Biohydrogen for Sustainable Energy Solutions

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Energy plays a crucial role in the sustainable development of modern nations. 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. 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.

Keywords: energy ; photofermentation ; dark fermentation ; microorganisms ; biohydrogen

1. 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. Numerous raw materials such as sugarcane and sugar beet molasses [1][2][3][4][5][6], cheese whey powder [7], coffee drink manufacturing wastewater [8], corn stalk [9], crude glycerol [10], rice slurry [11], starch wastewater [6], paper and pulp industry effluent [12], baggase [13], dairy wastewater [14], vegetable waste [15], palm oil mill waste [16], distillery wastewater, and waste barley [17][18] have been reported.

2. 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 beijerienckii*, *Clostridium amygdalinum*, *Clostridium cellolosi*, and *Clostridium acetobutylicum,* have been reported for fermentative hydrogen production [19][20][21][22][23]. 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 [24]. The optimum hydrogen production was obtained at pH 5.5.

Some facultative anaerobic bacteria (e.g., *Enterobacter aerogenes*) have also been recognized as H_2 producers since the hydrogenase enzyme was found in these bacteria [25]. The critical parameters, such as substrate concentration, temperature, pH, inoculum size, and yeast extract, were optimized to obtain a maximum H_2 yield of 0.21 L H_2 /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 H_2 production can be closer to the theoretical yield than mesophylls by overwhelming the thermodynamic barrier [26][27]. Some photofermentative bacteria require light energy to produce H_2 in anoxygenic conditions. Without O_2, these photoautotrophs, including cyanobacteria and green algae, produce H_2 through biophotolysis using their specific metabolic routes advantageously under defined conditions [26]. Mixed cultures are also considered the best choice for maximum H_2 yield. A study by Nicolau shows hydrogen production using heat-treated mesophilic anaerobic sludge inoculum instead of pure culture [28]. The hydrogen yield at pH 5.5 was 0.37 mol H_2/mol of carbohydrate, equal to 18.14 L H_2/kg of dry solid.

3. 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. The enzyme hydrogenase catalyzes the consumption and generation of H_2. 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 [29]. 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 [29][30][31].

Nitrogenase is another enzyme responsible for the production of H_2. It is found in purple non-Sulfur bacteria, archaea, and cyanobacteria [31]. Most atmospheric nitrogen is fixed by cyanobacteria and generates H_2 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 [30]. 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 [31].

4. Factors Affecting the Production of Hydrogen

4.1. 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 [32]. 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 H_2 generation 206 times at a pH level of 12 [33]. Meanwhile, acid 12 h hydrolysis enhances the production of H_2 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 [34]. The ultrasonication of food waste, assisted with hydrolysis, increases H_2 yield by 75–88% [35][36][37][38], but investment in equipment and energy cost are the major hurdles to commercializing this method.

The preheating of food waste is another pretreatment method [39]. The results depicted that, prior to starting fermentation, heating waste for at least 20 min at 90 °C could increase the H_2 yield.

4.2. Effect of Substrate Concentration

The substrate concentration plays a vital role in H_2 production by dark fermentation. When substrate concentration increases, it creates unfavorable conditions and consequently changes the pH, H_2 partial pressure, and the concentration of volatile fatty acids. Therefore, substrate inhibition may be minimized by arranging the optimum initial concentration of the substrate [40]. 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 [41]. 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 H_2 production [40]. The effect of substrate concentration on hydrogen production by *Lactobacillus casei* and *Clostridium butyricum* was also evaluated [42]. 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 H_2-generating bacteria.

4.3. 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 [43]. 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 [44]. 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 [34].

The effect of pH on hydrogen production by green algae was evaluated [45]. 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 H_2 generation in the media [46].

4.4. Effect of Operational Temperature

The operational temperature significantly affects the production of hydrogen and the activity of enzymes involved in hydrogen generation [47][48]. 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 [48][49]. Hence, it has been reported that the activity of H_2 producers is inhibited at a very extreme temperature of more than 60 °C. Only hyperthermophilic bacteria (*Pyrococcus furiosus* and *Thermotoga maritime*) can produce H_2 at extreme temperatures. These bacteria can produce H_2 at temperatures greater than 80 °C [50].

PNSBs are also sensitive to different ranges of temperature. For example, a study conducted to show the effect of cultural conditions on H_2 production by photofermentation described that the growth rate and the rate of H_2 production initially increased with an increase in temperature up to 30 °C. However, after 30 °C, the production rate of H_2 gas decreased rapidly because the higher temperature above 30 °C inhibits the activity of the enzyme nitrogenase [46].

4.5. Effect of Nutrients

The macronutrients also play a vital role in the growth of bacteria to produce H_2. The essential nutrients are sulfur, phosphorus, and nitrogen. The common form of inorganic sulfur in many organic wastes is sulfate (SO_4^{2-}) [51]. 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 H_2. The increased sulfide concentration also decreases the bioavailability of some trace elements [52].

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 H_2 production [53][54]. 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 H_2 production. The inhibition of nitrogen can be overcome by diluting the feedstock [55]. Besides nitrogen and sulfur, phosphorus is another nutrient required to enhance hydrogen production [56]. It was observed that a high rate of H_2 can be obtained in the presence of 600 mg L^{-1} K_2HPO_4 [57]. A 40% increase in the production of H_2 was observed at a 30% increase or decline in the respective chemical compound.

4.6. Effect of Light Intensity

Light intensity plays a significant role in producing H_2 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 [58]. It was also observed that the photosynthetic system of PNSB demanded more ATP and reduced power with increasing illumination intensity [58]. The enzyme nitrogenase also requires high ATP to sensitize the cells and produce H_2. Hence, the high intensity can become a limiting factor in photohydrogen production.

Photoinhibition in PNSB was also investigated [59]. 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 H_2 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 [60].

4.7. 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 [61][62]. 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 [44].

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 [63] 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 [64]. Ca^{2+}n is another trace element required for the growth of bacteria and H_2 production [65]. Mg^{2+} 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 Ni^{2+} has no inhibitory effect on the yield of H_2 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, electrodialysis, and cofermentation, can effectively overcome metal inhibition problems [66].

5. 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 [67][68][69]. The NPs also help produce biohydrogen by influencing the metabolic activities of microbes under aerobic conditions [70]. Nanoparticles prepared by different methods (biological, physical, and chemical) have been reported for H_2 production. The NPs of gold, silver, copper, nickel, iron, zinc oxide, palladium, titanium, silica, carbon nanotubes, and activated carbon have been used to enhance H_2 production [67][71][72][73]. These nanoparticles provide a larger surface area to adsorb electrons and, hence, enhance the production rate of H_2 by stimulating the hydrogen-producing enzymes [74].

Enhancement of Biohydrogen by Metallic Nanoparticles

Zhang's group was the first to use gold nanoparticles to enhance the biosynthesis of H_2 [74]. They used artificial wastewater for H_2 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 H_2 compared to the control. The cumulative hydrogen yield was maximum when 5 nm gold particles were used [74]. A study evaluating silver NPs for hydrogen production has also been conducted [75]. They used mixed culture and Ag-NPs to produce H_2 from glucose. When the concentration of the Ag-NPs was increased up to 20 nM, it affected the activity of the bacteria for enhancing H_2 generation. However, there was no increase in hydrogen production rate at more significant concentrations. The higher yield of H_2 observed at 20 nM Ag-NPs was 67.6%. The Ag-NPs also increased cell biomass and decreased the lag phase for H_2 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 H_2 yield was 3.21 mol H_2/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 [71]. The leaf extract of *Cortandrum sattvum* was used to synthesize palladium nanoparticles. They obtained a maximum H_2 yield of 1.48 mol H_2/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 H_2 via iron NPs. For example, Fe-NPs and iron ions were used to investigate their possible enhancement effect on the production of H_2 [76]. 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 phytogenic 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 $FeSO_4$. 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 H_2/mol^{-1} hexose) was observed under 100 mg L^{-1} Fe-NPs compared to the control (0.95 mol H_2/mol $^{-1}$ glucose). Meanwhile, Fe^{+2} ions helped to raise the yield of hydrogen to 1.45 mol/mol^{-1} glucose) [77].

Similarly, some other researchers have also reported Fe, Fe_2O_3, and Fe_3O_4 NPs prepared by different physical, chemical, and biological methods for the enhanced biosynthesis of hydrogen [20][78][79][80][81][82][83].

The effect of ZnO nanoparticles on hydrogen production was also reported [84]. 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 H_2. 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 H_2 [85][86][87][88][89].

Many studies have reported an enhancement of H_2 production via metallic NPs using the dark fermentation process, but few studies have been found in the literature for enhanced photofermentative H_2 production by nanoparticles. Zhao et al. investigated the effect of TiO_2-NPs on photofermentative H_2 production using the effluent of the dark fermentation process as a feedstock [86]. It was observed from the results that the TiO_2-NPs enhanced the activity of PNSB for the production of H_2 and reduced the activity of the uptake of hydrogenase enzyme. Another study by Pandey et al. reflected a similar enhancement effect of TiO_2-NPs for photofermentative H_2 production [85]. Meanwhile, Kanwal and colleagues investigated the effect of a phytofabricated nanoscale iron complex for H_2 production using photofermentative PNSB [90]. The use of carbon nanotubes (CNTs) has also been reported for improved hydrogen generation by H_2-producing bacteria [91].

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