

Biohydrogen—A Green Fuel for Sustainable Energy Solutions

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Abstract: Energy plays a crucial role in the sustainable development of modern nations. Today, hydrogen is considered the most promising alternative fuel as it can be generated from clean and green sources. Moreover, it is an efficient energy carrier because hydrogen burning only generates water as a byproduct. Currently, it is generated from natural gas. However, it can be produced using other methods, i.e., physicochemical, thermal, and biological. The biological method is considered more environmentally friendly and pollution free. This paper aims to provide an updated review of biohydrogen production via photofermentation, dark fermentation, and microbial electrolysis cells using different waste materials as feedstocks. Besides, the role of nanotechnology in enhancing biohydrogen production is examined. Under anaerobic conditions, hydrogen is produced during the conversion of organic substrate into organic acids using fermentative bacteria and during the conversion of organic acids into hydrogen and carbon dioxide using photofermentative bacteria. Different factors that enhance the biohydrogen production of these organisms, either combined or sequentially, using dark and photofermentation processes, are examined, and the effect of each factor on biohydrogen production efficiency is reported. A comparison of hydrogen production efficiency between dark fermentation, photofermentation, and two-stage processes is also presented.

Keywords: energy; photofermentation; dark fermentation; microorganisms; biohydrogen; microbial electrolysis cell



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1. Introduction

1.1. Global Energy Scenario and Global Warming

All the social, physical, and economic activities of human life are sustained by energy. The continual energy supply needed for increasing global demand creates a substantial challenge for our societies. According to the IEO reference case [1], the world energy need is expected to rise by 50% from 2018 to 2050. This energy requirement has been satisfied primarily by exploiting massive fossil fuels [1].

Carbon-rich energy carriers (fossil fuels) are produced in two steps. The first step consists of photosynthesis, while the second consists of decomposing organic matter, which has been compacted for millions of years under high pressure and temperature. The ability of fossil fuels to act as efficient energy carriers and their easy transferability into different types of energy has made them the motor of the industrial revolution [2]. However, the other side of the picture shows that burning these fossil fuels creates a major drawback in the form of greenhouse gas emissions, contributing to global warming and climate change.

Global warming is the central issue of today's world because it continuously raises the Earth's temperature and drastically affects agriculture and food security [3,4]. The developing regions of the world are principally affected. The expected results may include social and political uncertainty, mass migration, and military conflicts. Sea level rise and ocean acidification are the other significant problems [5,6]. In addition, greenhouse gas emissions and climate change affect the globe and require solutions for clean and sustainable energy usage [7–9]. Considering the danger of global warming to future generations, the leaders of most countries have united to put forward their efforts to

decrease the emissions of greenhouse gases and minimize average global warming to 2 °C above the preindustrial average world temperatures during the United Nations Climate Change Conference (UNFCCC) and the Conference of the Parties (COP) 2015 in Paris [2].

1.2. Sustainable Energy Economy

The states and governments of the world consider the possibility of sustainable development not only in the context of industrial advancements but also for social, economic, and environmental concerns. According to Davidson, the definition of sustainable energy is as follows [10].

“Sustainable energy is defined as energy providing affordable, accessible and reliable energy services that meet the economic, social and environmental needs within the overall developmental context of the society for which the services are intended while recognising equitable distribution in meeting those needs.”

Energy, economy, and environment are the three pillars of sustainable development that join in the form of a triangle. Although renewable energy is considered the critical component of sustainable energy, it does not fulfill the requirements of sustainable development without knowing its significance to the economy and environment [11] and fulfilling the five targets mentioned in Figure 1 [11].

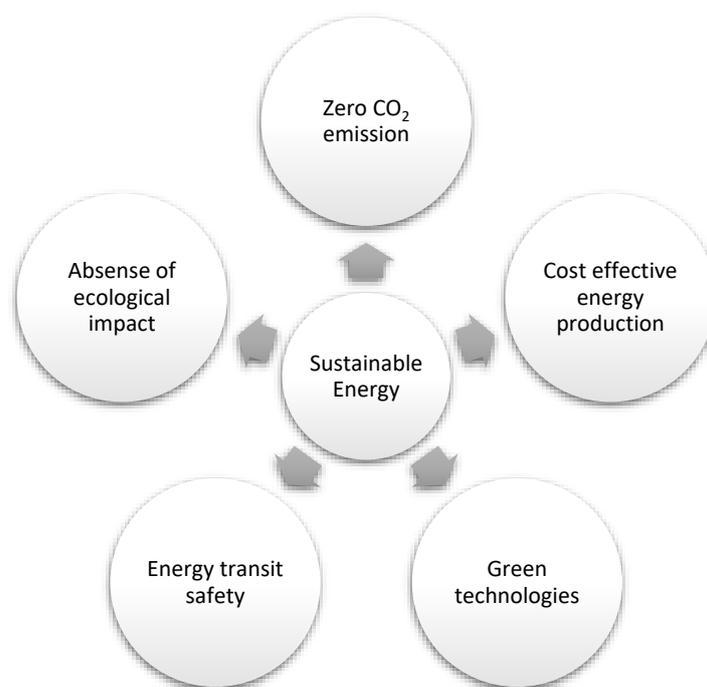


Figure 1. Five significant targets of sustainable energy.

Second-generation biofuels covered about 3% of the world’s energy requirements in 2013 [12]. They are considered more desirable fuels as there is no competition for land and food. Nevertheless, technical problems, such as low energy conversion efficiencies, are the foremost hurdle to their use. Microalgae are used as feedstock for the third generation of biofuels [13,14]. Microalgae are vigorous unicellular organisms that can be grown in wastewater or seawater and have higher energy efficiency than other fuels. However, a considerable amount of carbon dioxide is released during the burning and production of these biofuels, which is not compensated for by carbon dioxide fixation [2].

1.3. Sustainable Hydrogen Economy

Biohydrogen has become a promising biofuel in the modern era as it is a clean and efficient energy carrier. Hydrogen (H₂) has many benefits since it has the highest energy

per unit mass (142 kJ g^{-1}). Moreover, H_2 produces only water as a byproduct and is called zero-carbon fuel. It is also tasteless, odorless, colorless, and the lightest gas [15]. Therefore, H_2 gas is used in many applications, such as fuel cells for electricity generation and as fuel in rocket engines, including transportation applications [16].

Furthermore, H_2 is a significant industrial gas and raw material in various applications and processes [17]. Nevertheless, the high cost of production, storage problems, transportation, and an immature hydrogen infrastructure are the primary hurdles to economic sustainability using H_2 as a fuel source [17–21].

Biohydrogen can be produced in thermochemical [22–25] and biological ways [26–28]. The biological technologies for H_2 production are preferred due to their ecological benefits. These biological processes are also less energy intensive and more environmentally friendly concerning the global reduction of carbon dioxide [18,20,29]. Furthermore, renewable hydrogen production techniques can potentially become cost-effective because they can use raw biomass as feedstock, e.g., agricultural, municipal, and industrial wastewater and organic waste [18,20]. Biohydrogen production is classified into (i) the biophotolysis of water using algae, (ii) dark fermentation using anaerobic bacteria, (iii) photofermentation using photosynthetic bacteria, and (iv) microbial electrolysis cells (MECs). Dark fermentation has several advantages as compared to others. The process is less energy-intensive because it can be carried out at an ambient temperature and pressure [30].

2. Biological Routes of Hydrogen Production

The synthesis of hydrogen by biological methods involves the active use of different microbes under various environmental conditions (Figure 2). However, they have in common the presence of hydrogenase or nitrogenase enzymes, which play a significant role in metabolic pathways in the synthesis of hydrogen [31].

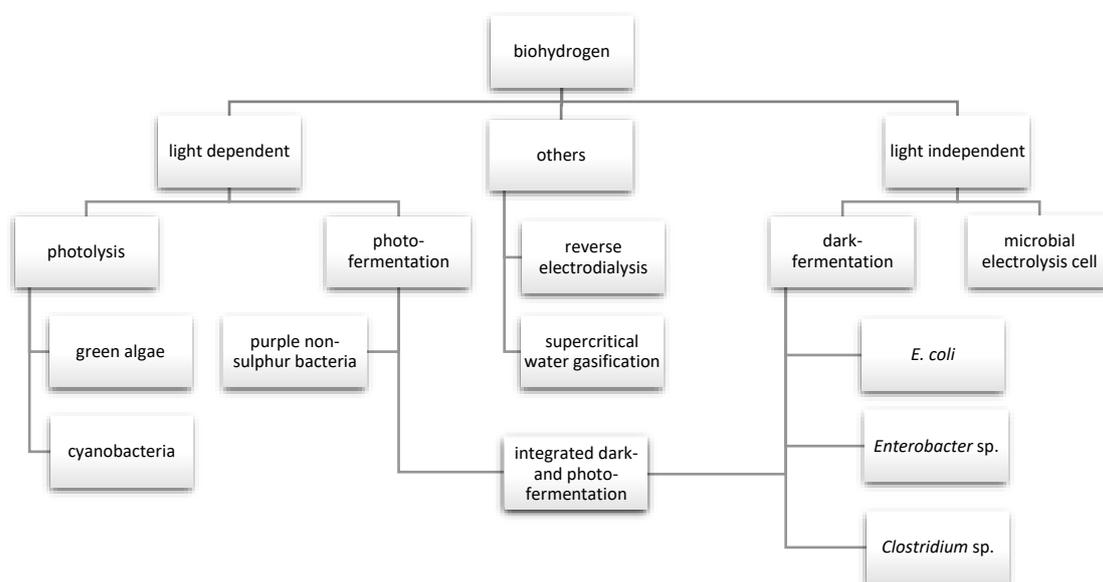


Figure 2. Methods of biological hydrogen production.

2.1. Biophotolysis

Hydrogen can be produced by photosynthetic microorganisms, such as green algae, cyanobacteria, and diatoms. These organisms integrate and store light energy as H-H bonds. Carbon dioxide (CO_2) and water are used in this process, thus reducing greenhouse gas emissions [26]. Biophotolysis can be divided into direct and indirect forms.

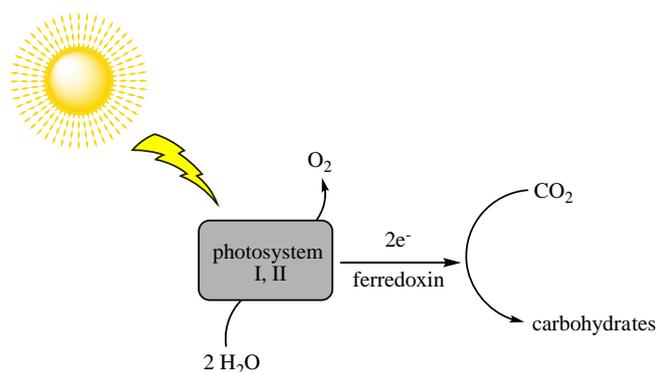
For direct biophotolysis, several green algae, such as *Chlamydomonas reinhardtii*, *Scenedesmus obliquus*, *Chlorella vulgaris*, and *Tetraspora* sp., have been extensively studied for their efficiency in hydrogen production [27,28]. During the process of direct photolysis, light radiation causes hydrogen evolution. Unique pigments in photosynthetic microor-

ganisms absorb light energy, which is mediated by nitrogenase and hydrogenase enzyme activity. The absorbed energy is then used to elevate the energy level of the electrons of the water molecules. Some atoms attain sufficient energy to split water molecules into H and OH atoms.

Consequently, the free hydrogen atoms combine to form hydrogen molecules and are released as hydrogen gas. Green algae were used to achieve 6–24% energy efficiency under low light intensities under laboratory scans during direct photolysis [16,26]. The sensitivity of the hydrogenase enzyme to oxygen generated during photosynthesis is the major drawback of this process. The evolved oxygen constrains the enzyme's activity, producing low H₂ yields [16,26]. Another limiting factor for microalgal cultures is the low biomass concentration because of reduced light penetration. The drying of algal biomass also makes it an energy-consuming process. The higher capital cost and the intensive care of microalgal farms are significant barriers to executing this process on a commercial scale [32].

Indirect biophotolysis needs photosynthetic bacteria (e.g., blue-green algae) to utilize CO₂ in the presence of light to produce carbohydrates, which are fermented in the presence of light to produce energy and, thus, liberate high yields of hydrogen molecules as byproducts (Figure 3) [33,34]. The cyanobacteria, *Anabaena variabilis*, was reported as a probable candidate for hydrogen production via indirect photolysis [35]. In this method, oxygen is the inhibiting factor for carbon dioxide-consuming bacteria. Besides, the requirement for a continuous light source is another limiting factor.

Aerobic phase



Anaerobic phase

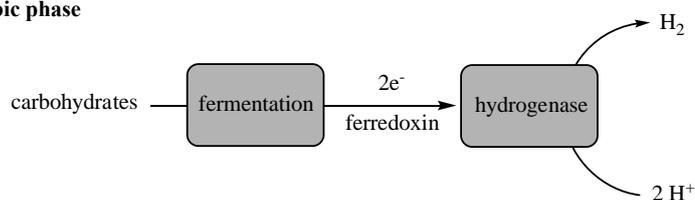


Figure 3. Process of hydrogen production via indirect photolysis.

2.2. Photofermentation Process

The process of photofermentation utilizes purple non-Sulfur bacteria to produce H₂ in the presence of light. These bacteria use organic acids (lactic acid, acetic acid, and butyric acid) as a carbon source and produce H₂ by nitrogenase enzyme action in N₂-deficient conditions. The purple non-Sulfur bacteria utilize organic acids as a source of electrons for photosynthesis, while the external source of light energy is utilized to oxidize the volatile organic acids to generate electrons for the synthesis of H₂ [36]. The released electrons are driven via electron carriers, and the protons are pushed across the membrane. A gradient is formed due to high and low concentrations of hydrogen ions. The energy produced by the protons is used to make adenosine triphosphate (ATP) from adenosine diphosphate (ADP) by the ATP synthase enzyme. The electrons are then transferred to the ferredoxin

by utilizing surplus ATP energy. The nitrogenase enzyme takes electrons derived from ferredoxin and accelerates the reduced protons into H_2 . All the reactions occur in the absence of nitrogen and the availability of excess energy of ATP [26]. This way, the organic compounds are entirely converted into H_2 and CO_2 .

The significant advantage of photofermentation is the 100% release of electrons from organic acids by photosynthetic bacteria to synthesize carbon dioxide and hydrogen. There is no inhibitory effect of oxygen on the performance of the nitrogenase enzyme, as this process occurs in the absence of oxygen. This technology uses a wide range of feedstock and generates fewer byproducts in the waste stream. The feedback on cost-effective photofermentation technology benefits waste management and the H_2 economy. Some studies have reported using industrial wastewater as an effective raw material [37]. Although much research has been reported on this process, some technical obstacles avoid its large-scale usage, including low light conversion efficiency, hydrogen production by nitrogenase, and low photosynthetic conversion efficiency [26]. A study using *Rhodobacter sphaeroides* with various volatile acids as the carbon source reported a maximum hydrogen yield of 17.8 L H_2 /mol-substrate for 2 g/L lactate and 2 g/L succinate [38]. The reactor design for photofermentation and the selection of biohydrogen specialists are the most critical factors for improving production yields [39]. Akroum et al. observed an optimal operational pH of 7.5 for hydrogen production in a column bioreactor using *Rhodobacter sphaeroides* [40]. The study reported a maximum hydrogen production rate of 0.04 L/L/h compared to 0.03 L H_2 /h studied in the dark fermentation process [41]. However, recent research shows that the combination of photofermentation with the dark fermentation process can boost hydrogen yield. An integrated photo and dark fermentation study investigated by Ghameri et al. resulted in a 40% rise in H_2 production when compared with the singular processes of photo and dark fermentation [20]. It was reported that different fermentation parameters, such as substrate concentration, pH, and temperature, are automatically controlled by the microbial cultures and help to enhance hydrogen yields [42]. A study conducted by Policastro et al. used the ethanol-rich wastewater produced during dark fermentation as a substrate for light-fermentative hydrogen production with a 0.31 L H_2 /g COD yield [43].

2.3. Dark Fermentation Process

Dark fermentation works with microorganisms that use various organic materials, such as industrial and agricultural wastes, to generate H_2 without oxygen and light [44,45]. These bacteria are either facultative or obligate anaerobes, but the process occurs without light when organic carbon is used as energy. The fermentation process involves the production of volatile or intermediate chemicals, including butyrate or propionate, ethanol, and acetate, depending on the mechanism used. The way of acetate byproduct generation is the most promising when compared to butyrate because it can give maximum yield. This is because, theoretically, four molecules of H_2 are produced per hexose in the acetate pathway, called the 'Thauer limit,' while two molecules of H_2 are formed in the butyrate pathway. The accumulation of H_2 as a metabolic inhibitor is a major drawback for the commercialization of bio- H_2 production [46–48]. The oxygen sensitivity of the hydrogenase enzyme in dark-fermentative bacteria is another limiting factor that reduces the H_2 yield [49].

The research on molasses wastewater gave a dominant pathway of acetate and ethanol with a maximum yield of 0.20 L H_2 /g-COD sucrose. The final gas composition from a reactor depends on the fermentation substrate and operating conditions [35,50].

Biohydrogen production involves the ethanol-type fermentation of molasses in an expanded granular sludge bed reactor. Another study showing a process dominated by the butyrate pathway observed a general decrease in butyrate concentration for reduced HRT, also marked by increased hydrogen productivity. Another study of the glucose substrate in a batch reactor observed a maximum conversion of 0.01 L of H_2 per g-COD/L, while the side products of hydrogen generation were 14–63% butyrate, 10–45% formate, and 16–40% ethanol [51].

In fermentative hydrogen production, three biochemical pathways may be used: (i) the utilization of pyruvate-formate lyase (PFL) and formate-hydrogen lyase (FHL), (ii) pyruvate-ferredoxin oxidoreductase (PFOR) and Ferredoxin (Fd)-dependent hydrogenase (HYD) under strict anaerobic conditions, and (iii) the utilization of NADH-ferredoxin oxidoreductase (NFOR) and HYD (Figure 4) [52].

Different strategies have emerged to overcome the limitations of the dark fermentation process. The single-stage fermentative hydrogen process produced large amounts of acids and alcohols as byproducts. These liquids can be used as feedstock in photofermentation and MECs to enhance H₂ generation. The integration of dark fermentation with other techniques has evolved to enhance the production of H₂ through the complete conversion of the substrate [53]. Metabolically or genetically engineered bacteria are other ways of producing H₂ [54]. However, this method is not economical at an industrial scale.

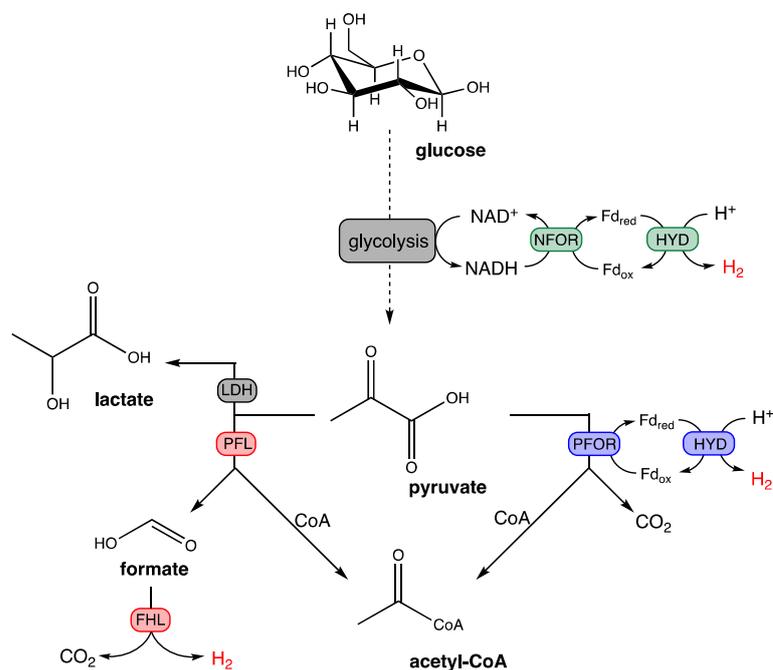


Figure 4. Dark fermentation metabolic pathways for hydrogen production. (red pathway) *E. coli* and *Enterobacteriaceae*. (blue pathway) *Clostridium* sp. and (green pathway) thermophilic bacteria). Abbreviations: NFOR = NADH-ferredoxin oxidoreductase, HYD = ferredoxin-dependent hydrogenase, PFOR = pyruvate-ferredoxin oxidoreductase, LDH = Lactate dehydrogenase, PFL = pyruvate-formate lyase, FHL = formate-hydrogen lyase.

2.4. Microbial Electrolysis Cell

Microbially catalyzed electrolysis cells produce H₂ during the fermentation of soluble organic matter found in wastewater when an electric current is passed through a small amount [55–57]. The simplest carbon source for H₂ generation in MECs is acetate. However, the presence of volatile fatty acids in the effluent of biohydrogen production via the dark process is considered favorable for producing hydrogen via microbial electrolysis cells [44,58]. The MEC consists of four main parts: the cathode and anode, the external electrical connection, and a cationic exchange membrane between the electrodes (Figure 5).

In the cell, bacteria inhabit the anode surface and oxidize the carbon sources of the waste to carbon dioxide, electrons, and protons over a series of redox reactions. The produced electrons are transferred to the surface of the anode by the bacteria. At the same time, the proton diffuses freely into the solution and migrates via the cation exchange membrane to the cathode compartment, contributing to the reduction reactions occurring on the cathode electrode surface. At the same time, the electrons are transported via an external power source to the cathode electrode, which couples with the free proton in the electrolyte solution to produce H₂ gas [59]. An AC/DC electricity source is required to

energize the MEC. The energy required by MEC is only 0.11V, which is lower than water electrolysis (1.8–2.0) [60]. Therefore, this technology is considered to have high potential and to surge, having few limitations, such as reduced mass transfer and energy loss. The five major challenges faced by MECs are the loss of methanogenic electrons, metabolic diversity, and electrode resistance (anode), complex wiring in cell design, power supply, membrane problems (high cost, biofouling, substrate, and gas crossover and long-term stability) and cathode related obstacles (long-term stability, high catalyst cost, side reaction, and electrode resistance). Future research in this field is based on the improvements in the challenges mentioned above [49]. Microbial electrolysis cells work efficiently when combined with dark fermentation techniques. The MEC–DF combined process produces about a 98% H₂ yield as compared to the single-stage process [61].

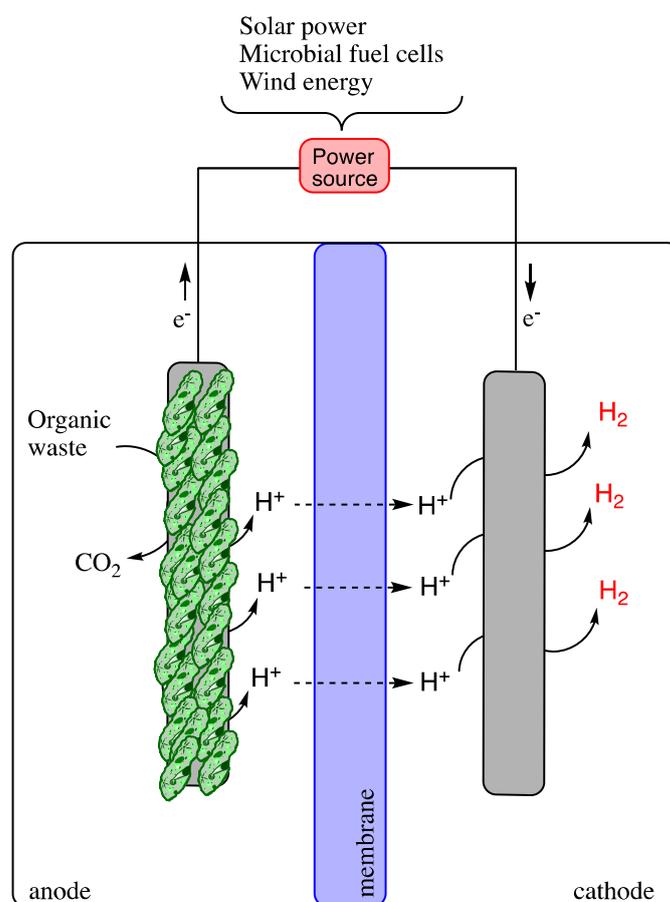


Figure 5. Schematic diagram of a microbial electrolysis cell for H₂ production.

3. Feedstock for Biohydrogen Production

Biological hydrogen production has been carried out using several waste materials and lignocellulosic materials, depending upon their availability and suitability in particular geographic situations. Table 1 shows the utilization of different feedstocks for hydrogen production. Numerous raw materials such as sugarcane and sugar beet molasses [62–67], cheese whey powder [68], coffee drink manufacturing wastewater [69], corn stalk [70], crude glycerol [71], rice slurry [72], starch wastewater [67], paper and pulp industry effluent [30], baggase [73], dairy wastewater [37], vegetable waste [74], palm oil mill waste [75], distillery wastewater, and waste barley [76,77] have been reported.

Table 1. Utilization of different feedstocks for hydrogen production.

Type	Feedstock	Fermentation Process	Substrate Conc.	pH	Hydrogen % Yield	Ref.	
Solid food waste	Wheat bran and flour	Anaerobic degradation	-	5.0	0.13 L/g	[78]	
		Dark fermentation	10.7 g/L	4.5	0.24 L/g glucose	[79]	
		Photofermentation	5 g/L	7.0	0.15 L/g glucose	[80]	
	Waste barley	Photofermentation	11 g/L	7.0	0.4 L/L culture	[77]	
	Cheese whey powder	Dark fermentation	-	-	0.11 L/g sugar	[81]	
	Oil palm waste	Photofermentation	-	7.0	0.02 L/L/h	[82]	
	Sugar cane bagasse	Photofermentation	-	7.0	0.04 L	[73]	
			10% w/v	7.0	0.35 L/L	[42,83]	
		Dark fermentation	22.8 g/L	6.0	6.98 L/L	[84]	
			-	6.8	0.76 L/L	[42]	
	Vegetable and fruit waste	Photofermentation	-	6.8	0.004 L/L/h	[74]	
			-	7.1	0.11 L/g	[85]	
		Dark fermentation	-	5.0	0.51 L/g volatile solid	[86]	
			-	5.9	0.12 L/g TOC	[87]	
	Food and beverage industry wastewater	Brewing industry wastewater	Anaerobic dark fermentation	-	5.95	0.15 L /g COD	[88]
			Photofermentation	10% v/v	7.2	2.24 L/L medium	[37]
		Sugarcane and sugar beet molasses	Photofermentation	28 g/L	7.0	1.01 L/L culture	[65]
Dark fermentation			10 mM	7.5	1.24 L/g sucrose	[66]	
			-	-	0.37 L/g glucose	[89]	
Distillery wastewater		Dark fermentation	-	5.5	0.02 L/g glucose	[90]	
		Photofermentation	-	5.8	0.04 L/g COD	[76]	
Soy sauce wastewater		Dark fermentation	-	6.5	-	[91]	
		Photofermentation	-	7.0	0.2 L	[73]	
Dairy industry wastewater		Dark fermentation	21.1 g/L	7.0	0.29 L/g COD	[92]	
		Photofermentation	-	7.0	2.2 L/L	[93]	
Tofu wastewater	Dark fermentation	-	7.2	-	[94]		
	Dark fermentation	20 g COD/L	5.5	0.11 L/g COD	[95]		
Palm oil mill wastewater	Photofermentation	-	6.0	0.66 L/L POME	[96]		
	Dark fermentation	48 g/L	4.5	0.06 L/g	[97]		
Algal biomass	Dark fermentation	50 g/L	7.5	0.07 L/L/h	[98]		
		5.0 g/L	7.4	0.31 L/g glucose	[99]		

4. Diversity of Biohydrogen-Producing Bacteria

There are numerous types of fermentative hydrogen-producing bacteria. *Clostridium* sp. is one of the most common anaerobic bacteria. Different species of *Clostridium*, such as *Clostridium butyricum*, *Clostridium beijerinckii*, *Clostridium amygdalinum*, *Clostridium cellulosi*, and *Clostridium acetobutylicum*, have been reported for fermentative hydrogen

production [100–104]. Anaerobic bacteria utilize glucose to produce hydrogen, while butyric acid or acetic acid is produced as the product—Chong et al. isolated *Clostridium butyricum* from POME [105]. The optimum hydrogen production was obtained at pH 5.5.

Some facultative anaerobic bacteria (e.g., *Enterobacter aerogenes*) have also been recognized as H₂ producers since the hydrogenase enzyme was found in these bacteria [106]. The critical parameters, such as substrate concentration, temperature, pH, inoculum size, and yeast extract, were optimized to obtain a maximum H₂ yield of 0.21 L H₂ /g glucose. The culture and maintenance of facultative anaerobes are more feasible than obligate anaerobes.

These microorganisms are further classified into mesophylls and thermophiles based on their growth temperatures. Although thermophiles are cultivated at elevated temperatures, with highly intensive energy requirements, their H₂ production can be closer to the theoretical yield than mesophylls by overwhelming the thermodynamic barrier [107,108]. Some photofermentative bacteria require light energy to produce H₂ in anoxygenic conditions. Without O₂, these photoautotrophs, including cyanobacteria and green algae, produce H₂ through biophotolysis using their specific metabolic routes advantageously under defined conditions [107]. Mixed cultures are also considered the best choice for maximum H₂ yield. A study by Nicolau shows hydrogen production using heat-treated mesophilic anaerobic sludge inoculum instead of pure culture [109]. The hydrogen yield at pH 5.5 was 0.37 mol H₂/mol of carbohydrate, equal to 18.14 L H₂/kg of dry solid.

5. Enzymes

Life depends on several chemical reactions, most of which are slow. Therefore, enzymes are naturally occurring catalysts to speed up biochemical reactions. The two enzymes involved in hydrogen production are hydrogenase and nitrogenase (Figure 6). The enzyme hydrogenase catalyzes the consumption and generation of H₂. After the discovery of this enzyme in 1930 by Stephenson and Stickland, numerous experiments have been conducted to learn more about it. Despite this, its crystal structure was elucidated approximately 20 years ago [110]. It is present in dark fermentative hydrogen-producing bacteria, green algae, and cyanobacteria. The hydrogenase enzyme is classified into three types based on the structure of active sites: NiFe-, Fe-Fe-, and Fe-hydrogenase. The NiFe-hydrogenase is only present in bacteria and archaea, while algae and bacteria have FeFe-hydrogenase. Hence, Fe-hydrogenase is a homodimer and is only present in methanogenic archaea [16,110,111].

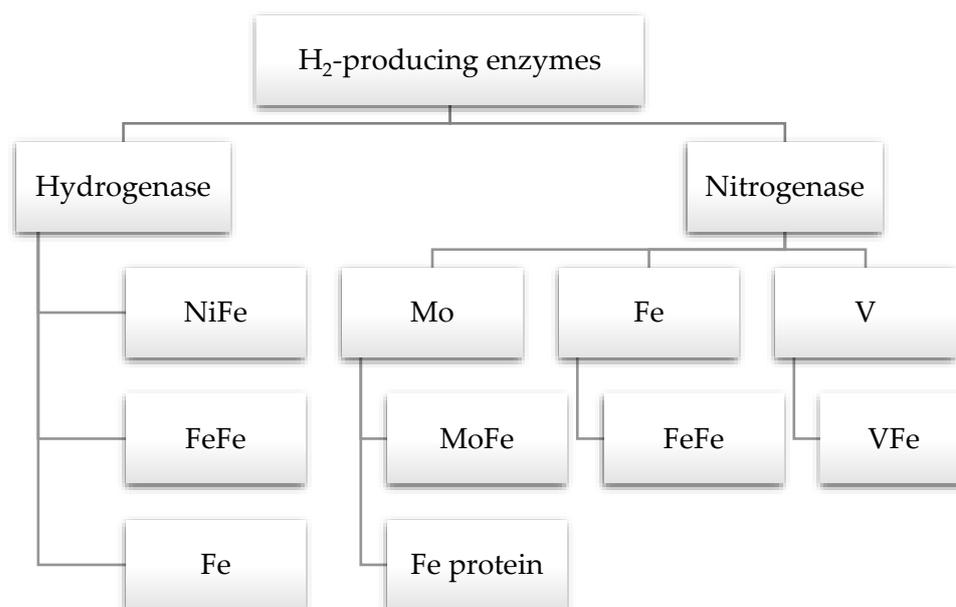


Figure 6. Enzymes involved in hydrogen production.

Nitrogenase is another enzyme responsible for the production of H₂. It is found in purple non-Sulfur bacteria, archaea, and cyanobacteria [111]. Most atmospheric nitrogen is fixed by cyanobacteria and generates H₂ as a byproduct. There are three forms of nitrogenase enzyme: Molybdenum, iron, and vanadium. They are located at the active sites of nitrogen reduction and bind with rare metal centers. Mo-nitrogenase consists of two proteins: dinitrogenase (MoFe protein) and dinitrogenase reductase (Fe protein). The nitrogenase helps to generate ammonium from nitrogen, but in nitrogen-deficient conditions, it starts producing hydrogen in an anaerobic environment [16]. The structure of iron and vanadium nitrogenases are similar to the structure of the Mo form, but they have FeFe and VFe cofactors, respectively. The FeFe and VFe nitrogenases enhance hydrogen production compared to Mo nitrogenase. Only one type of photofermentative bacteria, *R. palustris*, has been reported to have all three types of these nitrogenases [111].

6. Factors Affecting the Production of Hydrogen

The production rate and yield of hydrogen depend on many factors during dark and photofermentation processes, including the following.

6.1. Pretreatment Methods

The use of food waste and food processing wastewater as feedstock provides several organic compounds and nutrients with enhanced hydrogen production, but some inhibitory compounds affect the production and yield of hydrogen [112]. In addition, different pretreatment methods have been reported to increase the utilization of raw materials for successive hydrogen generation.

Among the pretreatment methods, hydrolysis and preheating are the most preferred methods. Hydrolysis can be acid/alkaline or ultrasound-assisted. The six-hour alkaline hydrolysis increases H₂ generation 206 times at a pH level of 12 [113]. Meanwhile, acid 12 h hydrolysis enhances the production of H₂ three-fold at a pH of 2. Hence, the main disadvantages are the utilization of chemicals in large quantities and the requirement of some other processes to neutralize the pH [114]. The ultrasonication of food waste, assisted with hydrolysis, increases H₂ yield by 75–88% [115–118], but investment in equipment and energy cost are the major hurdles to commercializing this method.

The preheating of food waste is another pretreatment method [119]. The results depicted that, prior to starting fermentation, heating waste for at least 20 min at 90 °C could increase the H₂ yield.

6.2. Effect of Substrate Concentration

The substrate concentration plays a vital role in H₂ production by dark fermentation. When substrate concentration increases, it creates unfavorable conditions and consequently changes the pH, H₂ partial pressure, and the concentration of volatile fatty acids. Therefore, substrate inhibition may be minimized by arranging the optimum initial concentration of the substrate [120]. Many researchers have reported inhibition by substrate concentration, but the main focus was on the sources of carbohydrates. The use of wastewater and organic waste as a substrate has rarely been reported in the literature [121]. The fed-batch reactors can be used to avoid substrate inhibition. Some bacteria, such as *Enterobacter aerogens*, can reduce substrate inhibition by stimulating the microbial activity of H₂ production [120]. The effect of substrate concentration on hydrogen production by *Lactobacillus casei* and *Clostridium butyricum* was also evaluated [122]. Glucose and galactose were used as carbon sources during the batch process. The results were based on the inoculum utilization of a single species or a mixture. It was observed that *L. casei* could not utilize galactose properly when used alone, while *C. butyricum* gave a fast response to galactose usage as a carbon source. On the other hand, the response for glucose utilization was faster by *L. casei* than *C. butyricum* under low concentrations of glucose, and, in turn, low hydrogen production was observed because *Lactobacillus* outcompeted the most significant H₂-generating bacteria.

6.3. Effect of Initial pH

Initial pH is another essential factor to be considered in the dark fermentation process, and it is noted that each microbe can function effectively in different conditions. For example, the effective pH for hydrogen production is 5–8. When the initial pH becomes lower than 5, hydrogen production reduces by half [123]. A pH range of 5–9 also has been used during the batch fermentation process, but a pH range of 5–6 has been reported as the initial optimum pH [124]. During fermentation, volatile fatty acids reduce the pH of the medium. Therefore, the initial pH was set from 6–7 to compensate for the end of the process [114].

The effect of pH on hydrogen production by green algae was evaluated [125]. The results showed that the pH of the medium affects the activity of the hydrogenase enzyme. They controlled the pH by adding NaOH and HCl over the range of 6.5–9.0, which does not affect algae growth. It was observed that an increased yield of 2.4% was obtained at a pH of 6.5. A considerable pH value is also required for Purple non-Sulfur bacteria (PNSB) to produce hydrogen via photofermentation. According to studies on hydrogen production during the photo-biological fermentation process, a pH of 7 is best for transporting electrons to the nitrogenase enzymes for H₂ generation in the media [126].

6.4. Effect of Operational Temperature

The operational temperature significantly affects the production of hydrogen and the activity of enzymes involved in hydrogen generation [127,128]. Thermophilic bacteria observed an enhanced biosynthesis of hydrogen at high temperatures compared to mesophilic bacteria during dark fermentation. The temperature range of 30–55 °C has been reported as optimal for enhanced biohydrogen production [128,129]. Hence, it has been reported that the activity of H₂ producers is inhibited at a very extreme temperature of more than 60 °C. Only hyperthermophilic bacteria (*Pyrococcus furiosus* and *Thermotoga maritima*) can produce H₂ at extreme temperatures. These bacteria can produce H₂ at temperatures greater than 80 °C [130].

PNSBs are also sensitive to different ranges of temperature. For example, a study conducted to show the effect of cultural conditions on H₂ production by photofermentation described that the growth rate and the rate of H₂ production initially increased with an increase in temperature up to 30 °C. However, after 30 °C, the production rate of H₂ gas decreased rapidly because the higher temperature above 30 °C inhibits the activity of the enzyme nitrogenase [126].

6.5. Effect of Nutrients

The macronutrients also play a vital role in the growth of bacteria to produce H₂. The essential nutrients are sulfur, phosphorus, and nitrogen. The common form of inorganic sulfur in many organic wastes is sulfate (SO₄²⁻) [131]. The sulfate-reducing bacteria reduce sulfate into sulfide during the process of fermentation. The sulfur-containing proteins also produce sulfide in the fermentation medium. Studies have been reported about the toxic effects of high sulfide levels in a medium, which inhibit the activity of microorganisms from producing H₂. The increased sulfide concentration also decreases the bioavailability of some trace elements [132].

Another essential nutrient for the growth of anaerobic bacteria is nitrogen. The high concentration of ammonia hydrogen decreases the activity of fermentative bacteria and the rate of H₂ production [133,134]. The degradation of proteins and amino acids also produces a high amount of ammonia in the fermentation media. The high concentration of nitrogen also interferes with the intracellular pH and affects the performance of microbes responsible for H₂ production. The inhibition of nitrogen can be overcome by diluting the feedstock [135]. Besides nitrogen and sulfur, phosphorus is another nutrient required to enhance hydrogen production [136]. It was observed that a high rate of H₂ can be obtained in the presence of 600 mg L⁻¹ K₂HPO₄ [137]. A 40% increase in the production of H₂ was observed at a 30% increase or decline in the respective chemical compound.

6.6. Effect of Light Intensity

Light intensity plays a significant role in producing H₂ by PNSB. It was shown that the performance of PNSB increases with an increase in light intensity from 2500–5000 lx, but a further increase in light intensity reduces the growth and production of hydrogen [138]. It was also observed that the photosynthetic system of PNSB demanded more ATP and reduced power with increasing illumination intensity [138]. The enzyme nitrogenase also requires high ATP to sensitize the cells and produce H₂. Hence, the high intensity can become a limiting factor in photohydrogen production.

Photoinhibition in PNSB was also investigated [139]. They suggested that hydrogen production decreased when light intensity was increased above 200 Wm⁻², while a study conducted by Cai and Wang found that the H₂ production decreased at an illumination intensity of 6000 lx. The favorable light source is LED because it has a wavelength range of 770–920 nm, which is considered best for the activity of PNSB. Furthermore, LED light is cost-effective in terms of heat generation, energy consumption, and life expectancy [40].

6.7. Effect of Metal Ions

Different metal ions are used for microbes' growth and to optimize the activity of the enzyme during dark- and photofermentation. These metal ions are required only in a moderate amount. When they are used in high quantities, they inhibit the fermentation process by inhibiting the growth of the bacteria. The effects of using higher concentrations of metals include destroying membrane function and eliminating the transmission of valuable ions and nutrients to the cell and intracellular accumulation of metals [140,141]. A study was conducted to describe the importance of Fe for metabolic changes and its involvement in the expression of non-Fe-S and Fe-S proteins in hydrogenase enzymes. However, when the concentration of Fe increases in the medium, it makes cell clumps and reduces the mass transfer activity. It has been reported that the pure culture requires very little Fe, while mixed culture can tolerate high doses of Fe without an inhibitory effect [124].

Trace metal ions, such as sodium, magnesium, and calcium, are also needed for the growth of bacteria. High amounts of these trace metals slow down the growth of microbes and become toxic at higher concentrations. The authors of [142] observed a high hydrogen yield in the absence of sodium. The higher concentrations of sodium raise the osmotic pressure, affect the activity of bacteria, and sometimes cause bacterial death. It has been recommended that the sodium concentration should be kept under 20 g L⁻¹ to achieve a maximum level of hydrogen production [143]. Ca²⁺ is another trace element required for the growth of bacteria and H₂ production [144]. Mg²⁺ is also responsible for cell function and reaction. It is the most demanding ion as a cofactor for 10 types of enzymes involved in the glycolysis process. The Ni²⁺ has no inhibitory effect on the yield of H₂ at a level of 0.1 mg L⁻¹. Hence, no significant measure has been taken to control metal inhibition during fermentation, but a few pretreatment techniques, such as biosorption, electro dialysis, and cofermentation, can effectively overcome metal inhibition problems [145].

7. Nanotechnology and Biohydrogen

The vast and newly emerging field of nanotechnology deals with nm-sized particles. The nanoparticles (NPs) have been utilized in several fields, such as biosensors, medicines, immobilization, and the production of biofuels [146–148]. The NPs also help produce biohydrogen by influencing the metabolic activities of microbes under aerobic conditions [149]. Nanoparticles prepared by different methods (biological, physical, and chemical) have been reported for H₂ production. The NPs of gold, silver, copper, nickel, iron, zinc oxide, palladium, titanium, silica, carbon nanotubes, and activated carbon have been used to enhance H₂ production [146,150–152]. These nanoparticles provide a larger surface area to adsorb electrons and, hence, enhance the production rate of H₂ by stimulating the hydrogen-producing enzymes [153].

Enhancement of Biohydrogen by Metallic Nanoparticles

Zhang's group was the first to use gold nanoparticles to enhance the biosynthesis of H₂ [153]. They used artificial wastewater for H₂ production via dark fermentation. The preheated and non-heat-treated cultures were used as inoculum. It was observed that the gold nanoparticles successfully increased the metabolic activity of the microbes, enhancing the rate of H₂ compared to the control. The cumulative hydrogen yield was maximum when 5 nm gold particles were used [153]. A study evaluating silver NPs for hydrogen production has also been conducted [154]. They used mixed culture and Ag-NPs to produce H₂ from glucose. When the concentration of the Ag-NPs was increased up to 20 nM, it affected the activity of the bacteria for enhancing H₂ generation. However, there was no increase in hydrogen production rate at more significant concentrations. The higher yield of H₂ observed at 20 nM Ag-NPs was 67.6%. The Ag-NPs also increased cell biomass and decreased the lag phase for H₂ production.

Han and colleagues investigated the effect of hematite NPs and initial pH on hydrogen production in mixed bacteria in an anaerobic fed-batch process. The maximum observed H₂ yield was 3.21 mol H₂/mol⁻¹ sucrose. A transmission electron microscope was used to check the slow discharge of hematite nanoparticles and their effect on the shape of bacteria. Furthermore, a study was conducted utilizing biogenic palladium nanoparticles and palladium ions [150]. The leaf extract of *Cortandrum sattvum* was used to synthesize palladium nanoparticles. They obtained a maximum H₂ yield of 1.48 mol H₂/mol⁻¹ glucose using a 5 mg L⁻¹ palladium nanoparticles concentration because of the higher activity of the hydrogenase enzyme. On the other hand, palladium ions showed a negative impact on the yield and lag phase of hydrogen.

Many bacterial cultures have been investigated for producing H₂ via iron NPs. For example, Fe-NPs and iron ions were used to investigate their possible enhancement effect on the production of H₂ [155]. Both showed a positive impact on the hydrogen yield compared to the control. However, Fe⁺² ions and Fe-NPs illustrated different behavior towards the generation of intermediate metabolites. The propionate production declined by 75% with Fe-NPs compared to a 35% reduction by the Fe⁺² ions. The enhancement effect of phytogetic iron nanoparticles and iron ions was also investigated. The green Fe-NPs were prepared using the extract of leaves and bark of *Syzygium cumini* and FeSO₄. The mesophilic bacterial strain of *Enterobacter cloacae* DH-89 was isolated from the soil and used to produce hydrogen. A 100% increase in hydrogen production (1.9 mol H₂/mol⁻¹ hexose) was observed under 100 mg L⁻¹ Fe-NPs compared to the control (0.95 mol H₂/mol⁻¹ glucose). Meanwhile, Fe⁺² ions helped to raise the yield of hydrogen to 1.45 mol/mol⁻¹ glucose) [156]. Similarly, some other researchers have also reported Fe, Fe₂O₃, and Fe₃O₄ NPs prepared by different physical, chemical, and biological methods for the enhanced biosynthesis of hydrogen [101,157–162].

The effect of ZnO nanoparticles on hydrogen production was also reported [163]. The ZnO-NPs were synthesized by the typical precipitation method. The pretreated biomass of water hyacinth was saccharified by the enzyme activity and used for the fermentative production of H₂. It was observed that the ZnO-NPs reduced the hydrogen yield compared to the control. On the other hand, metallic NPs (copper, nickel, silicon dioxide, and titanium dioxide) positively affected the rate of generation and yield of H₂ [164–168].

Many studies have reported an enhancement of H₂ production via metallic NPs using the dark fermentation process, but few studies have been found in the literature for enhanced photofermentative H₂ production by nanoparticles. Zhao et al. investigated the effect of TiO₂-NPs on photofermentative H₂ production using the effluent of the dark fermentation process as a feedstock [165]. It was observed from the results that the TiO₂-NPs enhanced the activity of PNSB for the production of H₂ and reduced the activity of the uptake of hydrogenase enzyme. Another study by Pandey et al. reflected a similar enhancement effect of TiO₂-NPs for photofermentative H₂ production [164]. Meanwhile, Kanwal and colleagues investigated the effect of a phytofabricated nanoscale iron complex for H₂

production using photofermentative PNSB [169]. The use of carbon nanotubes (CNTs) has also been reported for improved hydrogen generation by H₂-producing bacteria [170].

8. Future Perspective of H₂ Production

The selection of favorable biological H₂-producing technology for future research is based on different factors. Hossienzadeh et al. conducted techno-economic and life cycle assessments of H₂-producing techniques [49]. From the technological perspective, dark fermentation was chosen as a better process than others. The combination of dark fermentation with MECs and photofermentation generated around 1 L H₂/g organic waste. Among biological H₂-producing techniques, the dark-fermentative process (2.3 US \$/g) is the most cost-effective, followed by MECs (2.8 US \$/g) and photofermentation (3.5 US \$/g). According to an environmental impact assessment, low greenhouse gas emissions were observed from both fermentation processes compared to MECs [49].

According to EIA, the hybrid dark fermentation and MEC technology can biosynthesize 105 million tons of H₂ gas from 1.3 billion metric food waste. This represents a 120% higher potential than the actual demand in 2020 (90 million metric tons) [1].

9. Conclusions

Using biomass to harvest hydrogen gas is a promising and sustainable method for producing clean energy. Even though there are several renewable energy options, no single energy source can fully replace fossil fuels. However, hydrogen production, in this way, might be highly beneficial as it can reduce greenhouse gas emissions while future energy demands are met. Different routes of biological hydrogen production, such as photolysis, photofermentation, dark fermentation, and microbial electrolysis cells (MECs), represent the different categories of solid and liquid waste feedstock, microorganisms, and enzymes involved in biohydrogen production, which were discussed. In addition, various factors affecting the production rate (e.g., substrate concentration, temperature, and pH) were also examined.

Furthermore, several studies regarding the use of nanomaterials for the enhanced production of hydrogen were investigated. Of course, all techniques have some pros and cons. However, after analyzing various studies, dark fermentation was found to be the most suitable method when integrated with MECs. Besides, the utilization of engineered bacterial cultures and the role of nanotechnology have also enhanced hydrogen biosynthesis. However, the practical implication of these processes still requires further efforts from engineers and researchers.

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