light using sunlight. The hybrid MPEC achieved a hydrogen production rate of 5.1 μmol/h/cm^2 (113 L/h/cm^2) and attained a maximum current density of 0.8 mA/cm^2. The advantages of using MPECs include the treatment of organic-rich wastewaters without the need of consumable chemicals and high durability and stability of typical catalysts. In addition, the additional power supply is not required to drive the hydrogen evolution reaction. Nevertheless, MPEC technology is presently in the early stage of research and confronting significant difficulties such as high construction and operating costs and the need of bioanodes with large surface area conducting material [61]. Besides, it shows poor hydrogen production as compared with other methods. For examples, the maximum hydrogen production rates from brewery wastewater treatment by MPECs with black silicon photocathode and a microbial anode were between 0.31 and 0.43 L/L/d, while the maximum hydrogen production rates in dark fermentation of brewery wastewater was 8.67 L/L/d [34,62].

3.8. Comparison of biohydrogen production mechanisms

Table 2 shows a comparison of various biohydrogen production mechanisms based on the types of microorganisms, substrates utilized and end-products from the processes. Besides, the benefits and drawbacks of each process are highlighted in Table 2 as well. Generally, the synthesis of biohydrogen requires lesser energy than hydrogen production from fossil fuels because the biological mechanisms can occur at ambient temperature and pressure [21]. The formation of hydrogen can be as well promoted by the microorganisms by altering its cells' metabolic activities.

Table 3 summarizes the hydrogen yields and hydrogen production rates of various mechanisms of biological hydrogen generation. The hydrogen yields and hydrogen production rates have been converted into the units of mmol/L and μmol/L/h, respectively, to ease the comprehension. Bio-photolysis shows the lowest hydrogen production rate, while microbial electrolysis cell demonstrates the highest hydrogen production rate among other when comparing the microbial mechanistic processes. The low yield of hydrogen in bio-photolysis process is mostly related to the oxygen sensitivity of hydrogenase and poor light conversion efficiency.

4. Microalgal enzymes involve in biohydrogen production

The two main enzymes involve in producing biohydrogen from diverse microalgal species include hydrogenase and nitrogenases which served as the catalysts in biological reactions. Wang et al. [63] had reported that the effectiveness of [FeFe]-hydrogenase and [NiFe]-hydrogenase in producing biohydrogen was found to be greater than that of nitrogenase.

4.1. Hydrogenase

Hydrogenase is the enzyme that used to catalyze the reversible hydrogen oxidation reaction to produce protons and electrons. In addition, it is also served as terminal electron acceptor without oxygen in photosynthetic microorganisms [4]. Hydrogenase is able to produce hydrogen in the reversible reaction as follows [7]:

$$2H^+ + 2e^- \leftrightarrow H_2 \tag{16}$$

Hydrogenase can be catagorized into [Fe]-hydrogenases, [FeFe]-hydrogenases and [NiFe]-hydrogenases in which depending on the metal contents found in the active sites. [FeFe]-hydrogenases are usually participated in the reduction of proton, while [NiFe]-hydrogenases are mainly involve in oxidation of hydrogen [7]. The type of hydrogenase presents in the stroma of chloroplasts of eukaryotes is [FeFe]-hydrogenases [64]. Although [FeFe]-hydrogenases shows great performance in the hydrogen generation from green microalgae, it is higly sensitive towards oxygen [2]. Thus, it is challenging to generate hydrogen under the atmospheric environment [65].

On the other hand, the [NiFe]-hydrogenases are usually found in prokaryotes like cyanobacteria, archaea and anaerobic bacteria. The majority of [NiFe]-hydrogenases are uptake hydrogenases which involve in the oxidation of hydrogen. Based on Khetkorn et al. [65], the [NiFe]-hydrogenases are more tolerant towards oxygen as compared with [FeFe]-hydrogenases as an absolute anaerobic environment are not required. Oxygen-tolerant hydrogenases are hydrogenases that maintain the oxidation of hydrogen and/or proton reduction in the presence of oxygen.

In short, the [FeFe]-hydrogenases are typically used in production of hydrogen, while [NiFe]-hydrogenases are involved in consumption of hydrogen. Numerous [NiFe]-hydrogenases are reversibly inactivated by oxygen, and they can be reactivated after the removal of oxygen. Contrarily, [FeFe]-hydrogenases are irreversibly deactivated by a small quantity of oxygen because they are highly sensitive towards oxygen [66]. Nevertheless, [FeFe]-hydrogenases are recognized as the most potent enzymes to produce hydrogen as [FeFe]-hydrogenases are 1000 times more active than nitrogenases and approximately 10–100 times more active than [NiFe]-hydrogenases [4].

4.2. Nitrogenase

Nitrogenase is a metalloenzyme system that only exist in prokaryotes like cyanobacteria (blue-green microalgae) due to the present of heterocysts. There are three metal metallic cofactors in the structure of nitrogenase, namely, iron-sulfur cluster, P cluster, and Fe-Mo cluster. The iron–sulfur cluster is responsible to transfer electrons to the Fe-Mo cluster through the P cluster, while the Fe-Mo cluster acts as an active site for the reduction of dinitrogen into ammonia. The role of nitrogenanses is to transform atmospheric nitrogen into ammonia and produce hydrogen as a byproduct [65]. The biological reaction is explained by the equation below:

$$N_2 + 8H^+ + 8e^- \rightarrow 2NH_3 + H_2 \tag{17}$$

Hydrogen production by nitrogenase used a significant amount of energy and the reaction is irreversible. In order to produce one mole of hydrogen, at least four moles of adenosine triphosphate (ATP) are required. Li et al. [4] had stated that the basis of hydrogen generation via nitrogenase system was the nitrogenase continuously converting protons into hydrogen without the presense of additional chemicals. Each cycle involves the hydrolysis of two moles of ATP in response to each electron transport. Nitrogenase has a lower turnover rate than hydrogenase and it consumes more electrons, ATP molecules, and reductants from the photosynthesis or breakdown of internal substances. Hence, nitrogenase is not a particularly efficient mechanism to generate hydrogen from a metabolic approach due to the low turnover rate, the significant energy inputs required for biosynthesis, and the demand of ATP for catalysis [4]. Additionally, the generation of hydrogen requires the elimination of ammonia (product) and an anaerobic environment [67].

5. Key parameters to produce biohydrogen from microalgae

The generation of biohydrogen requires a certain pathway to activate particular metabolites and boost each step in order to allow the production process economical and sustainable [68,69]. The processes must meet certain requirements related to the light intensity, cell concentration, substrate utilization, pH medium, and temperature.

5.1. Light intensity

Light intensity is crucial in biophotolysis and biohydrogen production which is similar to photosynthesis. In general, photoysnthesis usually occurs in two succesive steps which are light reaction (light-dependent reaction) and dark reaction (light-independent reaction or Calvin cycle). During the light reaction, the ATP and NADPH are produced from the water splitting reaction in the presence of light. Then, both ATP and NADPH will aid in the generation of endogeneous substrates in the dark reaction. The dark reaction takes place continuously, and light is not required for dark reaction as water photolysis does not happened. Thus, the dark reaction can occur either with or without the presence of light. In indirect bio-photolysis, microalgae use light to facilitate photosynthesis and promote the formation of starch and lipids durring the light cycle. Consequently, excessive amounts of endogenous substrates are formed. Endogenous substrates are generated in the light cycle only and it is responsible in enhancing the production of biohydrogen. During the dark cycle, the endogenous substrates produced are consumed by PSII under anaerobic environment. In order to achieve the anaerobic condition quickly, the microalgal cells are momentarily exposured to the high intensity of light [70]. High light intensity induces photoinhibition and results in the low oxygen production. It is known that photoinhibition at high light intensities causes damage to PSII

Table 2
Comparison of various microbial mechanisms employed for biohydrogen production.

Mechanism	Microbial species	Substrate	Light requirement	Gas Produced	By-product	Advantage	Disadvantage
Direct bio-photolysis	Cyanobacteria and green algae	H_2O	Yes	• O_2 • H_2	• O_2	• H_2O is used as simple substrate • Consumption of CO_2	• Highly oxygen sensitivity • High light intensity is required
Indirect bio-photolysis	Cyanobacteria and green algae	H_2O	Yes	• O_2 • H_2 • CO_2	• O_2 • CO_2	• Separate O_2 production from hydrogen generation requirement • Conversion of metabolite by-products to hydrogen • N_2 fixing capability from air	• Highly oxygen sensitivity • High light intensity is required • Low yield of hydrogen • Production of CO_2
Dark fermentation	Fermentative bacteria such as *Escherichia coli, Clostridia, Enterobacter*	Simple sugars/organic waste	No	• H_2 • CO_2 • CH_4 • CO • H_2S	• Volatile fatty acids • Ethanol • Methanol • Acetate	• Light-independent process • No oxygen sensitivity issue • Utilization of different carbon sources	• Inefficient substrate conversion • Relatively low hydrogen yield • Low hydrogen purity in the mixture of gaseous products
Photo-fermentation	Photo-heterotrophic bacteria such as *Rhodopseudomona, Rhodobacter*	Simple sugars/organic waste	Yes	• H_2 • CO_2	• Volatile fatty acids • Ethanol	• Use of dark fermentation-produced organic acids • Microorganisms utilise organic waste and a broad light spectrum.	• Low light conversion efficiency • Requirement of sufficient ATP supply
Microbial fuel cell	Electrochemically active bacteria such as *Aeromonas hydrophila, Shewanella putrefaciens*	Acetate/glucose/organic waste/fermentation effluent	Yes	• H_2 • CO_2	• CO_2	• Able to convert substrate energy into electricity directly • No require gas treatment • Capable of treating a range of chemical substrates at different concentrations.	• High capital cost • Require external voltage to drive the hydrogen evolution reaction • Require the growth of microorganisms at high temperature
Microbial electrolysis cell	Exoelectrogenic bacteria such as *Geobacteraceae*	Organic waste/dark fermentation effluent/ acetate	Yes	• H_2 • CO_2 • CH_4 • H_2S	• CH_4 • Formic acid • Hydrogen peroxide	• High chemical oxygen demand elimination • High yield of biohydrogen • High substrate degradation	• High capital cost • Use of catalyst for the electrode. • Difficult to scale up • Require the growth of microorganisms at high temperature and low pH • Require external voltage to drive the hydrogen evolution reaction • Methane production reduces the production of hydrogen
Microbial Photoelectrochemical cell	Electrochemical active microorganisms	Organic waste	Yes	• H_2 • CO_2	• CO_2	• Utilize sunlight and treat organic pollutants without the use of extra chemicals • Able to treat a variety of wastewaters • Extra power supply is not required	• The current density is smaller than solar hydrogen conversion • Require photobio-anode with high surface area conducting substance • Low hydrogen yield

Table 3
Comparison of biohydrogen productive efficiencies among different microbial mechanisms.

Mechanism	Microbial species	Carbon substrate	Hydrogen yield	Hydrogen production rate	Reference
Direct bio-photolysis	Chlamydomonas reinhardtii Chlamydomonas moewusii SAG 24.91	–	2.69 mmol/L 0.78 mmol/L	17.02 μmol/L/h 5.12 μmol/L/h	[28]
Indirect bio-photolysis	Anabaena sp. UTEX 1448	–	9.73 mmol/L	67.07 μmol/L/h	[32]
Dark fermentation	*Enterobacter aerogenes*	Glucose	193.7 mL/g or 8.64 mmol/L	10.4 mL/g/h or 464 μmol/L/h	[36]
		Corn stover	36.08 mL H_2 /g raw material or 1.61 mmol/L	1.37 mL/(g TS)/h or 61.12 μmol/L/h	[35]
Photo-fermentation	*Rhodopseudomonas pseudopalustris* DSM 123	Tequila vinasses	97.6 mmol/L	11.7 mL/L/h or 522 μmol/L/h	[43]
	HAU-M1	Corn stover	141.42 mL H_2/g raw material or 6.31x 10^3 mmol/L	6.21 mL/(g TS)/h or 277 μmol/L/h	[35]
	Rhodobacter sp. KKU-PS1	Sugar manufacturing plant wastewater	2614 mL H_2/L or 117 mmol/L	5.24 mL/L/h or 234 μmol/L/h	[42]
Microbial fuel cell	Yeast *Cystobasidium slooffiae*	Xylose	23 L/m^3 hydrogen gas or 1.03 mmol/L	–	[48]
Microbial electrolysis cell	*Geobacter sulfurreducens*	Acetate	–	0.58 m^3/m^3/d or 1078.87 μmol/L/h	[52]
Microbial photoelectrochemical cell	Electroactive biofilm	Wastewater	–	5.1 μmol/h/cm^2 or 510 μmol/L/h	[60]

components, while PSI is generally stable to produce electrons from water splitting [9]. Besides, the enzymes are activated quickly in this environment by accelerating the consumption rates of sulphur and oxygen in culture medium and degrading the microalgal stored proteins and starch [2,23]. Typically, the amount of light present throughout the anaerobic process determines the optimal hydrogen production.

Chlamydomonas reinhardtii can utilize solar energy to generate hydrogen from water under anaerobic environment. Kim et al. [71] exposed *Chlamydomonas reinhardtii* to various light intensities, tranging from 60 to 300 μE/m^2/s, under sulphur-deprived environment. It was found that the amount of hydrogen generated had increased as the light intensities increased to 200 μE/m^2/s and started to decrease thereafter. The maximum hydrogen production was 225 mL Hydrogen/L under the light intensity of 200 μE/m^2/s. Besides, the time needed to intiate the hydrogen production had dramatically decreased from 66 to 22 h as the light intesity increased. Vijayaraghavan et al. [72] investigated the impact of alternating photoperiods for growing *Chlamydomonas reinhardtii* under light driven sulfur-deprived condition. The photoperiod cycles used were 2, 3, and 4 h with alternately light and dark periods. During these photoperiods, the corresponding hydrogen contents were 47 %, 86 %, and 87 %. This indicated that light is necessary for the production of hydrogen.

5.2. Microalgae cell density

Microalgal cell density is responsible for regulating the quantity of light that enters the cells. It is important for promoting microalgal growth and hydrogen production [2]. Cell density is determined by the type of cultivation methods which in turn influencing the hydrogen generations from microalgae. In addition, cell density is also influenced by other factors including the carbon dioxide concentration, the presence of organic or inorganic components, and the light penetration efficiency. Microalgae can be cultivated in autotrophic, mixotrophic, or heterotrophic modes [7]. Sajadian et al. [73] examined the production of biomass from the microalgae, i.e., *Chlorella sorokiniana*, in three various culture environments, and glucose was used as supplement in the heterotrophic and mixotrophic environments. Mixotrophic culture had produced the highest quantity of biomass dry weight which was 3.55 g/L, whereas photoautotrophic and heterotrophic cultures produced biomass dry weights of 0.68 g/L and 0.73 g/L respectively. In autrotrophic culture, microalgae use various inorganic chemicals and light energy to produce biomass. Autotrophic microalgae usually produce low density of biomass because the concentration of cells and light penetration are inversely correlated. Based on Jareonsin and Pumas [74], light only reached a few micrometres below the surface in the autotrophic culture which hindered cell growth. Heterotrophic microalgae have higher cell density and biomass production as they do not require light, while utilizing organic carbon as sources of energy and carbon. Mixotrophic microalgae grow by using both organic and inorganic carbon simultaneously. Microalgae that grow under mixotrophic condition can improve the cell density by fixing carbon dioxide and assimilating organic carbon [75].

It is crucial to have adequate cell concentration. When microalgae concentrations are too low, cultures cannot establish anaerobic conditions because total cell respirations are insufficient to absorb all dissolved oxygen [9]. According to Javed et al. [70], high cell density accelerated the rate of microalgal respiration, and subsequently, enhancing oxygen uptake and creating anaerobic environment. Nevertheless, if the microalgal culture has a very high cell concentration, the transition into anaerobic condition might happen quickly due to the intensive self-shading. Consequently, the accumulation of starch is limited, leading to the reduction of hydrogen productivity. When the cell concentration reaches 1.9 × 10^7 cells per mL, it is advisable to replace part of the culture with a new medium in decreasing the concentration to around 1–2 × 10^4 cells per mL to sustain an active growth phase whilst keeping microalgae as a preculture [2].

5.3. Substrate

Addition of substrates is ubiquitously required to increase the cell growth as well as production of hydrogen from microalage. The required substrates in a culture depend on the types of migroalgae growth modes which are heterotrophy, mixotrophy, and autotrophy. For instance, *Chlorella* can generate hydrogen through heterotrophic metabolism by utilizing both endogenous and exogenous substances [2,76]. Exogenous carbon source is crucial for growth of microalgae and it can enhance the production of hydrogen. The most significant endogenous substance is starch, that is produced during the initial phase of the photosynthesis, and is subsequently used to supply electrons for the formation of hydrogen. Moreover, microalgae can generate hydrogen from a variety of external carbon sources which include glucose, sucrose, fructose, acetate, and organic wastewaters. The initial concentration of glucose is important for the synthesis of biohydrogen. Based on El-Dalatony et al. [9], it had been found that 5 g/L of glucose is the ideal concentration for an effective biohydrogen generation. A report had found that 30 mM glucose was the optimum concentration because it shortened the lag time for the hydrogen production by around 10 h [77]. Besides, Sengmee et al. [78] had investigated the possibility to utilize crude glycerol as an external carbon source to generate hydrogen by *Chlorella* sp. under anaerobic enviroment. It was found that the *Chlorella* sp. produced the highest quantity of hydrogen which was 10.31 ± 1.85 mL/L at glycerol concentration of 16 g/L. In another study, Chlorella vulgaris MSU 01 strain was able to generate 220 mL/L of hydrogen by utilising corn stalk at the concentration of 4 g/L as the substrate [79]. Besides, the productions of hydrogen from Anabaena sp. CH3 by using different kinds of sugars such as glucose, sucrose, galactose and fructose as the substrates with a concentration of 2000 ppm each were investigated [80]. Fructose and sucrose were identified as the preferred substrates for Anabaena sp. CH3 to produce hydrogen which yielding 1.6 mmol and 1 mmol of hydrogen, correspondingly.

5.4. pH

The pH is the key physical parameter that affects the metabolic processes to generate biohydrogen from microalgae as a slight change in pH of the medium would influence the metabolic processes to generate hydrogen as well as the microalgae growth. In addition, pH also regulates the activities of enzymes and prevents the breakdown of endogenous substrate [2,23]. Optimum pH values are determined by the type of microalgae species. During photosynthesis, the initial pH drops as the carbonic acid is generated from the reaction of carbon dioxide and water. The pH value slowly increases later because the microalgae absorb the dissolved carbon dioxide [2,81]. When the pH level is low, hydrogen is generated by hydrogenase via the pyruvate-Fd-oxidoreductase pathway. However, ATP and acetate are produced instead of hydrogen because the [FeFe]-hydrogenase is inhibited [2,23]. In contrast, higher pH levels between 5.0 and 7.0 will speed up the hydrogen production process, resulting in a faster rate of hydrogen generation. Based on Javed et al. [70], microalgae could often grow in the pH range of 6.0 to 9.0 in which depending on the species. For instance, *Chlorella* sp. was observed to produce more hydrogen at a pH range of 7.0–9.0 and the maximal generation of hydrogen was found at a pH of 8.6. In the case of Chlamydomonas reinhardtii under sulfur deficiency environments, the pH of 7.7 had resulted in the highest rate and yield of hydrogen generation [82]. A pH value below 6 will shift the medium into an acidic environment in which may exert pressure on microalgae, and eventually causing the production of hydrogen to stop. For instance, the accumulation of acidogenic metabolites during dark fermentation results in decreasing of pH. Then, these acidogenic metabolites have the ability to penetrate the cell of hydrogen-producing microbes, leading to the growth discrepancy, increasing ionic strength, and cleaving the cell [83]. Consequently, the activities of hydrogen-producing microbes and hydrogen production are inhibited. On the other hand, the extreme basic medium with a pH greater than 9 also causes stress during the metabolic processes of microalgae. At these pH values, the photosynthesis will be hindered as inorganic carbon exists as carbonate (CO_3^{2-}) which cannot be consumed by microalgae. As a results, the growth of microalgae is retarded and the condition for hydrogen production may not be favoured [70].

5.5. Temperature

Temperature is among the physical factors that controls the microalgae growth and the capacity of a medium to produce hydrogen. It can increase the generation of biohydrogen by modifying the metabolic process [76]. Temperature regulates metabolism by facilitating enzymatic processes. Each enzyme has a range of ideal temperatures where it exhibits high activity. Intense temperatures would result in the denaturation of metabolic and life-sustaining enzymes. The biohydrogen synthesis metabolic process involves a number of hydrogenases, including uptake hydrogenase and hydrogen evolving hydrogenase. In a study, the activities of hydrogenases were investigated at four various temperatures which are 30 °C, 34 °C, 37℃, and 40 °C in fermentation hydrogen production by *Klebsiella pneumoniae* ECU-15 [84]. It was found that the cell growth, hydrogen production, and specific productivity of hydrogen increased with rising temperatures up to 37 °C, but subsequently declining with higher temperature. Besides, the activity of hydrogen evolving hydrogenase was observed to increase quickly and then declined over time. The hydrogen evolving hydrogenase achieved a maximum activity of 995.7 U/(G DCW) at 40℃ at fourth hour. In contrast, the uptake hydrogenase did not achieve maximal activity until the eighth to tenth hours. Hydrogenase was found to significantly enhance the production of hydrogen at 37 °C which obtained a maximal volume of 5363.8 mL/L.

The effective temperature ranges for biomass growth and hydrogen production are directly correlated with the types of microalgal species. Microalgae growth is exponentially increased by raising temperature to its optimum range, but an increase or drop in temperature beyond the optimum temperature slows or even eliminates microalgae activity [85]. High temperatures result in inhibition of cells' respiration, whereas low temperatures hinder with photosynthesis by reducing carbon. In addition, high temperature also inactivates the photosynthetic proteins, and subsequently hinders photosynthesis. As a result, the growth rate decreases as the photosynthesis decreases due to the decrease in the activity of enzyme ribulose-1,5-bisphosphate (Rubisco). Since enzymes are rudimentary proteins, they are denatured at high temperature. In addition, they are as well inactivated due to the disruption of active sites at high temperature. Basically, the temperature ranges can be divided into four categories which are ambient (15-30℃), mesophilic (30-39℃), thermophilic (50-64℃), and hyperthermophilic (greater than64℃) [86]. Based on Melitos et al. [87], the optimum temperature range for microalgae, cyanobacteria and PNS bacteria are 20-30℃, 25-55℃ and 25-35℃ respectively. For instance, microalgae such as *Chaetoceros* and *Anacystis nidulans* can survive temperatures of up to 40 °C in producing hydrogen [8].

6. Resolutions for challenges and future prospects of microalgal biohydrogen production

Pre-treatments of microalgae biomass are considered as an essential step to enhance the biohydrogen generation via dark fermentation. The pre-treatment processes primarily aim to disrupt the cell wall as well as release the organic materials from the cells. According to Shobana et al. [88], the microalgae biohydrogen generation through dark fermentation could be significantly increased to 50–70 % of theoretical values by using effective pre-treatment techniques. Besides, the methanogenic activity can be inhibited by employing pre-treatment such as thermal treatment. As a result, the hydrogen yield increases since the consumption of hydrogen has decreased [89]. There are various pre-

treatment methods in which can be divided into mechanical methods (bead milling, microwave, and ultrasonication), chemical methods (acid or alkaline treatment), thermal methods (steam explosion, and freezing and thawing) and biological method (enzymatic hydrolysis). The selection of pre-treatment techniques for employment is very much depending on the components in biomass feedstock. Table 4 shows the methods used for cell disruption as well as the benefits and drawbacks of each pre-treatment technology. Moreover, the addition of metals has been considered as another approach to improve the biohydrogen generation via dark fermentation. Trace metals are crucial for the anaerobic fermentation process, particularly for the activities performed by hydrogenase [67]. The addition of metals into fermentative medium will overall promote the transportation of intracellular electrons and provide alimentations for microbial developments. For example, the addition of nanoparticles such as nickel or iron could enhance the electron transport between ferredoxin and hydrogenase to initiate biohydrogen production. The introduction of metal ions, e.g. ferrous ions, ferric ions and nickel ions into fermentative medium can also spur the cell growth and production of biohydrogen via dark fermentation [67]. Furthermore, the cell immobilization is another technique used to promote biohydrogen production through fermentation processes. The fundamental of immobilization is that a solid support matrix is used to isolate the cells from the liquid growth medium [90]. The main techniques for immobilizing cells are adsorption, entrapment and encapsulation. Adsorption is the attachement of cells onto the surface of support materials; entrapment is a polymer assisted method in which the cells are entrapped into a polymer matrix; while encapsulation is incarcerating cells within a membrane that is semi-permeable, allowing chemicals and biochemcials to pass through it [91]. Among those, the entrapment method to immobilize cells is preferrable as it creates an anaerobic environment that encourages the biohydrogen generation. Liu et al. [92] discovered that the entrapment of photo-fermentative bacteria (*Rhodopseudomonas faecalis RLD-53*) into agar gel was not only could increase the biohydrogen production, but also improving its ability towards acid tolerance in which the biohydrogen still coud be produced at pH 5. The other advantages of cell immobilization include protecting the cells from external stress factors as well as increasing cell metabolic activities and cell densities, targeting to produce more biohydrogen [93].

Genetic engineering may aid to enhance the microalgae biohydrogen production in future. Mutation or modification of genetic by knocking in or out the particular genes are typically employed to increase the capacity and functions of the strains [10]. Modified microalgae strains have produced significantly more biohydrogen than the wild type. For example, the genetically modified strain of *Chlamydomonas reinhardtii* was observed to synthesize 5 to 13 times more hydrogen compared to the wild type [14]. Nonetheless, some non-specifuc strains might be produced occasionally during the genetic modifications and they might impact the growth and product yield [10]. Besides, genetic engineering may help to develope an oxygen-tolerant hydrogenase enzyme which is possible to achieve efficient hydrogen generation in both algae and cyanobacteria. Thus, further study is still required to prove the practicality of genetic engineering in enhancing the biohydrogen production from microalgae.

Dark fermentation is a promising approach to generate sustainable biohydrogen. It has advantages such as independent from light and low energy demands. Besides, the utilization of organic waste to produce biohydrogen by dark fermentation can significantly lower the cost of the process and handling these waste in a sustainable way. Moreover, it is also an approach to accomplish zero waste. However, dark fermentative biohydrogen production still faced some challenges. For instances, lignocellulosic biomass cannot be used directly by the microorganism to generate hydrogen. Pretreatment processes are required to break the lignocellulosic biomass into fermentable sugar before they are utilized by microbes to produce hydrogen. Furthermore, dark fermentation will produce a mixture of gases that require further a separation process. A membrane separation process is considered because of their low energy

Table 4
Advantages and disadvantages of various pre-treatment methods in disrupting the cells [88,89,96].

Pretreatment technique	Method of cell disruption	Advantages	Disadvantages
Bead milling	Cell disruption by collision with beads.	• High disruption efficiency. • Capable to treat wet biomass and ease the separation of components.	• Require high demand of energy. • Require to replace the beads periodically. • Thermal degeneration may result from heat generation.
Microwave	Cell disruption by heat and high pressure created by high-frequency shock waves and change in molecular interactions.	• High disruption efficiency. • Applicable for industrial scale. • Short pre-treatment time.	• High maintenance cost. • Require cooling due to excessive heat produced. • Change in non-covalent interactions could lead to alteration of component structures.
Ultrasonication	Cell disruption by heat and high pressure created by cavitation effects from ultrasonic waves.	• High disruption efficiency. • Minimum costs for maintenance. • Does not require additional component like beads or solvents. • Shorter pre-treament time.	• Require high demand of energy. • Difficult to scale up. • Require cooling due to excessive heat produced. • Not suitable to pre-treat all microalgal species.
Acid or alkaline treatment	Acid is used to hydrolyse cell wall polymers while alkaline is used to saponify cell wall lipids, resulting in cell disruption.	• Capable to pre-treat wet biomass. • Easy to scale up. • Low demand for energy.	• Denaturation of protein. • High possibility of corrosion. • Secondary pollution may occur.
Steam explosion	Cell disruption by explosion caused by steam exposure at high temperature and pressure, followed by depressurization.	• High disruption efficiency. • Low possibility of corrosion. • Low maintenance.	• Operate at high temperature and pressure. • Efficiency of cell disruptions varies by microalgal species.
Freezing and thawing	Large ice crystals are formed when biomass freezes, and it expands and damages the cell wall. Frequent freezing and thawing improve the cell disruption effectiveness.	• Operate at mild reaction conditions. • No requirement for homogenising the biomass. • Appropriate for removing sensitive components.	• Time consuming. • Cost and energy intensive. • Uncertain effectiveness of cell disruptions.

(*continued on next page*)

Table 4 (*continued*)

Pretreatment technique	Method of cell disruption	Advantages	Disadvantages
Enzymatic hydrolysis	Cell wall polymers and intracellular glucans are particularly destroyed by hydrolytic enzymes from bacteria and fungi.	• Operate at mild reaction conditions. • Low demand for energy. • Capable to pre-treat wet biomass. • High quality and purity of products.	• High cost of enzymes. • Long reaction time. • Require simple pre-treatment to increase the accessibility to polymers.

demand, mild operating requirements, and economix viability [94]. Nonetheless, the efficiency of separation with a particular membrane module in addition to the operating parameters is depending on the composition of gas [95]. The incomplete conversion of the substrate is also one of the limitations of dark fermentation but it can be solved by integrating it with other biological methods such as photofermentation or microbial electrolysis cells to enhance the hydrogen yield. To employ dark fermentation on a large scale, further investigation are required to make this process more effective, affordable and sustainable.

7. Conclusions

The biohydrogen production from microalgae has gained significant attention as a clean and sustainable fuel to achieve carbon neutrality and sustainability in nature. Biological methods are typically employed to produce microalgal biohydrogen. Besides, electrochemical approach are as well developed and improvised to generate biohydrogen from microalgae. The efficiency of biohydrogen production processes and yields are depending on several factors such as microalgae cell density, light intensity, pH, temperature and substrate introduced. However, these methods still encounter several major challenges such as the oxygen sensitivity of hydrogenase and low hydrogen yield which make the processes not sustainable. Genectic engineering, pre-treatment, chemical addition and cell immobilization are all effective ways to solve the isuues significantly. Generally, biohydrogen production from dark fermentation is more advantageous as compared with the other processes. Indeed, the low biohydrogen yield is the bottleneck suffered by dark fermentation for the adoption in large-scale application. Therefore, further research is needed to focus on improving the biohydrogen yields from microalgae before it can be deployed in a practical way.

CRediT authorship contribution statement

Jia Min Woon: Conceptualization, Writing – original draft, Investigation, Visualization. **Kuan Shiong Khoo:** Conceptualization, Writing – review & editing, Validation. **Mehdi Akermi:** Writing – review & editing, Funding acquisition. **Meznah M. Alanazi:** Writing – review & editing, Funding acquisition. **Jun Wei Lim:** Writing – review & editing, Conceptualization, Supervision. **Yi Jing Chan:** Writing – review & editing. **Pei Sean Goh:** Writing – review & editing. **Boredi Silas Chidi:** Writing – review & editing. **Man Kee Lam:** Writing – review & editing. **Juliana Zaini:** Writing – review & editing. **Muhammad Roil Bilad:** Writing – review & editing. **Yuguang Zhou:** Writing – review & editing. **Nurul Tasnim Sahrin:** Writing – review & editing. **Fatima Musa Ardo:** 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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