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Review

Microbes and Parameters Influencing Dark Fermentation for Hydrogen Production

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Abstract: Dark fermentation is a promising method for hydrogen (H_2) production utilizing the metabolic pathways of diverse microbial communities. This process can be carried out without the need for light, making it easier and more efficient to operate in different environments and at a lower cost. It also utilizes a wide range of substrates, making it highly adaptable to waste-to-energy applications. *Clostridium* spp. are particularly favored in this method due to their versatile metabolism, ability to utilize a wide range of substrates, and high H_2 yields. Anaerobes and facultative anaerobes are mostly used in studies due to their efficient hydrogenase enzyme activity and metabolic pathways. A pH range of 5.5–6.5 and a temperature of 30–37 °C for mesophiles and 55–60 °C for thermophiles are usually preferred in addition to the other parameters such as hydraulic retention time and substrate used. The highest H_2 yield of 9.39 mol H_2/mol sucrose consumed was obtained by *C. beijerinckii* using sucrose as a substrate under batch mode conditions at 37 °C and pH 6–7. The review analyzes different bacterial species and examines the influence of optimized parameters required on H_2 yield in different bioreactor operating modes.

Keywords: hydrogen production; anaerobic digestion; hydraulic retention time; metabolic pathways; organic substrates

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1. Introduction

The depletion of fossil fuels due to their continuous consumption is attracting attention. These fossil fuels adversely affect the environment due to the release of greenhouse gases. The United Nations Sustainable Development Goals, 7th and 13th, aim to develop affordable and clean energy to meet energy needs. Thus, various alternatives are being evaluated to switch to environmentally friendly resources [1], as investing in renewable energy reduces the overall generation of carbon dioxide (CO_2) [2].

Hydrogen (H_2) is a clean and environmentally friendly fuel that can replace current fossil fuel resources in the future. It is estimated that the global H_2 market will reach up to USD 1.6 trillion by 2050 [3]. Investment in H_2 production has increased significantly. The United States is expected to become the largest producer of clean H_2 by 2030, accounting for 37% of the global supply. It is estimated that clean H_2 policies in Europe, Japan, and Korea alone can support up to 1.6 Mt of energy production by 2030 [4]. Hydrogen is a high-energy-density gas that does not release greenhouse gases during combustion. Still, conventional methods of H_2 production, such as water electrolysis, require temperatures of around 50–100 °C, which increases energy demands and relies on freshwater, a resource that is becoming increasingly scarce. Photoelectrolysis, although being presented as an effective approach for H_2 production, has low photolytic efficiency [5], using complex

systems with a short lifetime [6]. The production of H_2 using renewable resources is considered a sustainable and environmentally friendly approach. Organic wastes, such as sewage sludge, organic wastewater, and lignocellulose, contain a huge amount of energy. It has been observed that each kilogram of chemical oxygen demand (COD) generates about $1.4 \cdot 10^7$ kJ of metabolic heat [7].

Biological methods of H_2 production are environmentally friendly and theoretically require low energy demands, as they occur under ambient conditions. Biological processes are being scaled up around the world. Cemvita, a startup based in Texas, USA, is pioneering H_2 production through microbial degradation of residual oil hydrocarbons in depleted wells. The company aims to achieve H_2 production at a cost of USD 1 per kg [8]. These methods include photofermentation, biophotolysis, microbial electrohydrogenesis, dark fermentation, and combined system methods. Figure 1 illustrates the various biological methods utilized for H_2 generation.

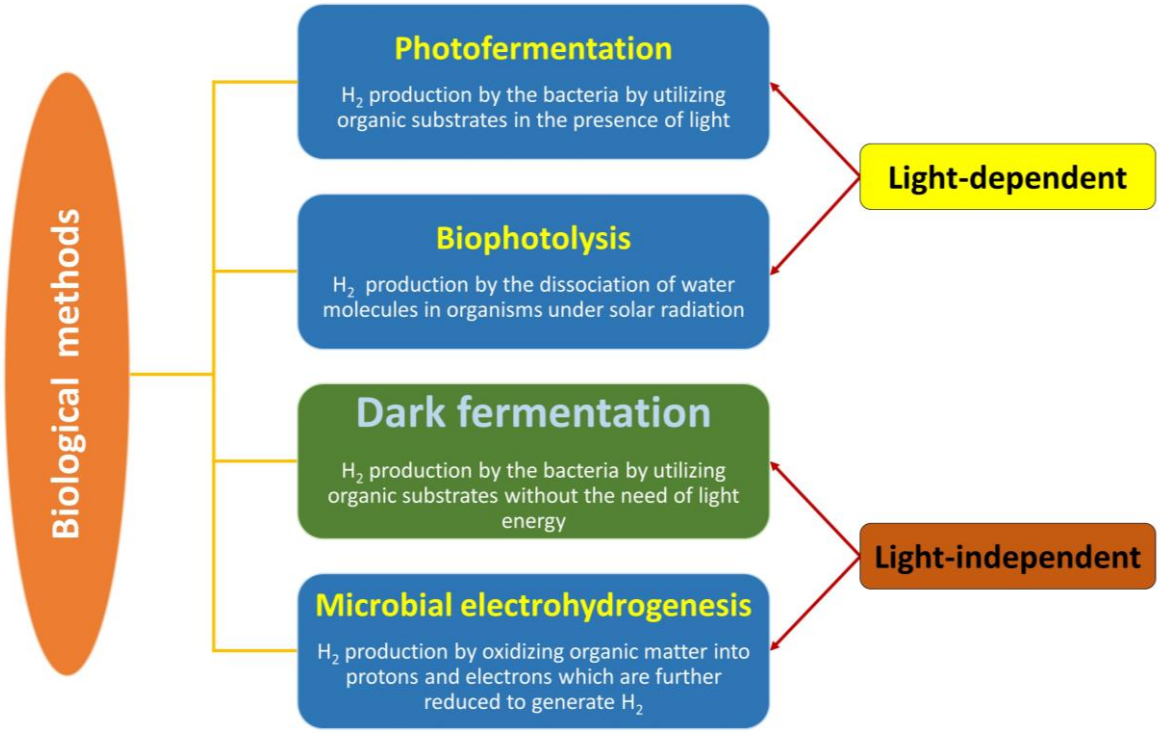

Figure 1. Biological methods of hydrogen production.

Photofermentation refers to the process through which bacteria, under light exposure, convert organic matter into H_2 gas. Purple non-sulfur bacteria can utilize a variety of substrates, including simple sugars, volatile fatty acids, and industrial and agricultural waste products, in the presence of light and anaerobic conditions. These bacteria have been employed in numerous photobioreactors (PBRs) for the enhancement of H_2 production [9]. The genera *Rhodobacter*, *Rhodobium*, *Rhodopseudomonas*, and *Rhodospirillum* are among the microbes involved in photofermentation. They convert organic acids into H_2 and CO_2 [10].

Biophotolysis is another biological light-dependent method for H_2 production. It entails capturing and transforming light energy into H_2 via photosynthesis, during which microbes split water molecules. There are two microbial photosynthesis pathways, one comprising microalgae that utilize a hydrogenase enzyme and the other involving cyanobacteria (blue-green algae), which employ a nitrogenase enzyme and sometimes a hydrogenase enzyme. This process creates an anaerobic environment that is necessary to sustain H_2 production [11]. In direct biophotolysis, the natural splitting of water molecules during photosynthesis is facilitated by the action of light. The production of H_2 is not continuous in this method, as the activity of water splitting is susceptible to oxygen (O_2) evolution

during photosynthesis, resulting in a brief period of H_2 production [12]. Both microalgae and cyanobacteria are capable of performing indirect photolysis, a process in which light is used to produce carbohydrates that are then converted into H_2. This method mitigates the issue of O_2 sensitivity by partially separating CO_2 fixation-based O_2 and H_2 generation by the nitrogenase enzyme [11].

Microbial electrohydrogenesis cells produce H_2 simultaneously with wastewater treatment. They promote the degradation of volatile fatty acids produced and are therefore considered a promising approach to optimize biomass conversion to H_2 [13]. In this method, a microbial anode is coupled to a hydrogen-evolving cathode, and the oxidation of water at the anode is replaced by the oxidation of low-cost compounds. Despite considerable efforts to scale up the system, this approach has faced major difficulties [14].

Among the different biological methods for H_2 production, dark fermentation is the main focus of this article. Dark fermentation, which is part of the acidogenic step of anaerobic digestion [15], offers several advantages as it does not require light and can use various raw materials as substrates for H_2 production by microbes [16]. In this process, microbes use organic compounds as an energy source and convert them into organic acids, alcohols, and gases, such as H_2, through fermentative pathways under anaerobic conditions [15]. The H2Boost project, led by the Biorenewables Development Centre at the University of York, has become the first initiative to successfully produce hydrogen on a large scale through dark fermentation. The project aims to develop a commercially viable and sustainable process for producing hydrogen from organic waste. It is believed that these new technologies have the potential to decarbonize the UK's transport sector and provide up to 35% of the country's energy needs by 2050 [17].

This biological method is used not only for H_2 production but also to convert solid residues into high-quality biofertilizers and to minimize the generation of residues when food waste is used as the substrate [18]. Studies in recent years have suggested that the introduction of synthetic materials or nanoparticles can enhance H_2 production during dark fermentation [19].

Doped composites of metal ions are characterized by a high surface area and exceptional electrical conductivity, which allows the enhancement of colony enrichment and the inter-species efficiency of electron transfer [20]. Dark fermentation is a promising technique for bioenergy production, offering a sustainable approach to generating H_2 through the anaerobic degradation of organic compounds. Parameters such as pH, temperature, microbes used, hydraulic retention time (HRT), substrate type and its concentration, and bioreactor design and its material play a key role in the efficiency of H_2 production using dark fermentation. This article aims to provide data on biological H_2 production via dark fermentation using different microbial strains under varying environmental conditions to compare and devise optimal conditions and microbes that can be used for efficient production.

2. Hydrogen Production Pathways Utilized by Dark Fermentation Bacteria

Dark fermentation is considered a promising alternative method for H_2 production. The general outline of the pathway followed by dark fermentation bacteria involves the breakdown of organic matter into simpler compounds and the release of H_2 as a byproduct under anaerobic conditions. The microbes involved in this process are either strict or facultative anaerobes. This fermentation does not require light energy and is mainly carried out by bacteria from the genus *Clostridium* and the family Enterobacteriaceae with the ability to produce H_2 [15]. The most suitable pathway for H_2 production depends on the bacterial species, the substrate type and concentration, pH, temperature, HRT, and other environmental factors used during the process. Figure 2 shows the general outline of the dark fermentation pathway, along with the key parameters related to dark fermentation, which will be discussed in detail in the following sections.

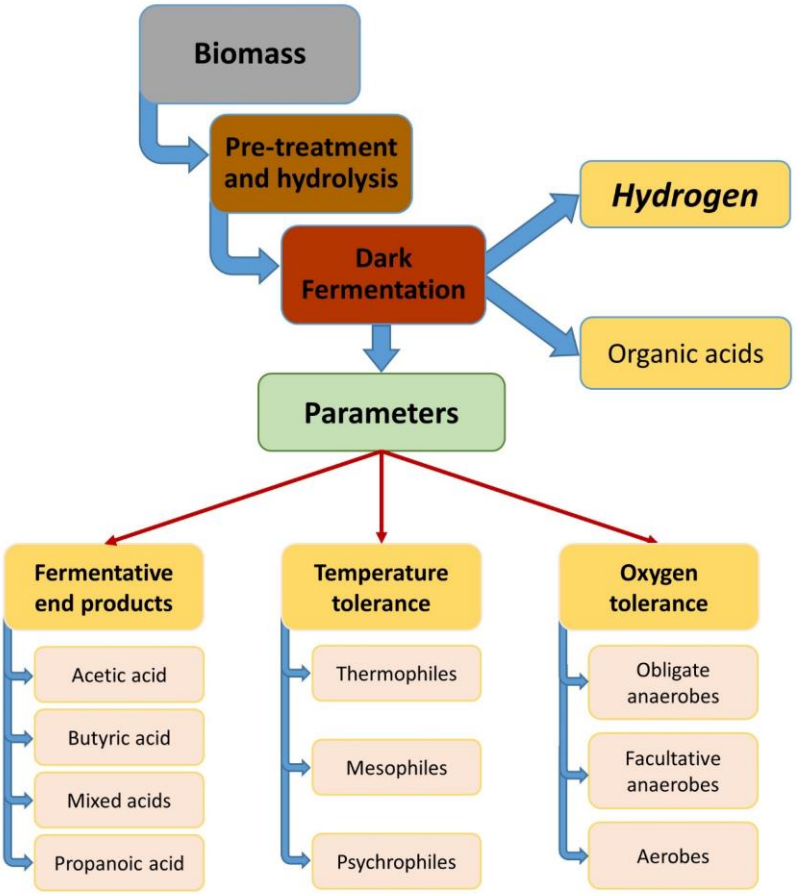

Figure 2. General outline of dark fermentation pathways and the parameters concerning dark fermentation. Adapted and reprinted with permission from [21] *Renewable Energy Conversion Systems*, Academic Press, M. Kamran, Chapter 8—Bioenergy, Copyright (2021), Elsevier, and [16] *Chemical Engineering Journal*, 481, Z.T Zhao J. Ding B.Y. Wang, M.Y. Bao, B.F. Liu J.W. Pang, J.Q. Ren, S.S. Yang, Advances in the biomass valorization in dark fermentation systems: A sustainable approach for biohydrogen production, 148444, Copyright (2024), Elsevier.

The pathway involves the uptake of organic substrates such as carbohydrates, organic acids, and other biodegradable compounds. These substrates undergo glycolysis in the cytoplasm of the bacterial cell, where glucose is converted to pyruvate, producing adenosine triphosphate (ATP) and reduced cofactors such as nicotinamide adenine dinucleotide (NADH). Pyruvate also undergoes acidogenesis, where it is converted into organic acids through the activity of enzymes such as pyruvate decarboxylase and formate hydrogenlyase to produce H^+ ions. Electrons are generated through the reduction of NADH, which is produced during glycolysis. Hydrogenase enzyme facilitates the release of molecular H_2 from the protons and electrons produced [22]. The fermentation pathways include the formate and NADH pathways, which can be further classified based on the bacteria involved. For example, pathways followed by *Clostridium* and *Enterobacter* spp. are classified as Clostridial-type and Enterobacterial-type fermentations based on specificity. In both Clostridial-type and Enterobacterial-type fermentations, H_2 is produced during the glycolytic pathways mentioned above. In Clostridial-type fermentation, pyruvate:ferredoxin oxidoreductase (PFOR) oxidizes pyruvate to acetyl coenzyme A (acetyl-CoA) in the presence of ferredoxin, which is simultaneously reduced. On the other hand, NADH:ferredoxin oxidoreductase (NFOR) catalyzes the reduction of ferredoxin by NADH. Electrons are released from reduced ferredoxin and are used in proton reduction by hydrogenases to produce H_2. In Enterobacter-type fermentation, NADH oxidation by NFOR forms H_2, similar to the process in Clostridial-type fermentation. In mixed-acid fermentation, how-

ever, pyruvate is converted to acetyl-CoA and formic acid by the activity of pyruvate formate lyase (PFL). Formate hydrogenlyase degrades formic acid into H_2 and CO_2 [23,24]. Hydrogen production by dark fermentative bacteria from biomass mainly occurs via the acetate and butyrate pathways [25]. The maximum H_2 yield through the acetate pathway is 4 mol H_2/mol glucose consumed, while the butyrate pathway offers a maximum theoretical H_2 yield of 2 mol H_2/mol glucose [26]. According to these studies, the type of bacteria, the environmental conditions, and the process parameters are critical in determining the optimal conditions for maximizing H_2 yield. In fact, the acetate pathway with glucose as a substrate gives better H_2 yields than the butyrate pathway, but performance may vary depending on environmental factors, according to other studies. Figure 3 shows the two main pathways utilized by the bacteria during dark fermentation.

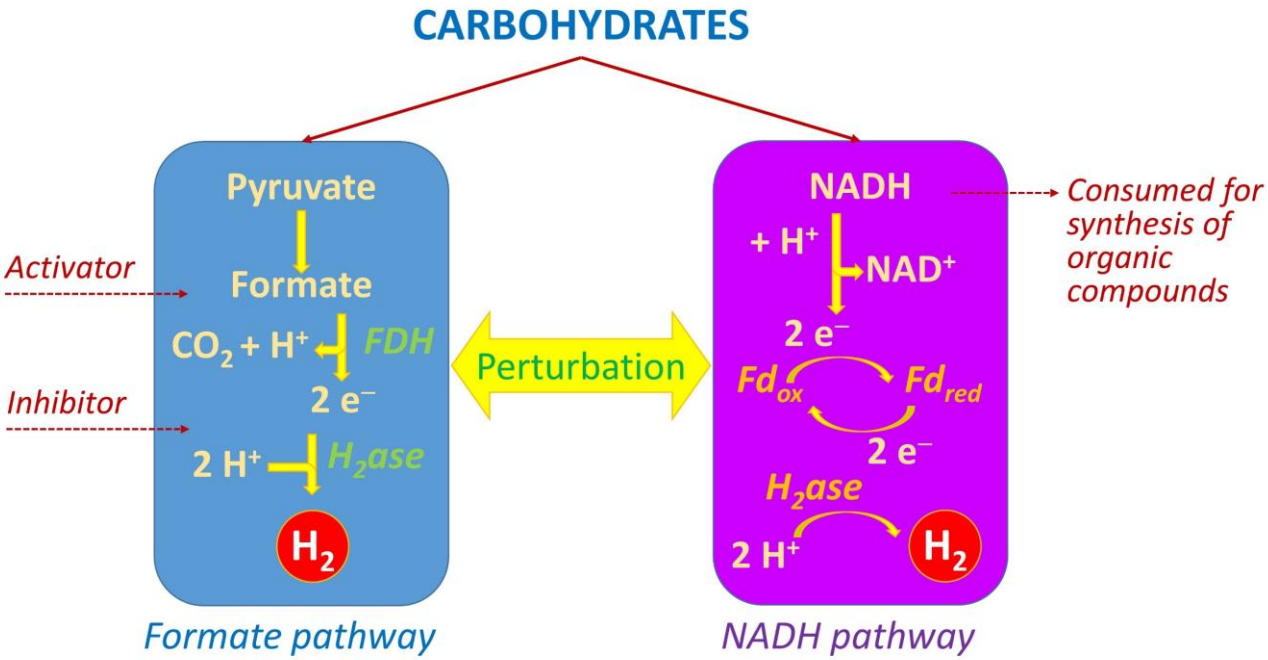

Figure 3. Two main hydrogen-producing pathways in dark fermentation [27].

3. Factors Influencing Dark Fermentation Reactions

Dark fermentation is influenced by many factors, including concentration, composition, and type of substrate. Different organic matter, such as carbohydrates, organic waste, and biomass, can be used as substrates at different concentrations. Based on these factors, the fermentation rate and H_2 yield will vary. Excessively high substrate concentrations can inhibit the activity of microbes due to substrate inhibition or osmotic stress [28]. Microbial consortia are another parameter to consider, as the choice of microbes for dark fermentation strongly influences the amount of H_2 produced, followed by the pH.

Different microbes have optimal pH ranges at which they have the highest affinity to produce H_2. In most cases, a slightly acidic to neutral pH is preferred by the dark fermentation microbes, as the fluctuations in pH can affect microbial activity and fermentation efficiency [29]. Alongside pH, temperature significantly affects the rate of dark fermentation. Certain microbes require optimal temperatures for their growth and activity. Typically, temperatures ranging from 30 to 40 °C are favorable for dark fermentation, although certain hydrogen-producing microbes can operate at 55 °C and even at 70 °C [30].

HRT, another factor affecting dark fermentation, is the average time that the substrate is in the bioreactor during the fermentation process. It affects the efficiency of substrate utilization and the amount of H_2 produced. HRT varies depending on the substrate and the microbial community. In addition, nutrient availability, inhibitors, agitation and mixing, and H_2 partial pressure affect H_2 production during dark fermentation. Nutrients aid in microbial growth and fermentation activity. A lack of nutrients can limit microbial activity,

while inhibitors such as heavy metals or phenolic compounds can inhibit microbial growth and reduce their H_2 production efficiency [31]. Figure 4 shows various factors that affect H_2 production in dark fermentation.

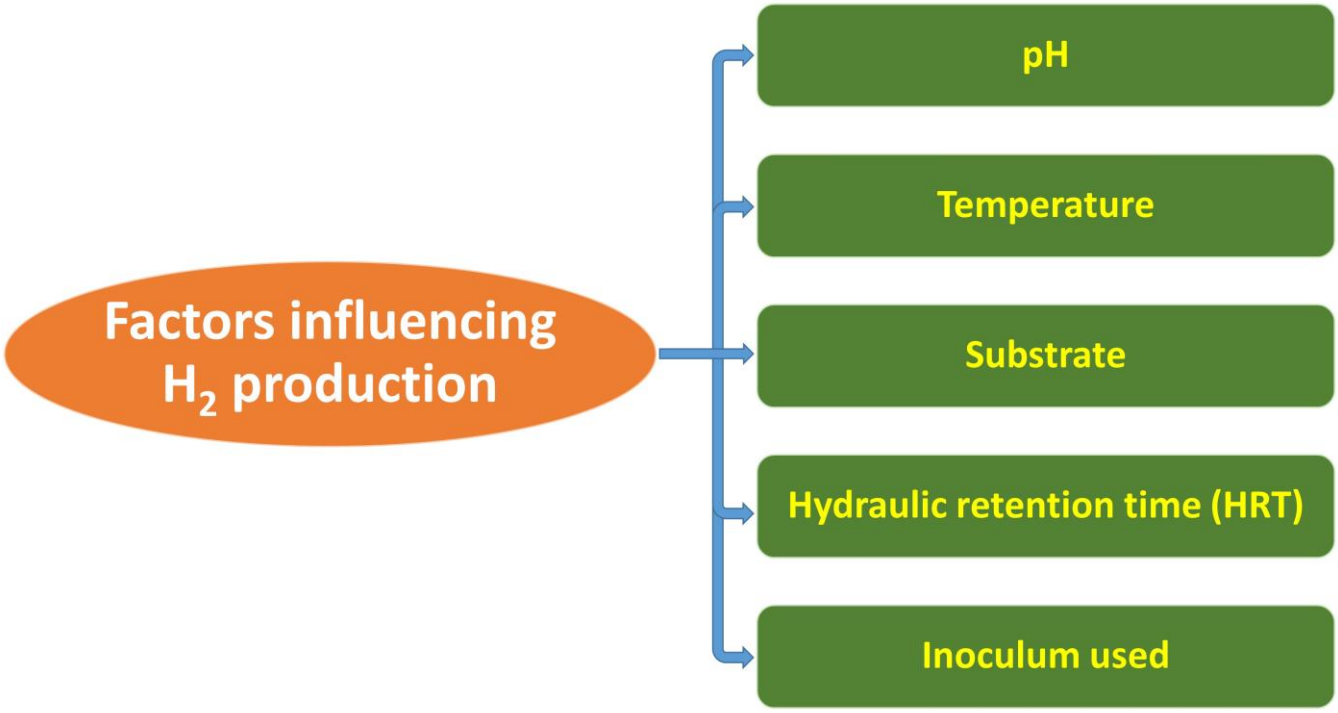

Figure 4. Factors influencing hydrogen production via dark fermentation.

3.1. Effect of pH

pH is an important parameter that influences the efficiency of H_2 production during dark fermentation. It affects the fermentation rate, the production of H_2 and organic acid, the metabolic activity and growth of bacteria, and substrate utilization during anaerobic substrate degradation [29]. Hydrogenase activity and the metabolic pathways in hydrogen-producing microbes are strongly influenced by pH but only within a reasonable range. Variations in pH can affect the conformation and activity of hydrogenase, thereby affecting the rate of H_2 production.

ATP is used to maintain neutrality in the cell, and pH values that are too high or too low can decrease the number of bacteria due to lower ATP levels in the cells [32,33]. Optimal pH enables higher H_2 yield, solving the issues related to solventogenesis and inhibiting methanogens. pH values between 5 and 6 are preferred for H_2 production, as the activity of methanogens is limited in the range between 6.5 and 7.0, although it has been observed that the optimal pH value varies depending on the type of inoculum and substrate used [34]. A study was conducted to analyze the effect of pH on the H_2 production ability of dark fermentation bacteria using organic wastewater with a high concentration of nitrogen-containing compounds [35]. A pH range from 4 to 11 was analyzed, with the best results achieved at pH 5, where the H_2 production rate was 0.053 mmol/h, the COD removal rate was 37.13 $\pm$ 1.86%, and the nitrate reduction rate was 1.57 $\pm$ 0.27 mg/L/h.

According to another research comparing different types of substrates, Spirulina platensis hydrolysate, which is a protein-based substrate, has an optimal pH range for H_2 production between 4.5 and 5.7 [36]. However, a pH range between 5.3 and 5.6 was found to be optimal for glucose-based substrates in dark fermentation [37], proving that variations in optimal pH can be related to substrate diversity, operating conditions, and inoculum sources [38]. Kim et al. [39] found that decreasing the pH to 5 negatively affected the H_2 yield compared to pH 8, with a decrease from 1.92 to 0.67 mol H_2/mol hexose. Another study defined that the inhibition of H_2 production due to a pH shift from 6 to 5 is caused

by the acidic environment, which limits the development of reducing metabolites [40]. On the contrary, the H_2 yield in continuous stirred tank reactors (CSTRs) was higher at a pH of 4.0–4.5 than at a pH of 6.0–6.5 [41]. Therefore, it can be stated that pH can influence the solubility and availability of organic substrates for microbial utilization. Variations in the solubility of substrates, such as carbohydrates and organic acids, can affect their accessibility to hydrogen-producing bacteria, making pH an important parameter to optimize for high H_2 yield.

3.2. Effect of Temperature

Temperature directly affects the metabolic activity of bacteria and their ability to produce H_2. The enzymatic reactions necessary to produce H_2 occur under optimal temperature conditions. In addition, the dynamic growth of bacterial populations also depends on temperature. At higher temperatures within an optimal range, the growth of these bacterial populations can be accelerated, leading to an increase in biomass and potentially higher overall H_2 production [42]. Hydrogen-producing microbes can operate in mesophilic, thermophilic, and even hyperthermophilic ranges. Sillero et al. [30] studied the effect of temperature on biohydrogen production using different mixtures of sewage sludge, vinasse, and poultry manure. In the biochemical H_2 production (BHP) experiments, temperatures of 35, 55, and 70 °C were tested in two mixtures. One of them consisted of sewage sludge and vinasse in a ratio of 50:50, while the other consisted of sewage sludge, vinasse, and poultry manure in a ratio of 49.5:49.5:1. The results showed that the addition of poultry manure at a temperature of 55 °C was ideal for biohydrogen production, with a yield of 0.522 mol H_2/mol volatile solids, which confirmed that H_2 production efficiency through dark fermentation is favorable at thermophilic temperatures. The choice of the optimal temperature for fermentation depends entirely on the microbes used, whether they are from a pure culture or a mixture, and on the substrates [43]. Efficient substrate hydrolysis has been reported under thermophilic conditions, but the presence of chemical compounds in the fermentation substrate may be affected by higher temperatures, reducing the conversion efficiency of substrates [44].

Zaira et al. [45] investigated the effect of temperature on the microbial community and biohydrogen production from lactate wastewater using dark fermentation. Temperatures of 35–45 °C were found to be favorable for H_2 production, indicating that hydrogen-producing bacteria in anaerobic sludge can grow within a narrow temperature range and that the enzymes catalyzing lactate conversion are also temperature-specific. The maximum H_2 yield of 0.85 mol H_2/mol lactate was obtained at 45 °C and pH 8.5. Additionally, the fermentation batch cycle length and the acclimation time for H_2 production are affected by temperature. The results showed that at 35 °C, the fermentation batch cycle length was about 65 h, while at 45 °C, it was 180 h. This suggests that different microbes are activated at different times due to variations in fermentation temperatures.

Another study on dark fermentation reported that mesophilic temperatures (37 °C), an HRT of 72 h, and a pH close to 7 are recurrent parameters of biomass fermentation [16]. Lin et al. [46] observed that H_2 yield was maximized with thermophilic bacteria due to their slow proliferation and lower cell densities, although efficient biohydrogen volumetric productivity was achieved with intermediate thermophiles, indicating thermophiles as very efficient species for high H_2 yield. In contrast, an analysis at temperatures of 50–55 °C showed 5–10 times higher H_2 production compared to 30–45 °C. A comparative analysis between mesophilic and thermophilic temperatures for H_2 production using cheese whey wastewater showed that 0.25 mol H_2/mol cheese whey was produced at 55 °C, while 0.29 mol H_2/mol cheese whey was produced at 35 °C [47]. These studies show that temperature alone is not responsible for high H_2 production; other parameters, such as substrate, pH, and other environmental factors, determine which bacteria are best suited for a particular fermentation process.

3.3. Substrates Used by the Dark Fermentation Bacteria

Substrates are critical for H_2 production in dark fermentation. The choice of substrate depends on its availability, the type of microbial inoculum used, cost, composition, and the desired end product. In addition, pretreatment methods such as acid or alkali treatment, milling, and enzymatic hydrolysis can improve the accessibility and digestibility of complex molecules. In an integrated microbial electrolysis cell (MEC) and dark fermentation system, a twofold increase in H_2 yield was achieved, rising from 0.12 to 0.23 mol H_2/mol substrate per day, and when 20 g/L corn stalk was used as the substrate in the same system, a simultaneous increase in H_2 production was observed [48].

In another H_2 production study, an MEC integrated with dark fermentation using cellulose as a feedstock resulted in a 42% increase in H_2 yield compared to dark fermentation alone [49].

Xue et al. [50] used food waste from a treatment plant as a substrate in dark fermentation experiments. The concentration of the substrate was slowly increased from 10 to 120 g of volatile solids per liter. Silt from the bottom of a lake was used as the inoculum, and the fermentation was carried out for 10 days. An H_2 yield of less than $1.16 \cdot 10^{-4}$ mol H_2/g of substrate was observed due to insufficient acidity. However, when a high substrate concentration was used, it facilitated rapid and strong acidification of the substrates, inhibiting only the non-hydrogen-producing bacteria, thus increasing the H_2 production capacity to $1.41 \cdot 10^{-3}$ mol H_2/g substrate.

In the analysis of the dynamics of bacterial communities and substrate conversion, olive mill waste, lactate, and acetate were used as substrates. Clostridium and Bacillus were the most abundant microbes during H_2 generation. The inoculum used was obtained from an anaerobic reactor treating cattle effluent, and the analyses indicated that lactic acid fermentation was the main hydrogen-producing pathway, although the amount of H_2 produced was not mentioned in the article [51].

In a study with *Clostridium butyricum*, 3 g/L of glucose was used as a substrate in an anaerobic vessel flushed with nitrogen (N_2). The experiments were performed in batch mode with an initial pH of 6.5 and a temperature of 37 °C. The conditions and substrate used allowed the production of 2.09 mol H_2/mol hexose [23]. In H_2 production studies with *Clostridium beijerinckii*, the bacteria produced 3.58 mol H_2/mol dextrose when 3 g/L of dextrose was used as a substrate in batch mode at 120 rpm and 37 °C [52].

In an analysis of thermophilic dark fermentation start-up for H_2 production, a bioreactor with a working volume of 5 L, 6 h HRT, and 55 °C temperature was set up. The results showed that a maximum of 1.64 mol H_2/mol glucose was obtained at a glucose concentration of 6 g/L using *Thermoanaerobacterium thermosaccharolyticum* strain TG57 [53]. In another study aiming at analyzing the influence of bisphenol A (BPA) and thermophilic bacteria, 20 g/L of food waste was used as a substrate in a 55 mL serum bottle. An experiment was conducted at 55 °C with a pH of 7, an inoculum ratio of 10% (*v*/*v*), and a stirring speed of 150 rpm. In the control experiment, without BPA, 1.36 mol H_2/mol substrate was produced, while with the addition of 100 mg/L BPA, H_2 production was reduced to 1.30 mol H_2/mol substrate and continued to decrease as the BPA concentration was further increased [54].

In a study with *Clostridium* spp. YM1, 1.19 mol H_2/mol sugar was produced when 27.73 g/L of rice straw hydrolysate, consisting of xylose and glucose, was used as a substrate, indicating that the butyric acid pathway was the main pathway followed by these bacteria. The analysis was conducted at a temperature of 30 °C with an initial pH of 6.5, and 20 mg of Mg^{2+} or Fe^{2+} was added to a 250 mL glass bottle to enhance the production of H_2 [55].

3.4. Hydraulic Retention Time (HRT)

HRT strongly influences microbial growth, substrate utilization, and H_2 yield [56]. Longer HRT allows for more substrate utilization by the microbes, influences the growth rate and metabolic activity of the bacteria, and provides sufficient acclimation and adaptation time for the bacteria to optimize their metabolic pathways for efficient substrate

degradation and biogas production. In addition, long HRT helps dominant species establish themselves and suppress less competitive species. It contributes to process stability by ensuring adequate substrate utilization rates, high efficiency, and improved microbial performance. However, on the other hand, in a continuous process, extended HRT can cause biomass washout, making longer HRTs not very adequate to achieve high H_2 yields [57]. For H_2 fermentation, shorter HRTs are preferred to reduce H_2 consumption, and in smaller reactors, they help reduce costs as well as wash out competing species [58].

In a study using molasses as a substrate in a vertical continuous stirred tank reactor with dark fermentation, an initial HRT of 8 h was gradually decreased to 2 h. The maximum H_2 yield was 0.6 mol H_2/mol substrate at 5 h HRT [59]. Pugazhendhi et al. [60] utilized 15 g/L glucose as a substrate in a mesophilic fixed-bed reactor with anaerobic digester sludge as the inoculum, gradually decreasing HRT from 12 to 1.5 h. Butyrate and acetate were the metabolic products during fermentation, with the highest H_2 yield of 2.3 mol H_2/mol glucose produced by *Clostridium butyricum* as the dominant species at all the HRTs analyzed. Another analysis using galactose and glucose in a continuously stirred tank reactor with inoculum from heat-treated digester sludge was performed for HRTs ranging from 6 to 24 h. The peak H_2 yield of 1.62 mol H_2/mol glucose was obtained using glucose as the feedstock at HRTs of 6 and 18 h, whereas with galactose, the H_2 yield was 1.00 mol H_2/mol galactose at HRTs of 12 and 24 h. In addition, in a mixture of galactose and glucose in a ratio of 8:2, an H_2 yield of 0.48 mol H_2/mol carbohydrate added was observed at HRTs of 6 and 18 h, respectively [61].

Hydrogen production in a continuously stirred tank reactor is most often studied because it provides more interaction between the substrate and the microbes. In a continuously stirred bioreactor at a temperature of 32 ± 1 °C and stirring at 60 rpm, the HRT was studied from 24 to 3 h under dark fermentation conditions. A mixture of 4 g/L each of cellobiose, xylose, and arabinose was used as the substrate to obtain the highest average H_2 yield of 7.84 mol H_2/mol substrate mixture at 3 h and pH 6, indicating the influence of HRT and pH on H_2 yield [62]. In another study using xylose in a dynamic membrane module bioreactor with a 444 µm pore polyester mesh, the H_2 production rate was analyzed from 12 to 3 h HRT at 37 °C. The results showed that the maximum yield of 1.40 mol H_2/mol xylose consumed was observed at 3 h HRT, indicating that lower HRTs are better for H_2 production because the bacterial population can shift from non-H_2 producers like *Lactobacillus* and *Sporolactobacillus* spp. to H_2 producers such as *Clostridium* spp. [63].

4. Bacteria Used for Hydrogen Production During Dark Fermentation

The rate of H_2 production by bacteria as part of their metabolism depends entirely on the conditions described above, namely pH, temperature, substrate used, HRT, and the type of bioreactor. Several bacterial species are well known for their ability to produce H_2 under dark fermentation conditions, including *Clostridium* and *Enterobacter* spp., *Escherichia coli*, and facultative anaerobes such as *Klebsiella pneumoniae*.

4.1. Clostridium

Clostridium spp. has been widely used in dark fermentation studies for H_2 production, especially *C. butyricum*, *C. beijerinkii*, *C. acetobutylicum*, and *C. thermocellum*. These strict anaerobic bacteria are found in soil, water, and animal intestines and are used in pure and mixed culture systems for dark fermentation H_2 production [64]. *Clostridium* spp. bacteria break down carbohydrates to produce organic acids, alcohols, and gases. H_2 production by these bacteria involves the conversion of pyruvate to acetyl-CoA by the enzyme PFOR, releasing CO_2 and transferring electrons to ferredoxin, which, combined with protons, forms molecular H_2. Hydrogenase is the key enzyme for H_2 production, catalyzing the reversible oxidation of molecular H_2. pH range of 5–7 and a temperature of 30–37 °C are optimal for H_2 production by these bacteria [65].

4.1.1. *Clostridium butyricum*

C. butyricum was first isolated from pig intestines by Prazmowski in 1880 [66]. The substrates used by this species to produce H_2 include glucose, fructose, xylose, starch, and glycerol. In addition, complex organic compounds (biomass and organic waste) can be used. Certain strains of *C. butyricum* are tolerant to alkaline [67] and phenol [68]. Table 1 presents several hydrogen-producing *C. butyricum* strains used for H_2 production under different conditions.

Table 1. Hydrogen production by different *Clostridium butyricum* strains.

Strain	Substrate (Concentration, g/L)	T (°C)	pH	Conditions Time (h)	More Details	Operation Mode (Reactor Volume, mL)	H_2 Yield as Mol H_2/mol Substrate *	Ref.
DSM2478, NCIMB8082	Glucose (3)	37	6.5		Anaerobic jars purged with N_2	Batch (250)	2.09	[23]
TM-9A	Glucose (10)	37	8.0	24	10.1 kPa	Batch (67)	3.10	[67]
RAK25832	Glucose (10)	30	8.0		Serum bottles flushed with N_2	Batch (75)	1.81	[69]
SP4	Hexose and pentose (5)	30			110 rpm, glass vials in N_2 flow	Batch (60)	0.93–1.52	[70]
TM-9A	Sugarcane molasses	37	7.5	24	Serum bottles	Batch (120)	3.34 (synthetic analytical grade glucose)	[71]
TISTR 1032	Synthetic food waste with volatile solids (28)	37	6.0		100 rpm	Semi-batch (5000)	0.02 (volatile solids)	[72]
BOH3	Fruit waste with sugar (10)		6.8	24	Purged with N_2	Batch (250)	2.30	[73]
CGS2	Glucose (5)	37	6.8		130 rpm	Batch (250)	0.95	[74]
CCT 7470	Cellulose and glucose (5)	37			Cellulose medium	Batch (250)	0.19 (cellulose), 0.58 (glucose)	[75]
INET1	Glucose (10)	35	7.0		10% *v*/*v* inoculum, purged with N_2, 100 rpm	Batch (150)	2.07	[76]
NH-02 (MT229351)	Maltose (10)	30	7.0	23	Purged with argon, 4% *v*/*v* inoculum	Batch (100)	1.90	[77]
CWBI1009	Glucose (5)		7.3		400 rpm, N_2 sparging	Batch (2500)	3.10	[78]
CWBI1009	Glucose (5)	30	7.3		N_2 flushing	Batch (270)	1.43	[79]
TERI BH05-2 in recombinant *E. coli*	Glucose (10)	37	7.0	48	N_2 flushing, 100 rpm	Batch (67)	3.20	[80]
TISTR 1032	Sucrose (25)		6.5		Sugarcane bagasse for immobilization	Batch	1.34	[81]
CGS5	Microalgal biomass hydrolysate (9)	37	5.5	30		Batch (20)	1.15	[82]
NRRL B-4112	Glycerol (10)			168	N_2 sparging		1.02	[83]
NRRL B-41122 with *Enterobacter aerogenes* NRRL B-407	Glycerol (10)	36	6.5		150 rpm	Batch (47)	1.25	[84]
TISTR 1032	Sugarcane juice	37	6	36	10% *v*/*v* inoculum, 150 rpm	Continuous (1000)	1.0	[85]
DSM 10702	Glucose (5)		5.5	12	N_2 purging	Batch + continuous (4500)	2.61	[86]
KCCM 35433 with *Sporolactobacillus vineae* KCCM 11493BP	Glucose (5)	35		32	200 rpm	Batch (160)	1.84	[87]

* In parentheses, the reference substrate for calculation when it differs from the cited one.

C. butyricum bacteria are widely used for H_2 production in pure culture as well as in mixed culture conditions. The studies indicated in Table 1 show that these bacteria are known to utilize a wide range of substrates, including glucose, sucrose, xylose, and even complex biomasses, and can work very efficiently for H_2 production around pH 7 and temperature of 30–37 °C in both batch and continuous mode of operation. *C. butyricum* TM-9A, when used with sugar molasses as a substrate at 37 °C and pH 7.5 for 24 h, produced

a maximum of 3.34 mol H_2/mol synthetic analytical-grade glucose. Therefore, these species are widely used in fermentation systems due to their high hydrogen-producing and substrate-degrading abilities.

4.1.2. *Clostridium beijerinckii*

C. beijerinckii has been extensively studied for the production of H_2 and butanol. This species has shown good tolerance to pH changes and various inhibitors and is known to utilize a variety of substrates, such as glucose, xylose, and cellulosic biomass [88]. During the dark fermentation process, the substrates are converted to pyruvate by glycolysis via the Embden–Meyerhof–Parnas (EMP) pathway. The pyruvate is further converted to acetyl-CoA by the enzyme PFOR, which produces reduced ferredoxin that donates electrons to hydrogenase enzymes and catalyzes the production of H_2 gas [89]. Table 2 presents a list of *C. beijerinckii* strains used to produce H_2 with different substrates and operational conditions.

Table 2. Hydrogen production by different *Clostridium beijerinckii* strains.

Strain	Substrate (Concentration, g/L)	Conditions				Operation Mode (Reactor Volume, mL)	H_2 yield as Mol H_2/mol Substrate	Ref.
		T (°C)	pH	Time (h)	More Details			
DSM 1820	Glucose (2)	37			10% *v*/*v*, flushed with N_2, 100 rpm	Batch (180)	2.70 ± 0.20	[24]
Not specified	Dextrose (3)	37	7.5	96	Flushed with N_2, 120 rpm	Batch	3.58	[52]
DSM 791	Glycerol (11)	37	7.5	96		Batch (120)	1.21	[90]
G117	Glycerol (20–80)	39	6.8	12–72	Purged with N_2, 150 rpm	Batch (120)	1.18–1.45	[91]
NCIMB 8052	Glucose (20)	37	6.5		Cathodic electrofermentation, graphite felt electrode, 130 rpm, sparged with N_2	Batch (150)	1.51	[92]
ATCC 8260	Sucrose (7.5)	37	6.0–7.0		1 mL inoculum	Batch (15)	9.39	[93]
NCIMB-8052	Glucose (3)	32	6.5	120	Sparged with N_2, 10% *v*/*v* inoculum, 120 rpm, light illumination	Batch (60)	2.47	[94]
NCIMB 8052	Glucose:xylose 1:5 *w*/*w* (3)	30			Flushed with N_2, 3% *v*/*v* inoculum	Batch (26)	0.09	[95]
RZF-118	Glucose (9)	35	7.0	20	Flushed with N_2, 8% *v*/*v* inoculum, 140 rpm	Batch (100)	1.97	[96]
ATCC 8260	Glucose (3)	30	6.3		Flushed with argon, 3% *v*/*v* inoculum, 180 rpm	Batch (100)	0.191	[97]

It was observed from Table 2 that when *C. beijerinckii* was inoculated in batch mode of operation, it showed a maximum H_2 production of 9.39 mol H_2/mol sucrose consumed. These bacteria have mostly been studied with glucose as the substrate, with a pH range from 6 to 7 and a temperature of 37 °C. *C. beijerinckii* NCIMB-8052 yielded an H_2 yield of 2.47 mol H_2/mol glucose using a 10% *v*/*v* inoculum, pH 6.5, 32 ± 1 °C, 120 rpm, over 120 h under light illumination. However, when the same strain was fed a mixture of glucose and xylose as the substrate at 30 °C, it produced a lower H_2 yield of 0.086 mol H_2/mol substrate, indicating that using only glucose as the substrate provided a better H_2 yield.

4.1.3. *Clostridium pasteurianum*

C. pasteurianum follows a similar pathway to other *Clostridium* spp. It is known as an iron-reducing bacterium used for biofuel production via fermentation. Discovered in 1890 by Sergei Winogradsky, this bacterium can survive in mesophilic conditions and can fix atmospheric N_2. *C. pasteurianum* can utilize various substrates [98], release various products, and show rigorous growth in basic media under non-sterile conditions. The *C. pasteurianum* strains DSM525, CH5, H4 (DSM 525), and MTCC 116 produce H_2 with greater

efficiency in the presence of iron oxides [99]. This bacterium is highly efficient in anaerobic H_2 production. Strict anaerobic bacteria of this species contain Fe–Fe hydrogenases in their cytoplasm, and when they use organic matter as a substrate, electrons are released. These electrons are used by the Fe–Fe hydrogenase to form H_2 with the concomitant reoxidation of reduced ferredoxin produced during degradation [100].

In a study, the effect of $ZnFe_2O_4$ nanoparticles on H_2 production by *C. pasteurianum* DSM 525 was investigated [101]. Optimal operational conditions were determined using the response surface method, which provided 2.14 mol H_2/mol glucose, corresponding to a 116% increase in H_2 production compared to the control after 48 h of batch culture. The metabolic pathway for H_2 production shifted from the butyric acid type to the ethanol type. These bacteria were also used as a coculture with *Geobacter sulfurreducens* in glucose fermentation [102]. This study showed that the maximum rate and yield of H_2 production in the coculture increased by 122.2% and 28.92%, respectively, compared to monoculture, using 50 mL serum vials at 37 °C, without agitation. Table 3 presents a list of *C. pasteurianum* strains used to produce H_2 with different substrates and operational conditions. Most of these bacteria were used at 35–37 °C since they are mesophilic, with the maximum H_2 production rate observed at pH 6.6–6.8.

Table 3. Hydrogen production by different *Clostridium pasteurianum* strains.

Strain	Substrate (Concentration, g/L)	Conditions				Operation Mode (Reactor Volume, mL)	H_2 Yield as Mol H_2/mol Substrate	Ref.
		T (°C)	pH	Time (h)	More Details			
	Glucose (0.12)	30	7.0	60		Batch (100)	2.34 ± 0.02	[103]
CH5	Glucose	35	7.0		120 rpm	Batch (120)	1.61	[104]
MTCC116	Glucose (54.18)	37	6.6	96	10% *v*/*v* inoculum, 192 rpm	Batch (150)	$3.60 \cdot 10^{-3}$	[105]
DSM525	Glucose	37			Flushing with N_2, 5% *v*/*v* inoculum, ferrihydrite nanorods	Batch (25)	3.55	[106]
	Glycerol (7.4)	36	6.7		10% *v*/*v* inoculum, 150 rpm	Batch	0.63	[107]
MTCC 116	Glycerol (7.4)	36	6.7		10% *v*/*v* inoculum, flushing with N_2, 150 rpm	Batch (100)	2.00–3.00	[108]
CH5	Xylose (40)	35	6.8		Flushed with argon, 120 rpm, 400 mg/L nanometal particles for bacteria immobilization	Batch (120)	0.16	[109]
CH5	Xylose (40)	35			10 mL inoculum, nanometal and soluble iron, 120 rpm, flushed with argon	Batch (120)	1.46	[110]

C. pasteurianum has been studied using glucose, glycerol, and xylose as substrates. Some of the studies have incorporated nanoparticles for bacterial immobilization, increasing the hydrogen-producing ability of this species [106]. Strain DSM525 provided the highest H_2 yield of 3.55 mol H_2/mol of glucose as a substrate under batch mode at 37 °C.

4.1.4. *Clostridium thermocellum*

C. thermocellum is a thermophilic anaerobe first isolated in 1926. This bacterium is known to grow at 60–65 °C and pH 6.5–7 and to form cellulosomes on its cytomembrane. *C. thermocellum* is attracting attention for H_2 production as it can convert cellulose and hemicellulose directly into ethanol and H_2 [111]. This bacterium is widely used for H_2 production from lignocellulosic biomass and paper waste [112]. In a study to determine the cellulose-degrading ability of these bacteria, cellobiose was used at three different substrate concentrations. When the substrate concentration was low at 0.1–4.5 g/L, H_2 yields of 1–1.5 mol H_2/mol glucose were obtained at 60 °C, pH 7.3 in batch mode with

10 mL working volume [113]. Table 4 shows a list of *C. thermocellum* strains with their H_2 production ability using different substrates and operational conditions.

Table 4. Hydrogen production by different *Clostridium thermocellum* strains.

Strain	Substrate (Concentration, g/L)	Conditions				Operation Mode (Reactor Volume, mL)	H_2 Yield as Mol H_2/mol Substrate *	Ref.
		T (°C)	pH	Time (h)	More Details			
DSM 1237	Cellulose (25)	60	7.3–7.4			Batch (20)	1.30 (hexose)	[114]
DSM 1313	Cellulose (5)	60	7.0		Argon flushing, 60 rpm	Continuous (2000)	0.76–1.21 (hexose)	[115]
27405	Cellulose (5)	55	7.0	168	Purged with N_2, 150 rpm	Batch (120)	1.92 (hexose)	[116]
KJC315	Cellobiose (5)	60	7.0	24	Purged with N_2, 100 rpm, 33% CO_2	Batch (130)	3.26 (hexose)	[117]
DSM1313	Cellulose (10)	55		192	Purged with N_2, 10% *v*/*v* inoculum, 150 rpm	Batch (130)	1.15 (hexose)	[118]
ATCC 27405	Cellulose (3)	55		168	20 mM $CaCO_3$, 10% (*v*/*v*) inoculum, 150 rpm	Batch (120)	5.87 (hexose)	[119]

* In parentheses, the reference substrate for calculation when it differs from the cited one.

Studies have shown that these anaerobic bacteria are very capable of degrading cellulose. Most of the studies have used a temperature of either 55 or 60 °C, which is optimal for the activity of *C. thermocellum*. The most commonly used strains include *C. thermocellum* ATCC 27405 and DSM1313. The maximum H_2 production of 5.87 mol H_2/mol hexose was obtained with 3 g/L of cellulose at 55 °C, 168 h, 20 mM $CaCO_3$, and 150 rpm, indicating the influence of $CaCO_3$ in obtaining higher H_2 yields. In addition, it was observed that these bacteria have been used at higher HRTs, indicating that their metabolic activity and growth can be maintained for longer periods compared to other species to produce H_2.

4.2. *Enterobacter*

Enterobacter spp. are known to be potential strains for large-scale H_2 production due to their high growth rates, adaptability to environmental conditions, utilization of waste biomass such as feedstock, and resistance to variations in pH, dissolved O_2, and H_2 pressure [120]. Metal nanoparticles have also been used to increase H_2 production by enhancing ferredoxin–oxidoreductase activity [121]. The addition of iron or nickel oxides can help the bacteria produce H_2 by promoting their cell proliferation and enzyme production [122].

4.2.1. *Enterobacter aerogenes*

E. aerogenes is a facultative anaerobe that produces ATP through oxidative phosphorylation. In the presence of O_2, it grows via aerobic respiration, whereas in the absence of O_2, it switches to anaerobic respiration using alternative electron acceptors. These bacteria produce more H_2 when nanoparticles are added. In a study performed by Lin et al. [123], ferric oxide nanoparticles facilitated dark fermentation by Enterobacter aerogenes and increased the H_2 yield from 1.32 to 1.55 mol H_2/mol glucose when the ferric oxide nanoparticle concentration was increased from 0 to 200 mg/L. Table 5 presents a list of *E. aerogenes* strains with their H_2 production abilities using different substrates and operational conditions.

Table 5. Hydrogen production by different *Enterobacter aerogenes* strains.

Strain	Substrate (Concentration, g/L)	Conditions				Operation Mode (Reactor Volume, mL)	H_2 Yield as Mol H_2/mol Substrate *	Ref.
		T (°C)	pH	Time (h)	More Details			
	Fermentable sugar	37		72	Anaerobic, Fe_2O_3 nanoparticles	Batch (100)	2.60	[122]
ATCC13408	Glucose (3)	37	6		Aerobic, 220 rpm, purging with N_2, Fe_2O_3 nanoparticles 200 mg/L	Batch (200)	1.32–1.55	[123]
ATCC 13048	Mahogany wood hydrolysate and preculture medium (45)	37		48	120 rpm, 15% *v*/*v* inoculum	Batch (250)	0.03 (glucose)	[124]
IAM1183	Glucose (15)	37		20	200 rpm, purging with N_2	Batch (100)	1.34 ± 0.21	[125]
2822	Cheese whey (32.5)	31	6.5	104	250 rpm, 10% *v*/*v* inoculum, flushing with argon	Batch (2000)	0.26 (lactose)	[126]

* In parentheses, the reference substrate for calculation when it differs from the cited one.

Few studies have been performed with *E. aerogenes*, as summarized in Table 5. The bacteria require the addition of nanoparticles to increase H_2 production, which might be one of the reasons why it has not been widely used for dark fermentation H_2 production studies. It has been observed that this microbe grows well on a variety of substrates and can produce H_2 around 37 °C. All studies show that continuous agitation in the bioreactor is required to increase bacterial interaction with the substrate, thus yielding more H_2. The maximum H_2 yield of 2.6 mol H_2/mol sugar was observed using fermentable sugar as the substrate, with the addition of ferric oxide nanoparticles, at 37 °C for 72 h.

4.2.2. *Klebsiella pneumoniae*

K. pneumoniae has been studied for its growth rate, growth conditions, and production of valuable byproducts. This bacterium produces H_2 through a mixed-acid fermentation pathway, converting glucose or other substrates into various end products. Formate, produced via the formate H_2 lyase (FHL) pathway, is broken down by the FHL complex through the activity of formate dehydrogenase and hydrogenase to produce H_2 and CO_2. In a study with biodiesel waste using *K. pneumoniae* DSM2026, an H_2 production rate of 0.532 mol H_2/mol glycerol was obtained by optimizing the medium components. Using Plackett–Burman and uniform design methods, the optimized medium contained 20.4 g/L glycerol, 5.7 g/L KCl, 13.8 g/L, NH_4Cl, 1.5 g/L $CaCl_2$, and 3 g/L yeast extract, resulting in a 5-fold increase in H_2 levels [127]. Table 6 presents a list of *K. pneumoniae* strains used to produce H_2 under different substrates and operational conditions.

As shown in Table 6, *K. pneumoniae* can utilize different substrates, such as glucose and glycerol. Studies were performed in batch mode over a temperature range of 33–40 °C, between 12 and 72 h HRT, with a main pH range of 5.5 to 8. In a study, strain TR17 produced an H_2 yield of 0.26 mol H_2/mol glycerol using 11.14 g/L crude glycerol as a substrate at 40 °C and pH 8 in batch mode. However, when an up-flow anaerobic sludge blanket reactor was used with this same strain TR17 and 10 g/L glycerol as the substrate at 40 °C, pH 8 for 12 h in batch mode, a H_2 production of 4.08 mol H_2/mol glycerol was observed, indicating that this reactor is an optimal setup for achieving higher H_2 yields with this strain of *K. pneumoniae*.

Table 6. Hydrogen production by different *Klebsiella pneumoniae* strains.

Strain	Substrate (Concentration, g/L)	Conditions				Operation Mode (Reactor Volume, mL)	H_2 Yield as Mol H_2/mol Substrate *	Ref.
		T (°C)	pH	Time (h)	More Details			
DSM 2026	Glycerol (20.4)	37	6.5	24	10% *v*/*v* inoculum	Batch (5000)	0.80	[127]
ECU-15	Glucose (10), Xylose (2), and Cellobiose (1.5)				150 rpm, N_2 flushing	Batch (1000)	2.07 (glucose)	[128]
MGH 78578	Brewery wastewater (3–4)	35	5.5	72	10% *v*/*v* inoculum, 90 rpm	Batch (250)	0.80–1.67 (glucose)	[129]
ECU-15	Glucose (35.62)	37	6.0		150 rpm, flushed with N_2	Batch (1000)	1.22	[130]
HE1	Glycerol (50)	35	6.0		200 rpm	Batch (2500)	0.34	[131]
TR17	Glycerol (20)	40	8.0	24		Batch (60)	0.25	[132]
	Sucrose (3.588)	37	5.5	48	20% *v*/*v* inoculum, 8500 rpm	Batch (100)	0.80 (xylose)	[133]
TR17	Glycerol (11.4)	40	8.0		10% *v*/*v* inoculum, flushed with N_2	Batch (60)	0.26	[134]
TR17	Glycerol (10)	40	8.0	12	Up-flow anaerobic sludge blanket reactor	Batch (1000)	4.08	[135]
Y7-3	Corn straw (50)	37		24	5% *v*/*v* inoculum, 220 rpm	Batch (100)	0.18	[136]
ABZ11	Glucose (9.15)	34	6.8	48	150 rpm	Batch (2000)	2.71	[137]

* In parentheses, the reference substrate for calculation when it differs from the cited one.

4.3. *Escherichia Coli*

E. coli is a facultative anaerobic mesophilic bacterium that grows optimally around 37 °C [138]. During dark fermentation, in the absence of O_2, *E. coli* must find an alternative terminal electron acceptor. Protons serve this role, with H_2 being produced when electrons are transferred to protons, while organic acids and ethanol are produced to maintain redox balance [139]. In *E. coli*, four [Ni–Fe]-hydrogenases are involved in H_2 metabolism. The activity and reversibility of these enzymes depend on various conditions, such as pH, substrate type and its concentration, O_2 availability, and oxidation–reduction potential [140]. The co-production of H_2 with ethanol is more profitable than separate fermentation stages [141], as it improves the energy balance of biorefinery designs [142]. Table 7 presents a list of *E. coli* strains and their H_2 production abilities using different substrates and operational conditions.

The analysis determined the efficiency of *E. coli* in producing H_2 in monoculture and coculture. These bacteria can utilize a wide range of substrates, and the studies showed that they can produce H_2 at temperatures ranging from 31 to 37 °C. A minimum pH of 5.5 and a maximum pH of 8 were applied, with all studies performed in batch mode. As can be seen in Table 7, the maximum H_2 production of 2.82 mol H_2/mol glucose was observed with *E. coli* strain WDH-LF using glucose as a substrate at 31 °C, pH 8.2, and 400 rpm, proving optimal H_2 production under these certain conditions.

Table 7. Hydrogen production by different *Escherichia coli* strains.

Strain	Substrate (Concentration, g/L)	Conditions				Operation Mode (Reactor Volume, mL)	H_2 Yield as Mol H_2/mol Substrate *	Ref.
		T (°C)	pH	Time (h)	More Details			
K-12	Garden waste (cellulose, 13)	33			Purged with argon	Batch (100)	2.73 (cellulose)	[143]
W3110	Acetate (10)	33	6.3		Purged with 95% N_2	Batch (69)	0.21	[144]
W3110	Hemi cellulosic hydrolysates (10–15)	31	8.2		200 rpm	Batch (10)	1.15–1.73 mol H_2/mol substrate	[145]
WDHL	Glucose (15) Lactose (15) Galactose (15)	37	6.0		175 rpm	Batch (1000)	0.30 1.02 (hexose) 1.12	[146]
BW25113	Glucose	37	7.5			Batch (100)	0.05	[147]
WDH-LF	Glucose	31	8.2		400 rpm	Batch (10,000)	2.82	[148]
XL1-Blue	Fructose (5) Glucose (5) Xylose (5)	35	6.5		Purged with argon, 150 rpm	Batch (130)	1.17 (fructose) 0.96 (glucose) 0.69 (xylose)	[149]
CECT432, CECT434 and *E. cloacae* MCM2/1	Glycerol (20)	37	6.3	72	10% *v*/*v* inoculum, purged with argon	Batch (1200)	$4.40 \cdot 10^{-3}$	[150]
BH20	Glucose (4)	37		16	120 rpm	Batch (200)	0.32 ± 0.01	[151]
E. coli and *Enterobacter aerogenes*	Acetate (0.563), butyrate (0.537), propionate (0.059), and lactate (0.214)	37	5.5		12.5 g/L biochar, purged with N_2	Batch (500)	0.33	[152]
E. coli and *Enterobacter aerogenes*	Acetate (0.608), butyrate (0.516), propionate (0.051), and lactate (0.191)	37	5.5		Purged with N_2, 50 mL inoculum, gas sampling every 24 h, 10 mg/L copper	Batch (500)	0.15	[153]
WDHL	Cheese whey powder	37			175 rpm	Batch (1000)	$1.50 \cdot 10^{-3}$	[154]
HD701	Mixture of glucose, sucrose, starch, acid-hydrolyzed sucrose, and starch	35	7.0		10% *v*/*v* inoculum, sparged with N_2	Batch (1000)	2.00 (glucose)	[155]

* In parentheses, the reference substrate for calculation when it differs from the cited one.

5. Discussion

This study aims to analyze the important parameters required for bacteria to produce H_2 through dark fermentation. The analysis showed the influence of pH, temperature, HRT, substrate used, and its concentration on the ability of bacteria to produce H_2 as a product of their metabolism. Table 8 shows the advantages and disadvantages observed during the analysis of each strain for H_2 production.

Table 8. Advantages and disadvantages of dark fermentation species.

Bacteria	Advantages	Disadvantages
C. butyricum	– Ability to metabolize a wide range of substrates – Ability to grow in a wide range of environmental conditions, such as slightly acidic to neutral pH [67]	– Low total substrate conversion efficiency – Sensitive to pH fluctuations [156]
C. beijerinckii	– Ability to utilize a wide range of substrates – Resistant to inhibitory compounds [88]	– Sensitive to very acidic pH [157]
C. pasteurianum	– Ability to utilize a wide variety of substrates [98] – Produce H_2 with greater efficiency in the presence of iron oxides [99] – Nanoparticles can be used to immobilize bacteria and increase H_2 productivity [106] – Can convert cellulose and hemicellulose directly to ethanol and H_2 [111]	– Complex feedstocks require pretreatment to make the sugars available for fermentation, increasing operating costs – Production of organic acids during fermentation can lower pH, inhibiting bacterial growth and H_2 production [101]

Table 8. *Cont.*

Bacteria	Advantages	Disadvantages
C. thermocellum	– Have high hydraulic retention time [116]	– Require higher temperatures of 60–65 °C for their growth and activity [111]
E. aerogenes	– Can grow through aerobic respiration or, in the absence of O_2, switch to anaerobic respiration using alternative electron acceptors [123] – pH around 6–6.5 has been used for H_2 production [120,123]	– Require the addition of nanoparticles to increase H_2 production [123]
K. pneumoniae	– Produces H_2 via a mixed-acid fermentation pathway [127] – Temperature range of 33–40 °C, 12 to 72 h HRT, and a distinctive pH range of 5.5 to 8 are observed to optimize H_2 production [129,135,137]	– It is a pathogen responsible for several human infections, thus raising biosafety concerns [158]
E. coli	– Flexibility in genetic engineering – Have faster growth rate compared to other species [159] – Can utilize wide range of carbon sources [155]	– Relatively lower H_2 yields compared to Clostridium species [90,145]

The treatment of wastewater and simultaneous production of H_2 using a biological method is a very realistic approach, especially when operating costs, energy losses, or fees per volume of water treated are critical factors. *Clostridium* spp. has been preferred for H_2 production, as shown in several studies, due to its ability to grow under varied conditions. A pH range of 5–7, a temperature of around 30–37 °C for mesophiles, and around 60 °C for thermophiles are widely used for the dark fermentation process. The highest H_2 production obtained from different species is shown in Table 9.

Table 9. Maximum hydrogen production by different species.

Bacteria	Substrate (Concentration, g/L)	Conditions				Operation Mode (Reactor Volume, mL)	H_2 Yield as Mol H_2/mol Substrate	Ref.
		T (°C)	pH	Time (h)	More Details			
C. butyricum TM-9A	Sugarcane molasses	37	7.5	24	Serum bottles	Batch (120)	3.34 (synthetic analytical grade glucose)	[71]
C. beijerinckii	Sucrose (7)	37	6.0−7.0		1 mL inoculum	Batch (15)	9.39	[93]
C. pasteurianum DSM525	Glucose	37			Flushing with N_2, 5% inoculum, ferrihydrite nanorods	Batch (25)	3.55	[106]
C. thermocellum ATCC 27405	Cellulose (3)	55		168	10% (*v*/*v*) inoculum, 150 rpm, 20 mM $CaCO_3$	Batch (120)	5.87 (hexose)	[119]
E. aerogenes	Fermentable sugar	37		72	Anaerobic, Fe_2O_3 nanoparticles	Batch (100)	2.60	[122]
Klebsiella species TR17	Glycerol (10)	40	8.0	12	Up-flow anaerobic sludge blanket reactor	Batch (1000)	4.08	[135]
E. coli WDH-LF	Glucose (16)	31	8.2		400 rpm	Batch (10,000)	2.82	[148]

C. beijerinckii attained the highest H_2 production yield, 9.39 mol H_2/mol sucrose, at 37 °C and pH 6–7 [93], demonstrating that this species is very efficient for H_2 production. *C. thermocellum* strain 27,405 follows *C. beijerinckii* with an H_2 production yield of 5.87 mol H_2/mol hexose, using cellulose as a substrate, at 55 °C for 168 h, with the addition of 20 mM $CaCO_3$ [119]. *C. pasteurianum* DSM525 achieved a maximum H_2 production of

3.55 mol H_2/mol glucose at 37 °C [106], whereas *C. butyricum* TM-9A attained 3.34 mol H_2/mol synthetic analytical-grade glucose, using sugarcane molasses, also at 37 °C [71]. *E. aerogenes*, using fermentable sugar, had a maximum H_2 production of 2.6 mol H_2/mol sugar at 37 °C with an HRT of 172 h [122]. The only drawback observed with this species was the need for the addition of nanoparticles in all studies. *Klebsiella* spp. TR17 provided a maximum H_2 production of 4.08 mol H_2/mol using glycerol at 40 °C and 12 h HRT [122], whereas the *E. coli* WDH-LF strain produced up to 2.82 mol H_2/mol glucose [148]. These results allowed the identification of the most suitable strain from each species.

In addition, the analysis of each bacterial species identified several conditions and factors essential to obtain maximum H_2 production. In a study with *C. butyricum* TM-9A, using sugarcane molasses as the substrate at 37 °C, pH 7.5, and an HRT of 24, 3.335 mol H_2/mol synthetic analytical-grade glucose was produced [71]. However, when the same strain with 10 g/L of glucose as the substrate at 37 °C at a pH 8, it produced 3.1 mol H_2/mol glucose [67], indicating no significant differences between the results. Another strain of *C. butyricum*, TISTR 1032, using sucrose at a pH of 6.5 in a batch mode, produced 1.34 mol H_2/mol hexose [81]. However, when sugarcane juice was used as the substrate at 37 °C, pH 6, in continuous mode, only 1.0 mol H_2/mol hexose was produced [85], confirming that pure sucrose is a better substrate for this strain to produce H_2. *C. butyricum* CWBI1009 was studied using glucose monohydrate as the substrate under different operational conditions. When this strain was used in batch mode at 400 rpm and pH 7, 3.1 mol H_2/mol glucose was obtained [78], while at 30 °C and pH 7.3, the production was 1.43 mol H_2/mol glucose [76], proving that the latter conditions are better for higher H_2 production. The analysis with *C. beijerinckii* in batch mode showed a maximum H_2 yield of 9.39 mol H_2/mol sucrose when 7.5 g/L of sucrose was used at 37 °C and pH 6–7 [93], followed by dextrose. When using 3 g/L of dextrose with the same strain, it produced 3.58 mol H_2/mol dextrose in batch mode at 37 °C and 120 rpm [52]. Glucose resulted in an H_2 productivity of 2.7 ± 0.2 mol/mol glucose at 37 °C and 100 rpm with the *C. beijerinckii* strain DSM 1820. The *C. beijerinckii* NCIMB-8052 strain produced an H_2 yield of 2.47 mol H_2/mol glucose using 10% v/v inoculum, at pH 6.5, 32 ± 1 °C, 120 rpm, 120 h, and light illumination [24]. When using a mixture of glucose and xylose as the substrate at 30 °C, the same strain produced a lower H_2 yield of 0.086 mol H_2/mol substrate [95], indicating that the use of glucose alone as the substrate provides higher H_2 yields.

C. pasteurianum was analyzed using glucose, glycerol, and xylose as substrates. The DSM525 strain produced the highest H_2 yield of 3.55 mol H_2/mol using glucose as substrate at 37 °C in batch mode [106]. When *C. pasteurianum* MTCC 116 was used with glycerol as the substrate at 37 °C, pH 6.7, and 150 rpm, following the acetate pathway for metabolism, a maximum H_2 yield of 3 mol H_2/mol glycerol [108]. In contrast, when the same strain was used with glucose as the substrate at 37 °C, pH 6.6, and 192 rpm, the H_2 yield decreased to 0.0036 mol H_2/mol glucose, indicating that glycerol is a better substrate for H_2 production by this strain. *C. pasteurianum* CH5 was studied with xylose and glucose. When xylose was used as the substrate, a maximum H_2 yield of 1.46 mol H_2/mol xylose was obtained at 35 °C and 120 rpm [110], whereas a higher H_2 yield (1.61 mol H_2/mol glucose) was achieved using glucose at 35 °C, pH 7, and 120 rpm [104], indicating glucose as a better substrate for H_2 production by this strain.

Finally, *C. thermocellum* was also considered for the production of H_2. The DSM 1313 strain of *C. thermocellum* used cellulose (Avicel) as a substrate. In continuous mode at 60 °C, pH 7, with argon flushing and 60 rpm, it produced 0.76–1.21 mol H_2/mol hexose [115], whereas when the studies were performed in a batch mode at 55 °C, with 8 days of incubation and 150 rpm agitation, 1.15 mol H_2/mol hexose was produced [118]. The highest H_2 production rate of 1.92 mol H_2/mol hexose was observed with *C. thermocellum* strain 27405, using 5 g/L of cellulose in batch mode at 55 °C, pH 7, flushed with N_2, 150 rpm for 168 h [116]. Various conditions, such as an optimum temperature of 55 °C and 150 rpm agitation, were used in all studies, proving this strain to be efficient for the production of H_2. When 5 g/L of sucrose was used as a substrate with an HRT of 168 h,

the H_2 production rate was 1.92 mol H_2/mol hexose [116], whereas when the substrate concentration was increased to 18 g/L with an HRT of 10 days, 11.19 mol H_2/mol cellulose was produced [160], indicating the effect of substrate and its concentration, as well as HRT, on H_2 yields. *Clostridium* spp. has shown great potential, as most of the dark fermentation studies have used strains from these species to produce H_2.

In addition to these bacteria, some other facultative anaerobic bacteria have shown the potential to produce H_2 through dark fermentation. *E. aerogenes*, *K. pneumoniae*, and *E. coli* are facultative anaerobes used to produce H_2 via dark fermentation. *E. aerogenes* requires the addition of nanoparticles to increase H_2 production. The maximum H_2 yield of 2.6 mol H_2/mol sugar was observed with fermentable sugar as the substrate at 37 °C for 72 h, along with the addition of ferric oxide nanoparticles [120]. This was followed by *E. aerogenes* strain ATCC13408 using glucose as a substrate at 37 °C, pH 6, 220 rpm, and ferric oxide nanoparticles, which yielded H_2 in the range of 1.32–1.55 mol H_2/mol glucose [123].

In the case of *K. pneumoniae*, the ECU-15 and TR17 strains were mainly used to produce H_2. The ECU-15 strain, when used with a mixture of glucose, xylose, and cellobiose at 150 rpm in batch mode, produced up to 2.07 mol H_2/mol substrate [128], which was the maximum H_2 produced by this strain. When the same strain was used with only glucose as the substrate at 37 °C, pH 6, and 150 rpm, it produced 1.22 mol H_2/mol glucose [130], indicating that the mixture of glucose, xylose, and cellobiose is a better substrate for high yields of H_2. In another analysis with *K. pneumoniae* strain TR17, lower H_2 yields were observed with crude glycerol. At 40 °C, pH 8.0 for 24 h in batch mode, 0.25 mol H_2/mol glycerol was produced [132]. In a similar study with crude glycerol at 40 °C and pH 8 in batch mode, an H_2 yield of 0.26 mol H_2/mol glycerol was observed [134], but when an up-flow anaerobic sludge blanket reactor was used with strain TR17 and glycerol as the substrate at 40 °C, pH 8 for 12 h in batch mode, an H_2 yield of 4.08 mol H_2/mol glycerol was observed [135], indicating that this reactor is optimal for higher H_2 yield with this strain of *K. pneumoniae*.

Finally, in the analysis of *E. coli*, a maximum H_2 production of 2.82 mol H_2/mol glucose was observed with strain WDH-LF, using glucose as a substrate at 31 °C, pH 8.2, and 400 rpm [148]. For the *E. coli* strain W3110, hemicellulosic hydrolysates were found to be a better substrate than acetate. When hemicellulosic hydrolysates were used at 31 °C, pH 8.2, and 200 rpm, H_2 production of 1.15–1.73 mol H_2/mol substrate was obtained [142], but when acetate was used as the substrate at 33 °C and pH 6.3, H_2 production of 0.21 mol H_2/mol acetate was achieved [144]. When *E. coli* strain WDHL was analyzed with different substrates, the highest H_2 production of 1.12 mol H_2/mol was observed with galactose at 37 °C, pH 6, and 175 rpm, followed by glucose, which produced 0.30 mol H_2/mol glucose produced under the same conditions, proving that galactose is a better substrate for this strain [146]. This comparative analysis allowed for the determination of the most suitable bacterial strain and the advantages and disadvantages of using each strain for dark fermentation studies to produce H_2 under specific conditions available in the particular laboratory or industry at that particular time of the year since temperature plays a major role in H_2 production efficiency, along with other conditions, such as bioreactor design and material, type of bioprocess, and the substrate used.

6. Conclusions

H_2 production is a promising way to generate clean and green energy. Biological methods such as dark fermentation have enabled the production of renewable and carbon-neutral H_2. Dark fermentation studies using anaerobic and facultative anaerobic bacteria have been widely identified, as these bacteria can utilize simple sugars and even food and agricultural wastes. Optimization of parameters such as pH, temperature, type of substrate used and its concentration, type of bioreactor, HRT, type of inoculum, and its metabolic pathway regulation can significantly improve H_2 production. In addition, pretreatment, purification, and storage methods can be employed to make H_2 more efficient for potential

applications in industry and transportation. However, the method still requires certain technological advances to be adequately scaled up for industrial applications.

Bacteria play a critical role in the efficiency and feasibility of H_2 production. *Clostridium* spp. has been widely preferred in studies. These bacteria possess highly active hydrogenase enzymes, which are crucial for the efficient conversion of reduced ferredoxin to H_2. This review provides a detailed analysis of the parameters and bacteria strains used in dark fermentation studies. Each strain is compared to identify the most suitable bacteria for specific environmental and process conditions, as preferred in several studies over the years and those available during individual research. This will allow researchers to obtain more reliable and easier data in the future by monitoring available results that still need to be optimized. In addition, it will provide a cost-effective waste treatment process that addresses energy and waste disposal issues and serves as a cleaner alternative to fossil fuels.

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