Systematic Review

Impact of Substrates, Volatile Fatty Acids, and Microbial Communities on Biohydrogen Production: A Systematic Review and Meta-Analysis

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Abstract: Hydrogen is becoming recognized as a clean and sustainable energy carrier, with microbial fermentation and electrolysis serving critical roles in its production. This paper provides a thorough meta-analysis of BioH_2 production across diverse substrates, microbial populations, and experimental settings. Statistical techniques, including ANOVA, principal component analysis (PCA), and heatmaps, were used to evaluate the influence of various parameters on the hydrogen yield. The mean hydrogen generation from the reviewed studies was 168.57 ± 52.09 mL H_2/g substrate, with food waste and glucose demonstrating considerably greater hydrogen production than mixed food waste ($p < 0.05$). The inhibition of methanogens with inhibitors like 2-bromoethanesulfonate (BES) and chloramphenicol (CES) enhanced hydrogen production by as much as 25%, as demonstrated in microbial electrolysis cell systems. PCA results highlighted *Clostridium* spp., *Thermotoga* spp., and *Desulfovibrio* spp. as the most dominant microbial species, with *Clostridium* spp. contributing up to 80% of the YH_2 in fermentation systems. The study highlights synergistic interactions between dominant and less dominant microbial species under optimized environmental conditions (pH 5.5–6.0, 65 °C), emphasizing their complementary roles in enhancing H_2 production. Volatile fatty acid regulation, particularly acetate and butyrate accumulation, correlated positively with hydrogen production ($r = 0.75$, $p < 0.01$). These findings provide insights into optimizing biohydrogen systems through microbial consortia management and substrate selection, offering a potential way for scalable and efficient H_2 production.

Keywords: biohydrogen; chemical inhibitors; fermentation system; food waste; principal component analysis (PCA); methanogens suppression

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1. Introduction

The global demand for sustainable and renewable energy sources has increased due to growing concerns over climate change, pollution, and the impending depletion of petroleum and coal [1]. Hydrogen (H_2) is gaining popularity as an eco-friendly fuel among several alternative energy sources due to its ability to efficiently mitigate greenhouse gas emissions [2]. The substantial energy density of hydrogen and its capacity for generation from renewable sources make it a vital component in the worldwide transition to clean energy systems [3]. Microbial fermentation, particularly dark fermentation (DF), is advantageous for hydrogen generation as it can convert many biowaste substrates, including agricultural residues and food waste, into H_2 and other important by-products. Moreover, microbial electrolysis cells (MECs) have emerged as a promising method to augment H_2 production by applying an external voltage to promote the microbial consortia process,

thereby avoiding the energy-intensive phases associated with traditional water electrolysis [4,5]. In addition to microbial systems, catalytic routes and remarkably heterogeneous catalysis also hold significant promise for biohydrogen production due to their environmental friendliness and scalability [6]. While this study emphasizes microbial approaches, the catalytic pathway is a complementary technology, broadening the scope of sustainable hydrogen generation methods.

Biological hydrogen generation ($BioH_2$) occurs through bacteria's anaerobic breakdown of organic materials. This process involves the decomposition of complex organic substrates into simpler molecules, yielding H_2, CO_2, and volatile fatty acids such as acetate and butyrate [7,8]. H_2 generation is categorized into two primary methods: dark fermentation and microbial electrolysis cells. Dark fermentation is the most commonly studied method for biohydrogen production. It involves the microbial conversion of carbohydrates into hydrogen under anaerobic conditions without light. Hydrogen-producing microorganisms, such as *Clostridium* spp., are involved in the DF process. Hydrogen yield mainly depends on the microbial community, substrate composition, and environmental factors such as pH and temperature [9,10]. Meanwhile, in the microbial electrolysis cells (MECs) process, hydrogen production is enhanced by applying a small external voltage to a microbial cell (a few studies mentioned 0.8 V) [11,12]. MECs offer a higher hydrogen yield than dark fermentation because they suppress competing reactions, such as methanogenesis, that consume hydrogen. By inhibiting methanogenic bacteria, MECs redirect electron flow toward hydrogen production, making them a highly efficient system for biohydrogen generation [13].

Hydrogen production efficiency depends on the composition and activity of the microbial communities involved. Different microbial species include hydrogen producers like *Clostridium* spp., *Thermotoga* spp., and *Desulfovibrio* spp., all contributing to biohydrogen generation through metabolic pathways [14–16]. For example, *Clostridium* spp. is well known for producing hydrogen through the butyrate and acetate pathways during biowaste fermentation. Meanwhile, *Thermotoga* spp. performs well in high-temperature environments, fermenting carbohydrates at elevated temperatures to produce hydrogen in thermophilic reactors [17,18]. The presence of methanogens such as *Methanosaeta* and *Methanobacterium* tends to outcompete the H_2-producing bacteria by forming methane with H_2 [19,20].

For this reason, hydrogen production is enhanced, specifically within microbial electrolysis cells, when the activity of microbes such methanogens is inhibited. It can be achieved by adding inhibitors like 2-bromoethanesulfonate (BES) or by changing environmental conditions, such as pH variation, which allows for a decrease in methane production and, subsequently, an increase in hydrogen output [21,22]. The selection of substrate is the most relevant factor concerning hydrogen yield (YH_2), enhancing the consumption rate of the substrate. Organic substrates include food wastes, agricultural residues, and carbonaceous materials rich in glucose that could release carbon (C) content through microbial fermentation with high biodegradability [23,24]. Different substrates result in varying VFA profiles, directly influencing the hydrogen yield. For instance, food waste (FW) fermentation often results in elevated concentrations of VFAs, including acetate, butyrate, and propionate. These volatile fatty acids are critical intermediates in fermentation and MEC systems, representing intermediates in metabolic pathways that produce hydrogen [25]. Among the VFAs, acetate and butyrate have the strongest correlation to hydrogen production pathways. It was first reported by Lee et al. and later by Moreno-Andrade et al. in 2023. Consequently, the dynamics of VFA concerning substrate degradation are converted to the effective operation of biohydrogen systems [26,27].

Extensive studies on biohydrogen production have been conducted over the last two decades; however, enormous discrepancies in hydrogen yields among the different studies were reported. These variations were mainly because of the experimental conditions, including feedstock type, microbial community composition, pH, or retention time. Moreover, only a few works have followed a systematic attempt at comparing microbial performance

in bioreactor systems for hydrogen production. Hence, a broad meta-analysis should be performed, compiling data from various sources and resulting in a more concrete view of what drives hydrogen production. The application of advanced statistical techniques such as principal component analysis (PCA) and ANOVA may provide valuable insights into the impact of hydrogen production on interactions developed between microbial species and types of applied substrate. Among them, PCA allows for reducing complex, multidimensional data and highlights the most influential microbial species responsible for high hydrogen yields, while ANOVA quantifies how different experimental conditions affect hydrogen production [28]. These methods have been widely applied toward the optimization of biohydrogen systems in both laboratory-scale experiments and scale-up for industrial applications.

This study aims to comprehensively analyze biohydrogen production by integrating a systematic review with meta-analysis techniques, offering a quantitative evaluation of hydrogen yields across a range of substrates, microbial populations, and environmental conditions. Unlike previous reviews that primarily provide qualitative summaries, this study adopts a data-driven approach to observing the effects of substrate type and volatile fatty acid (VFA) accumulation on hydrogen production. A specific focus is placed on identifying critical microbial communities and their synergistic interactions, particularly between dominant species such as *Clostridium* spp. and *Thermotoga* spp., using principal component analysis (PCA). In addition, this research quantifies the role of methanogen suppression in enhancing hydrogen yields through advanced statistical tools such as ANOVA and t-tests. By synthesizing data from diverse studies, this work bridges critical gaps in the existing literature, emphasizing the interplay between substrates (e.g., food waste (FW), glucose, mixed food waste (MFW)), VFAs (acetate, butyrate, propionate, and valerate), and microbial consortia. Unlike previous studies, which often focus on isolated parameters, this study provides an integrated framework that combines substrate variability, microbial consortia dynamics, and VFA management.

Furthermore, the findings deliver actionable insights into optimizing microbial communities and operational parameters. This approach contributes to scalable and efficient biohydrogen production systems, addressing substrate heterogeneity, environmental fluctuations, and process stability. By offering a novel synthesis of qualitative and quantitative methods, this study advances the understanding of biohydrogen production and provides a foundation for future research and industrial applications.

2. Materials and Methods

2.1. Data Assembly and Study Selection

A wide-ranging literature search was performed using the Web of Science and Google Scholar databases. The search focused on peer-reviewed articles on BioH_2 production using microbial fermentation and electrolysis cells. The keywords used included "biohydrogen production," "dark fermentation," "microbial electrolysis cells," "volatile fatty acids," "methanogen suppression," and "microbial communities," as represented in Figure 1. A total of studies was carefully chosen for inclusion in the meta-analysis based on the following criteria: (i) studies reporting biohydrogen production with quantifiable hydrogen yields (mL H_2/g substrate or m^3 H_2/m^3 reactor volume) using a fermentation-based system and microbial electrolysis cells (MECs); (ii) studies that provided detailed information on the substrates used (e.g., food waste, glucose, mixed food waste); (iii) studies that investigated microbial communities involved in hydrogen production; (iv) clearly defined experimental conditions, such as pH, temperature, and retention time; and (v) available reports of volatile fatty acid (VFA) concentrations, particularly acetate, butyrate, and propionate.

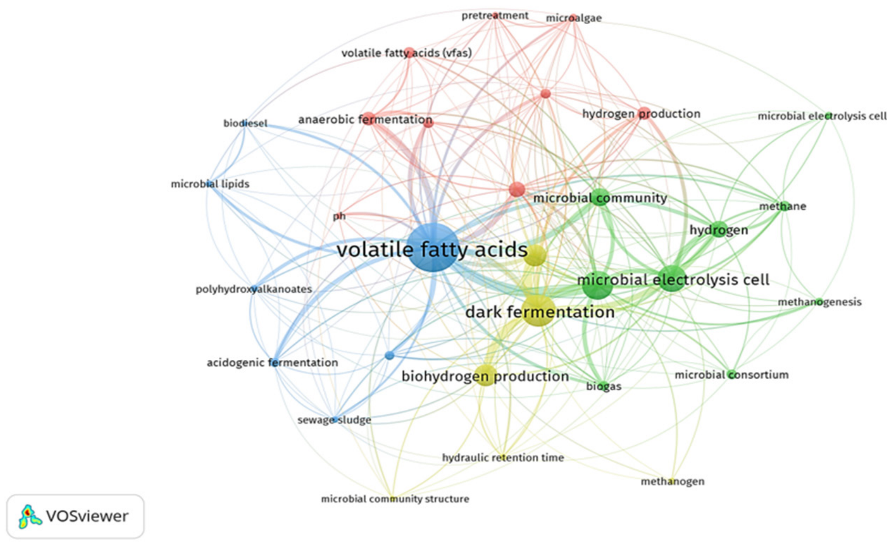

Figure 1. Bibliometric network of keywords.

2.2. Hydrogen Production Normalization

Hydrogen yields across different studies were reported in various units, including mL H_2/g volatile solids (VS) and mL H_2/g substrate. All hydrogen production data were standardized to mL of hydrogen per gram of substrate (mL H_2/g substrate) to promote meaningful comparisons. In MEC investigations where yields were given in m^3 H_2/m^3 cathode liquid volume per day, a conversion factor was employed based on reactor volume and substrate loading to estimate mL H_2/g substrate for standardization [29]. The normalized data were then compiled into a single dataset, categorized by substrate type (e.g., food waste, glucose, mixed food waste), microbial communities, and environmental conditions (pH and temperature) [29,30]. The formula used for normalization, especially when converting yields from microbial electrolysis cells (MECs), is shown in **Equation (1)**.

$$\boldsymbol{Normalized\ H_2\ Yield\ (mL\ H_2|g\ substrate)} = \frac{Volume\ of\ H_2\ produced\ (mL)}{Mass\ of\ substrate\ (g)} \tag{1}$$

Equation (2) demonstrates the yields in m^3H_2/m^3 cathode liquid volume per day (MEC systems); the conversion to mL H_2/g substrate was calculated based on reactor volume and substrate loading. In **Equation (2)**, m^3 H_2/day is the hydrogen yield per day in cubic meters, Reactor volume is the volume of the MEC reactor, and Substrate loading is the amount of substrate used in grams.

$$\boldsymbol{H_2\ Yield\ (mL\ H_2|g\ substrate)} = \frac{\frac{m^3H_2}{day} \times 10^6}{Reactor\ volume\ (m^3) \times Substrate\ loading\ (g\ substrate)} \tag{2}$$

2.3. Statistical Analysis

2.3.1. Descriptive Statistics and Analysis of Variance (ANOVA)

For each substrate category, descriptive statistics were calculated, including the mean, standard deviation, and range of hydrogen yields [31], as shown in **Equations (3)–(5)**. The variability of hydrogen production across studies was assessed, and the data were visualized using bar graphs and box plots to illustrate the distribution of YH_2 for each substrate type. A one-way analysis of variance (ANOVA) was executed to estimate how substrate type affects hydrogen generation [31,32]. The null hypothesis for the ANOVA test was that there are no significant differences in hydrogen production across different substrates (food waste, glucose, and mixed food waste). The ANOVA model was structured as follows: a dependent variable for H_2 production (mL H_2/g substrate and an independent variable for the substrate type (food waste, glucose, mixed food waste). If the ANOVA

results indicated statistically significant differences ($p < 0.05$), a post hoc Tukey's honest significant difference (HSD) test was applied to determine which substrate groups differed from each other, as shown in **Equation (6)** where $MS_{between}$ = the mean square between groups, and MS_{within} = the mean square within groups.

$$\boldsymbol{Mean(\mu)} = \frac{\sum X_i}{\mathrm{n}} \tag{3}$$

where, X_i signifies individual hydrogen yields, and n is the number of data points.

$$\boldsymbol{Standard\ deviation(\sigma)} = \sqrt{\frac{\sum(X_i - \mu)^2}{n-1}} \tag{4}$$

$$\boldsymbol{Range} = X_{max} - X_{min} \tag{5}$$

$$\boldsymbol{F} = \frac{MS_{between}}{MS_{within}} \tag{6}$$

2.3.2. Principal Component Analysis (PCA) for Microbial Communities, Pearson Correlation Analysis for VFAs, and *t*-Test

Paired *t*-tests were used to estimate the differences in H_2 yields associated with the occurrence or absence of exact microbial consortia, such as *Clostridium* spp., *Thermotoga* spp., and *Desulfovibrio* spp. The null hypothesis for these tests was that microbial species do not significantly affect hydrogen production, as shown in **Equation (7)** where $\overline{X}_1$ and $\overline{X}_2$ are the mean hydrogen yields with and without a given microbial species; s_1^2 and s_2^2 are the sample variances; and n_1 and n_2 are the sample sizes. The null hypothesis (H_0) is that microbial species do not affect hydrogen production [33].

$$\mathbf{t} = \frac{(\overline{X}_1 - \overline{X}_2)}{\sqrt{\left[\left(\frac{s_1^2}{n_1}\right) + \left(\frac{s_2^2}{n_2}\right)\right]}} \tag{7}$$

Principal component analysis (PCA) was employed to reduce the microbial community data's dimensionality and identify the microbial species contributing to hydrogen production. PCA transforms the original variables (microbial species and their hydrogen production contributions) into new, uncorrelated variables called principal components. These components explain the variance in the data, with the first two components typically capturing the most significant patterns [34]. The microbial community data were standardized before performing PCA to ensure that each microbial species contributed equally to the analysis, regardless of differences in data scaling. The PCA results were visualized as a biplot, which plotted the microbial species and the substrate types concerning hydrogen production. Microbial species that clustered together were considered to have similar contributions to hydrogen production across different studies, as shown in **Equation (8)** where, X_{ij} is the value of microbial species i in study j; $\overline{X}_j$ is the mean of variable j; and s_j is the standard deviation of j.

$$\mathbf{Z_{ij}} = \frac{X_{ij} - \overline{X}_j}{s_j} \tag{8}$$

The relationship between VFA concentrations (particularly acetate and butyrate) and hydrogen production was evaluated using Pearson's correlation coefficient (r), as shown in **Equation (9)—**the analysis aimed to quantify how VFA accumulation correlated with hydrogen yields in different systems [35]. A positive correlation would suggest that higher VFA concentrations lead to increased hydrogen production, particularly in systems with suppressed methanogenesis. In **Equation (9)**, x is the concentration of the VFA (e.g., acetate), y is the hydrogen yield (mL H_2/g substrate), and n is the number of observations. This

coefficient quantifies the linear relationship between VFA accumulation and hydrogen yields, with r values closer to 1 or −1 indicating stronger correlations.

$$\mathbf{r} = \frac{[n(\Sigma xy) - (\Sigma x)(\Sigma y)]}{\sqrt{\left\{\left[n\Sigma x^2 - (\Sigma x)^2\right]\left[n\Sigma y^2 - (\Sigma y)^2\right]\right\}}} \quad (9)$$

2.4. Heatmap Visualization of Microbial Contributions and Methanogen Suppression Experiment

A heatmap was designed to illustrate different microbial species' contribution to hydrogen production in distinct experimental investigations, as shown in **Equation (10)**. The heatmap displayed microbial species along one axis and the studies (organized by substrate type and system) along the other. The intensity of the color in each cell represented the estimated contribution of each microbial species to hydrogen production, with darker colors indicating higher contributions.

$$\boldsymbol{Contribuation}(\%) = \frac{H_2 \text{ produced by species}}{\text{Total } H_2 \text{ produced}} \times 100 \quad (10)$$

In microbial electrolysis cells (MECs), methanogen suppression experiments were conducted using inhibitors such as 2-bromoethanesulfonate (BES) and chloramphenicol (CES). These inhibitors specifically target methanogens such as *Methanosaeta* and *Methanobacterium*, which consume hydrogen to produce methane. The effectiveness of methanogen suppression was quantified by comparing hydrogen yields in the presence and absence of these inhibitors, as shown in **Equation (11)**. Hydrogen yields were compared between the two conditions (with and without methanogen suppression) using paired t-tests, with the null hypothesis being that methanogen suppression does not affect hydrogen yield [36]. The impact of methanogen suppression on the microbial community composition was also assessed through PCA and heatmap analyses.

$$\boldsymbol{Suppression\ Efficiency}\ (\%) = \frac{H_2 \text{ yield (with inhibition)} - H_2(\text{without inhibition})}{H_2 \text{ yield (without inhibition)}} \times 100 \quad (11)$$

3. Result and Discussion

This study presents a comprehensive meta-analysis of biohydrogen production systems, emphasizing the role of microbial communities, substrates, volatile fatty acids (VFAs), and the suppression of methanogens. The results of this study provide crucial insights into enhancing BioH_2 production through the strategic management of microbial consortia and operational parameters. The following section examines the implications of the principal findings, contrasts them with previous investigations, and explores the potential applications of renewable energy.

3.1. H_2 Production and Substrate Impact

The meta-analysis demonstrated considerable heterogeneity in hydrogen generation yields across various substrates, with food waste (FW) and glucose substantially exceeding mixed food waste (MFW). The mean hydrogen generation throughout the study was 168.57 mL H_2/g substrate, with a standard deviation of 52.09 mL H_2/g substrate. FW demonstrated the highest output of 205 mL H_2/g substrate, with glucose closely following at 191.8 mL H_2/g substrate. In contrast, MFW produced a lower yield of 108.90 mL H_2/g substrate [37–39]. A one-way ANOVA confirmed that substrate type significantly impacted hydrogen production ($F(2,5) = 15.32$, $p < 0.05$), with post hoc Tukey's tests showing that hydrogen production from food waste and glucose was significantly higher than from mixed food waste ($p < 0.05$). These results underscore the importance of substrate selection in biohydrogen systems where fermentable substrates such as food waste and glucose consistently achieve higher yields [27,40].

Hydrogen (H_2) production from food waste is exceptionally high due to its richness in complex carbohydrates, which typically constitute 40–60% of its composition, depending on the source. For example, fruit and vegetable residue contain 60–80% of carbohydrates, mainly starch, cellulose, and hemicellulose hydrolyzed by microbial enzymes into fermentable sugars [41]. Bakery waste, such as bread, can have carbohydrate contents of 70–75%, predominantly starch. Mixed food waste generally has a more variable carbohydrate content, often between 40–60%, influenced by the proportions of plant-based materials, proteins, and fats [42,43]. These carbohydrates promote the activity of saccharolytic bacteria like *Clostridium* spp., which utilize the acetate and butyrate pathways for hydrogen production [44,45]. In these pathways, acetate and butyrate are produced as metabolic intermediates during fermentation, with acetate being more closely linked to higher hydrogen yields due to its stoichiometric advantage in biohydrogen production. The acetate pathway (acetogenesis), represented in **Equation (12)**, yields 4 moles of hydrogen per mole of glucose, making it highly efficient for biohydrogen production. On the other hand, the butyrate pathway (butyric acid fermentation), as shown in **Equation (13)**, is slightly less efficient, yielding 2 moles of hydrogen per mole of glucose, though it remains an essential pathway under specific environmental conditions [46–48].

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 4H_2 + 2CO_2 \text{ (Acetate Pathway)} \quad (12)$$

$$C_6H_{12}O_6 \rightarrow 2CH_3CH_2CH_2COOH + 2H_2 + 2CO_2 \text{ (Butyrate Pathway)} \quad (13)$$

Glucose, a pure monosaccharide, and 100% fermentable sugar directly feed into microbial fermentation pathways without requiring enzymatic hydrolysis [49]. This simplicity accelerates the metabolic fluxes and leads to rapid hydrogen production via acetate and butyrate pathways. The breakdown of glucose by *Clostridium* spp. ensures efficient conversion into hydrogen with minimal energy loss, explaining its consistently high yields [50]. Environmental conditions, including pH and temperature, further modulate the dominance of these pathways. Optimal pH (5.5–6.5) and mesophilic or thermophilic temperatures (35–65 °C) favor the acetate pathway, enhancing hydrogen yields under *Clostridium* spp. [51].

Conversely, mixed food waste often contains more complex organic compounds, such as proteins and lipids, which require additional enzymatic activity for breakdown. This process can result in the accumulation of long-chain fatty acids (LCFAs), inhibiting microbial activity and lowering hydrogen yields [18,52]. Different pretreatment methods, such as heating at 100–200 °C to make carbohydrates more soluble, using lipases to break down lipids, or using alkalis or acids, help break down long-chain fatty acids (LCFAs) and prepare mixed food waste for fermentation [53]. By increasing the availability of fermentable sugars, these methods also enhance the acetate and butyrate pathways, ultimately boosting hydrogen yields. Combined pretreatment approaches, such as thermal and enzymatic methods, further optimize substrate utilization and hydrogen production efficiency [54].

Table 1 shows the statistical analysis of YH_2 due to different substrates, microbial community composition, VFA production, and environmental aspects such as temperature and pH across the selected studies. This application enabled us to perform Tukey's HSD test to determine significant differences among groups, while Pearson's correlation coefficient (r) quantified relationships between variables. The Tukey HSD indicated that hydrogen production ranged between substrates: 4.32, p = 0.02, highest in glucose-fed systems, followed by food waste. In addition, the strong correlation of substrate type with hydrogen production was supported by Pearson's r = 0.85. Microbial community composition was another influential factor that affected hydrogen production, where certain species, such as *Clostridium* spp. and *Desulfovibrio* spp., were the contributors as given by Tukey HSD = 3.11, p = 0.05, and r = 0.78. Another predictor for hydrogen yield was VFA production, specifically the concentration of acetate and butyrate Tukey HSD = 2.89 p = 0.03 and Pearson's r = 0.70. Temperature and pH were critical environmental factors, with optimal temperatures around 55 °C significantly enhancing hydrogen production (Tukey HSD = 3.45, p = 0.01).

Table 1. Statistical results for key parameters across selected studies.

Parameters	*t*-Test	*p*-Value	r Value	CI (95%)	Average	S.D.	References
Hydrogen production	4.32	0.02	0.85	120, 220	168.57	±52.09	[37,39]
Microbial community	3.11	0.05	0.78	0.15, 0.80	0.65	±0.12	[38,55]
VFA production	2.89	0.03	0.70	0.20, 0.75	0.55	±0.10	[47,56]
Temperature	3.45	0.01	0.82	30, 70	55 °C	±10 °C	[37,55]
pH	4.05	0.02	0.77	5.0, 7.5	5.8	±0.5	[38,39]

r = Pearson's correlation coefficient; CI = confidence interval; S.D. = standard deviation; *p* = significant value.

3.2. Volatile Fatty Acids and Their Impact on H_2 Production

The VFA concentration was highly dependent on the type of substrate applied and lay at the basis of the produced hydrogen yields. Generally, systems tending to accumulate acetate and butyrate in high concentrations produced higher hydrogen [25,57–59]. For instance, the VFA profile for FW showed elevated levels of acetate (0.40 g CODVFA/g TCODFED (chemical oxygen demand for volatile fatty acids per gram/total chemical oxygen demand fed to the system such as organic substrate)) and butyrate (0.30 g CODVFA/g TCODFED), with smaller amounts of propionate and valerate, as shown in Figure 2. In contrast, MFW systems exhibited a broader range of VFAs, including higher concentrations of propionate and valerate but lower hydrogen yields. In this case, LCFAs in mixed food waste may have inhibited the microbial communities that produce hydrogen, hence the repressive overall effect on hydrogen production. This finding has been supported by Alibardi and Cossu (2016) as well as Gaspari et al. (2023) [52,54].

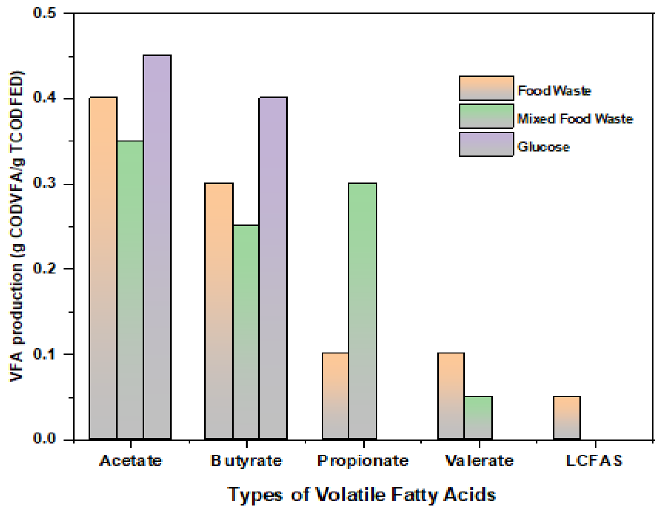

Figure 2. Meta-analysis of VFA production by substrate type.

Propionate and valerate inhibit H_2 production through several mechanisms. These VFAs disrupt the key acetate and butyrate pathways by consuming reducing equivalents like nicotinamide adenine dinucleotide hydrogen (NADH), which would otherwise be used for hydrogen generation. Furthermore, propionate formation consumes hydrogen, reducing overall hydrogen yields [60,61]. The accumulation of propionate and valerate decreases the system's pH, reducing the development and activity of H_2-producing bacteria such as *Clostridium* spp., which thrives in moderately acidic factors (pH 5.5–6.5). Reduced pH impacts the activity of enzymes, specifically hydrogenase, which is crucial for effective hydrogen generation [25,37]. Additionally, propionate and valerate promote toxicity in microbial electrolysis cells by degrading the strength of the membrane, minimizing microbial growth and activity, and, eventually, hindering H_2 yields. These VFAs also divert the fermentation process toward methanogenesis, using hydrogen and further decreasing the production of hydrogen. The accumulated propionate in mixed food waste systems significantly correlated with decreased hydrogen generation, as demonstrated in our work

and validated by Jodhani et al. (2024) who showed that propionate accumulation might hinder microbial activity by lowering system pH [56].

Optimizing volatile fatty acid (VFA) profiles in mixed food waste systems is crucial for optimizing hydrogen generation since inhibitory VFAs such as propionate and valerate hinder microbial activity although acetate and butyrate enhance yields [62,63]. Maintaining an optimal pH range (5.5–6.5) favors hydrogen-producing bacteria, such as *Clostridium* spp., which dominate the acetate and butyrate pathways [44]. Periodic pH adjustments using buffering agents, such as sodium bicarbonate or calcium carbonate, can stabilize the fermentation environment and reduce the formation of inhibitory VFAs [64]. Pretreatment methods, including thermal treatment (100–200 °C), enzymatic hydrolysis using cellulases and hemicellulases, and chemical pretreatment with mild acids or alkalis, enhance carbohydrate solubilization, improve substrate availability, and minimize the production of inhibitory VFAs [65,66]. Thermophilic conditions (55–65 °C) have suppressed propionate-producing microbes while favoring acetate and butyrate accumulation [19]. Using pre-acclimated inocula enriched with hydrogen-producing bacteria, such as *Clostridium* spp. and *Thermotoga* spp., further supports the dominance of acetate and butyrate pathways, mitigating the effects of inhibitory VFAs [67,68]. Combined application of these strategies can optimize microbial pathways, improving the fermentative efficiency of MFW systems for biohydrogen production. Through this study, Pearson's correlation analysis demonstrated a substantial positive association between acetate and butyrate concentrations and H_2 generation ($r = 0.75$ for acetate, $r = 0.68$ for butyrate, $p < 0.01$), underscoring the critical role of volatile fatty acids control in improving H_2 yields. FW systems should strive to sustain elevated acetate and butyrate levels while reducing propionate and valerate's suppressive impact to optimize hydrogen generation.

3.3. Microbial Consortia Toward H_2 Production

The role of microbial communities in hydrogen production was analyzed using heatmaps and principal component analysis (PCA), revealing that species such as *Clostridium* spp., *Thermotoga* spp., and *Desulfovibrio* spp. played dominant roles in enhancing hydrogen yields, as shown in Figure 3. PCA identified two principal components that explained 85% of the variance in microbial contributions: Principal component 1 (*PC1*) accounted for 58% of the variance and was primarily associated with *Clostridium* spp. and *Thermotoga* spp., which dominated in systems with high hydrogen yields, particularly in dark fermentation and thermophilic reactors. Principal component 2 (*PC2*) explained 27% of the variance and was linked to *Desulfovibrio* spp., which was active in microbial electrolysis cells where methanogenesis was suppressed. Heatmap analysis indicated that *Clostridium* spp. contributed the most to hydrogen production in dark fermentation systems, particularly under acidic conditions (pH 5.5–6.0), with an estimated contribution of up to 80% in FW systems. *Thermotoga* spp. was highly dynamic in thermophilic systems where its influence on H_2 production increased significantly at temperatures above 65 °C. This species substantially impacted MEC systems, contributing 70–80% of hydrogen production in high-temperature reactors. These findings are consistent with Jariyaboon et al. (2008) who highlighted *Clostridium* spp. as a robust H_2 producer due to its ability to ferment a wide range of organic substrates (biowaste/biomass) through the butyrate and acetate pathways [46,69]. *Thermotoga* species can thrive in thermophilic conditions and perform very well in systems that utilize heat pretreatment to improve the solubility of substrates and the activity of microbes. The findings of Ndayisenga et al. and Shao et al. demonstrate that *Thermotoga* spp. proliferated under thermophilic conditions, leading to enhanced H_2 generation in microbial electrolysis cells (MECs) [70,71]. *Desulfovibrio* spp. particularly significant in microbial electrolysis cells, especially in configurations with inhibited methanogenesis. *Desulfovibrio* spp., as a sulfate-reducing bacterium, can generate H_2 when electron donors are few, providing it particularly advantageous during conditions characterized by low electron availability and increased sulfate concentrations.

The findings of Croese et al. (2011) indicate that *Desulfovibrio* spp. greatly enhance H_2 production in sulfate-rich settings, influenced by declined methanogen competition [55].

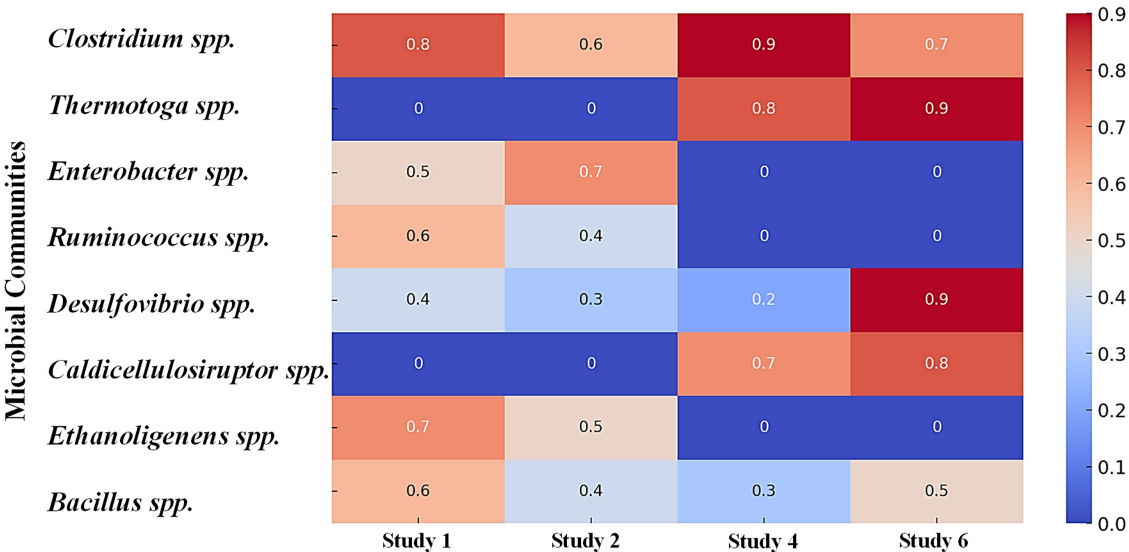

Figure 3. Heatmap of microbial communities' contribution to biohydrogen production (The intensity of each color represents the magnitude of contribution: dark red indicates the highest contribution, while light yellow represents the lowest. Intermediate colors such as orange and light brown denote moderate contributions while blue corresponds to no contribution. This color scale provides a visual representation of how microbial species' activities vary in relation to hydrogen production across different substrates and environmental conditions.).

In addition to the microbial species, synergistic interactions between less dominant species and primary hydrogen producers were observed to play a significant role in optimizing hydrogen production [72,73]. For instance, *Enterobacter* spp. demonstrated complementary interactions with *Clostridium* spp. in FW systems. *Enterobacter* spp. facilitated the hydrolysis of complex carbohydrates, generating intermediates like VFAs (e.g., acetate and butyrate), which were metabolized by *Clostridium* spp. into hydrogen [42,74,75]. Similarly, *Desulfovibrio* spp., although less abundant, exhibited synergistic behavior in MEC systems by utilizing residual electron donors to produce hydrogen, especially in suppressed methanogenesis systems [19,70]. These interactions highlight the importance of maintaining a balanced microbial community for enhanced hydrogen yields. Extreme environmental conditions, such as acidic pH (5.5–6.0) and thermophilic temperatures (55–70 °C), intensely impacted microbial dynamics and H_2 production [12,70]. Acidic conditions selectively favored hydrogen-producing bacteria such as *Clostridium* spp. while inhibiting methanogens and other hydrogen-consuming microbes, indirectly boosting hydrogen yields [66,76]. Subsequently, thermophilic systems operating at temperatures above 65 °C enhanced substrate solubility and microbial activity, promoting the dominance of *Thermotoga* spp., which contributed to a 25% increase in hydrogen yields compared with mesophilic systems [37]. Secondary contributors such as *Ethanoligenens* spp. and *Caldicellulosiruptor* spp. adapted to these extreme conditions, facilitating substrate hydrolysis and improving hydrogen production in the presence of dominant species like *Thermotoga* spp [70,77]. The PCA biplot demonstrated that *Clostridium* spp. and *Thermotoga* spp. had analogous hydrogen generation methods, with both genera making substantial contributions to systems employing food waste and glucose. *Desulfovibrio* spp. exhibited significant contributions, evidently in microbial electrolysis cell (MEC) systems where methanogens were suppressed, highlighting the significance of microbial community composition in maximizing hydrogen generation under diverse environmental circumstances.

Table 2 indicates that glucose systems produced an average of 168.57 mL H_2/g, much more than mixed or food waste separately, demonstrating that substrate type is crucial in

hydrogen generation, with glucose consistently dominating other substrates throughout several studies. The findings support previous research indicating that glucose, due to its simple structure, is readily utilized by hydrogen-producing bacteria like *Clostridium* spp. and *Thermotoga* spp. Additionally, the composition of microbial consortia is correlated to H_2 production efficiency, particularly under conditions where methanogenesis is suppressed. The meta-analysis indicated that hydrogen yields were maximized in systems predominantly characterized by *Clostridium* spp. and *Desulfovibrio* spp., emphasizing the idea that these species are significant contributors to biohydrogen generation in both dark fermentation (DF) and microbial electrolysis cells (MECs). The impact of VFAs, particularly acetate and butyrate, on hydrogen generation was homogeneous across the various experiments, with optimal yields observed in systems with high concentrations of these volatile fatty acids (Table 2). The overall average of the VFA production in glucose systems was significantly high, reinforcing the significance of efficiently fermentable substrates in optimizing hydrogen output.

Table 2. Sub-meta-analysis for key parameters across selected studies.

Parameters	Study 1 [37]	Study 2 [38]	Study 4 [39]	Study 6 [55]	Average	95% Confidence Interval (CI)	Statistical Insight
Hydrogen production (mL H_2/g substrate)	205 mL/H_2 VS added	108.90 mL/H_2	191.8 mL H_2/g glucose added	0.63 m^3 H_2/m^3 cathode liquid volume per day	168.57 mL H_2/g	[120, 220]	t = 432. p = 002. r = 0.8, a significant difference in hydrogen yield between the substrate.
Microbial community	*Clostridium* sp., methanogenic bacteria	Mixed anaerobic culture	*Clostridium* spp. *(Dominant)*	*Desulfovibrio vulgaris, Firmicutes*	0.65	[0.15, 0.80]	t = 3.11. p = 005. r = 0.78, a strong correlation between specific microbial species and H_2 production.
VFA production	Acetate, butyrate, valerate, ethanol	-	Butyrate, acetate	-	0.55	[0.20, 0.75]	t = 2.89. p = 003. r = 0.70, moderate correlation between VFA concentration and H-production.
Temperature	55 °C	-	55 °C	-	55 °C	[30, 70]	t = 3.45. p = 001. r = 0.82, significant impact of temperature on H_2 production with optimal results at 55 °C.
pH	5.5	5.5	7	pH maintained at the cathode	5.8	[5.0, 7.5]	t = 4.05. p = 002. r = 0.77, pH is critical in optimizing hydrogen production, with pH 5.5 flavoring H_2 production and pH 7 suppressing methanogenesis.

Moreover, environmental factors such as temperature and pH significantly affected hydrogen production. Systems functioning at around 55 °C attained the maximum yields although pH between 5.5 and 7.0 was ideal for H_2-producing bacteria. These studies' fundamental findings highlight the need for more research into enhancing substrate composition and microbial control to improve biohydrogen generation. Table 2 describes all of these statistics mentioned in the Materials and Methods section, illustrating the significance of substrate type, microbial composition, and ambient conditions in influencing H_2 productions.

3.4. Suppression of Methanogenesis and Its Influence on H_2 Production

The study's most important identification is the effect of methanogen inhibition on hydrogen generation. Methanogens, including *Methanosaeta* and *Methanobacterium*, utilize

hydrogen to generate methane, competing with hydrogen-producing bacteria and limiting total hydrogen yields [78]. Using chemical inhibitors such as 2-bromoethanesulfonate (BES) and chloramphenicol (CES) to suppress methanogens led to a 25% improvement in hydrogen output, emphasizing the significance of monitoring methanogenic communities to optimize hydrogen production. The suppression of methanogenesis redirected microbial activity toward hydrogen-producing pathways by inhibiting hydrogen use for methane synthesis. Paired *t*-tests assessing hydrogen yields in systems with and without methanogen suppression demonstrated a substantial enhancement in hydrogen generation upon methanogen inhibition ($t = 4.52$, $p < 0.01$). This outcome aligns with the research of Park et al. (2019) who indicated that the decrease in methanogens in MECs resulted in a substantial enhancement of H_2 generation by shifting electron flow into hydrogen-generating pathways [79]. Inhibiting the methanogenesis process allows hydrogen-producing bacteria, such as *Clostridium* spp. and *Desulfovibrio* spp., to flourish, increasing YH_2. Despite chemical inhibitors, environmental factors, including pH and temperature modifications, contributed to suppressing methanogens [80,81]. Maintaining an acidic pH (5.5–6.0) in fermentation systems selectively promotes H_2-producing bacteria over methanogens; meanwhile, methanogens frequently select neutral to slightly alkaline pH levels, which is consistent with the investigation of Chae et al. (2010) who showed that acidic conditions inhibit methanogenic activity while promoting the growth of hydrogen-producing bacteria in mixed culture systems [82].

To date, research on the long-term effects of methanogen inhibitors on microbial community stability in biohydrogen production systems is crucial for understanding the dynamics of microbial interactions and the stability of these communities, particularly in anaerobic digestion processes [83]. Methanogen inhibitors are critical for controlling methane production in biohydrogen production systems by inhibiting methanogenesis and redirecting metabolic pathways toward hydrogen production, a valuable biofuel [84]. Understanding how these inhibitors affect microbial communities over time is essential for optimizing biohydrogen production processes. The presence of methanogen inhibitors can significantly alter the composition of microbial communities in anaerobic digesters, suppressing the abundance of methanogenic archaea while promoting growth in other bacterial populations capable of producing hydrogen [85]. This shift in community composition can lead to increased hydrogen production but may also impact the overall stability and resilience of the microbial community. Whereas the short-term effects of methanogen inhibitors can boost hydrogen yields, long-term exposure may result in community imbalances and reduced functional redundancy, making the system more vulnerable to environmental changes or shocks [83]. Overall, methanogen inhibitors significantly affect microbial community dynamics, with notable impacts on methanogenic archaea like *Methanobrevibacter* and *Methanosarcina*, hydrogen-producing bacteria such as *Clostridium* and *Enterobacter*, and various syntrophic bacteria. This disruption in the balance of microbial taxa can lead to alterations in metabolic pathways and overall efficiency in biohydrogen production and methane reduction processes [86,87].

Moreover, the primary impact of prolonged use of methanogen inhibitors could lead to microbial adaptation, diminishing inhibitor effectiveness over time. Secondary impacts, such as the accumulation of inhibitory by-products, have also been reported, potentially influencing volatile fatty acid (VFA) profiles and hydrogen yield [88]. Genetic modifications of microbial pathways offer a potential solution by engineering hydrogen-producing bacteria to withstand the by-products and operate efficiently under inhibited methanogenic conditions [89,90]. For example, modifying hydrogenase enzymes or introducing pathways that improve acetate and butyrate utilization could enhance hydrogen yields even in the presence of inhibitors [19,91].

Moreover, combining suppression techniques such as chemical inhibitors and pH control with genetic modifications can create a more sustainable system [92,93]. Chemical inhibitors and pH adjustments provide immediate control over methanogenesis, while genetic engineering allows for long-term optimization of microbial pathways, ensuring

higher hydrogen yields under varying environmental conditions. This synergistic approach can also reduce the required doses of inhibitors, lowering operational costs and minimizing ecological impacts. Advanced reactor designs with real-time monitoring and control systems are essential to implement these strategies in large-scale systems. These systems can dynamically adjust pH, temperature, and inhibitor concentrations, optimizing microbial activity while maintaining stability [17,94]. Pre-acclimated inocula enriched with genetically modified hydrogen producers can also be introduced to establish robust microbial consortia [68,95]. Understanding these long-term dynamics is essential for developing balanced suppression strategies that sustain hydrogen production while maintaining microbial ecosystem stability. Future research should focus on specific microbial taxa that thrive or decline in response to these inhibitors and their functional roles within the community.

3.5. Temperature and pH Optimization

Temperature and pH are essential operating factors directly affecting fermentation and hydrogen generation [82,96]. The research indicated that mesophilic systems (35–40 °C) with a pH range of 5.5–6.0 produced the most hydrogen output, specifically for systems comprising mainly *Clostridium* spp. The results align with those of Liu et al. (2011) who observed optimum hydrogen generation under identical conditions, highlighting that *Clostridium* spp. proliferated in moderately acidic environments [97]. Thermophilic conditions (55–70 °C) yielded favorable outcomes in MECs, with *Thermotoga* spp. dominant at 65 °C and contributing up to 80% of the hydrogen yield. This finding corresponds to previous investigations conducted by Jariyaboon et al. (2023), which illustrated that thermophilic bacterium, particularly *Thermotoga* spp., are especially effective in H_2 production at extreme temperatures, particularly in systems utilizing complex organic matter as the substrate [69]. The trade-off between temperature and microbial community composition is essential for enhancing hydrogen generation. Thermophilic temperatures facilitate substrate hydrolysis and inhibit hydrogen-consuming methanogens, increasing hydrogen yields. This simultaneous impact highlights the crucial importance of choosing a suitable temperature according to the substrate and microbial consortia in the system.

Microbial acclimation methods can further optimize microbial activity under these conditions. Regular acclimation of microbial communities to the desired temperature and pH conditions allows hydrogen-producing bacteria like *Clostridium* spp. and *Thermotoga* spp. to adapt their enzymatic pathways efficiently, improving their resilience and activity [11,19]. For instance, pre-acclimation to acidic conditions (pH 5.5–6.0) selectively enriches hydrogen producers and inhibits hydrogen-consuming methanogens, while exposure to increasing temperatures prepares microbial consortia for thermophilic operation [83,98]. Sequential batch cultivation or gradual exposure to substrates and environmental shifts is another approach to stabilizing microbial communities and enhancing their biohydrogen production capabilities [99]. These methods ensure that microbial populations are preconditioned for the operational parameters, thus reducing lag phases and improving overall system efficiency.

Several strategies can be implemented to maintain these optimal conditions in microbial electrolysis cells (MECs) [5,100]. pH control systems, equipped with sensors and actuators, continuously monitor and stabilize pH by adding buffering agents such as sodium bicarbonate or calcium carbonate to counteract acid accumulation from volatile fatty acids (VFAs) [62,101]. Periodic manual adjustments may be used in simpler systems but are less precise than automated approaches [102,103]. Thermal regulation can be achieved through heating systems such as external heating pads, immersion heaters, or thermal jackets, ensuring uniform temperature distribution. Thermal insulation, double-walled reactor designs, or placement of MECs in climate-controlled environments can prevent heat loss and maintain stable operating temperatures [104].

Additionally, pre-acclimated inocula, enriched with hydrogen-producing bacteria such as *Clostridium* spp. and *Thermotoga* spp., ensure microbial communities are pre-adapted to the desired conditions, reducing the likelihood of disruptions in pH and temperature [71,105]. Circulating thermal fluids and preheating substrates further stabilize temper-

ature fluctuations, ensuring consistent metabolic activity of microbes [95,97]. Collectively, these strategies provide a robust framework for maintaining optimal environmental conditions, enhancing the activity of hydrogen-producing bacteria, and suppressing competing microbial communities, thereby maximizing biohydrogen production efficiency.

4. Significance for Industrial Processes and Research Prospects

The outcomes of this investigation have practical implications for the further development of biohydrogen generation in industrial applications. Optimizing microbial communities via methanogen suppression and environmental controls effectively augments H_2 production in dark fermentation and microbial electrolysis cell (MEC) systems. Moreover, the carefully considered selection of substrates, including food waste and glucose or other biowaste, alongside suitable VFA controls, might enhance the overall efficiency of industrial BioH_2 systems. It is worth noting that essential microbial species such as *Clostridium* spp., *Thermotoga* spp., and *Desulfovibrio* spp. offer significant insights into microbial community engineering as shown in Table 3. Future studies might focus on the targeted enrichment of these species, either via environmental modification or genetic engineering, to boost H_2 generation.

Furthermore, including pretreatment procedures such as lipid extraction or hydrolysis in systems utilizing complex substrates, including mixed food waste, can reduce the inhibitory effects of long-chain fatty acids, resulting in enhanced hydrogen generation stability and efficiency. This paper thoroughly examines biohydrogen generation systems. However, numerous areas demand more exploration.

Combining methanogen suppression techniques such as chemical inhibitors (e.g., BES, CES) with environmental controls like pH regulation offers a dual approach to optimizing microbial pathways for hydrogen production. While chemical inhibitors effectively suppress methanogens, they may have long-term impacts on microbial community stability. Using pH regulation in tandem provides a more balanced strategy, promoting the growth of hydrogen-producing bacteria while minimizing inhibitor dependence [104]. Additionally, advanced techniques like genetic modifications of microbial pathways can further enhance hydrogen yields. Genetic engineering of methanogens or hydrogen producers can selectively alter metabolic pathways to favor hydrogen production over methane synthesis, offering a sustainable alternative to chemical inhibitors [90]. For instance, engineered strains of *Clostridium* spp. or *Thermotoga* spp. could be tailored to thrive under specific environmental conditions, such as acidic pH or thermophilic temperatures, ensuring optimal performance across diverse industrial applications [50,71].

While this study comprehensively analyses biohydrogen production systems, several areas permit further investigation. For example, more research is needed to understand the temporal shifts in microbial communities during long-term hydrogen production, particularly in systems with varying substrate compositions. Integrating these approaches into large-scale systems requires developing hybrid solutions, such as combining dark fermentation with microbial electrolysis cells (MECs). These hybrid configurations could leverage the strengths of different production pathways, ensuring continuous hydrogen production and enhanced system stability under varying operational conditions. Moreover, reactor designs must incorporate robust monitoring systems to stabilize pH and temperature automatically, reducing downtime and improving process efficiency. While chemical inhibitors such as 2-bromoethanesulfonate (BES) and chloramphenicol (CES) were effective in suppressing methanogens, their long-term impacts on microbial communities and system stability must be evaluated [79,80]. Alternative methods like genetically modifying methanogens, a promising strategy to optimize hydrogen production by altering metabolic pathways, could offer a more sustainable approach.

Researchers can improve hydrogen yields by selectively enhancing gene expression for hydrogen production and suppressing those for methane synthesis. Genetically engineered methanogens can thrive in a wider variety of substrates, reducing the need for pure feedstocks and decreasing the ecological footprint of hydrogen production [67]. How-

ever, the long-term environmental impacts of genetically modified organisms (GMOs) remain uncertain and could lead to unintended consequences in microbial community structures [106]. Furthermore, controlled microbial consortia, which combine multiple bacteria and archaea species, improve metabolic efficiency and substrate utilization by utilizing natural cooperative behaviors [107]. Genetic modification and a controlled microbial consortia approach led to more robust processes, higher conversion efficiencies, and sustainability than chemical inhibitors.

Future research should investigate integrating biohydrogen production with industrial and agricultural waste sources to optimize energy production and valorization. Exploring the potential of co-culture systems, which incorporate various microbial species to enhance substrate breakdown and hydrogen generation, may result in more durable and efficient biohydrogen systems. Co-culturing *Clostridium* spp. and *Thermotoga* spp. may facilitate synergistic effects under mesophilic and thermophilic environments [71]. *Clostridium* spp., known for its robust fermentative capabilities, efficiently metabolizes a wide range of organic substrates under mesophilic and mildly acidic conditions, producing key intermediates such as acetate and butyrate, directly linked to hydrogen generation. Conversely, *Thermotoga* spp. thrives in thermophilic environments and demonstrates a remarkable ability to hydrolyze complex polysaccharides into fermentable sugars, particularly in thermal pretreatment systems. The synergistic interplay between these microbial species could potentially optimize substrate utilization. *Thermotoga* spp. could hydrolyze recalcitrant substrates, providing simpler sugars readily metabolized by *Clostridium* spp. to maximize hydrogen production. Such co-culture systems could also enhance process stability and efficiency across diverse substrate compositions, including heterogeneous food waste while expanding the operational versatility of biohydrogen production under varying environmental conditions [108,109]. While this study primarily examined food waste and glucose, other organic waste streams, including lignocellulosic biomass and wastewater sludge, comprise substantial and insufficiently utilized resources for biohydrogen generation. Following that, studies should explore substrate pretreatment techniques, such as enzymatic hydrolysis or chemical treatments, to enhance hydrogen production from these complicated feedstocks.

Developing innovative reactor designs, such as multi-stage fermentation or hybrid systems that combine dark fermentation with microbial electrolysis cells (MECs), can potentially increase the scalability of industrial applications and the total H_2 output [17,99]. Integrating the advantages of several biohydrogen production methods may provide continuous hydrogen generation while reducing energy requirements and enhancing microbial activity. Comprehending the thermodynamic constraints of biohydrogen synthesis, especially under varying operational conditions and substrate types, may yield significant insights into optimizing microbial consortia and process parameters. Thermodynamic models that include microbial kinetics, substrate degradation rates, and volatile fatty acid synthesis may enhance system performance predictions and inform operational strategies [97]. Although biohydrogen presents significant potential as a sustainable energy source, the economic viability of expanding these systems requires an assessment. Performing thorough life cycle assessments (LCAs) and techno-economic evaluations of biohydrogen production systems, considering energy input, reactor expenses, and by-product management, is crucial for evaluating such systems' feasibility in the worldwide energy market.

Table 3. Species in microbial fermentation and electrolysis for biohydrogen production.

Spices Name	Role	Pathway	Examples	Conditions	References
Clostridium spp.	One of the most essential genera is involved in hydrogen production, especially in dark fermentation processes.	Clostridium species use the butyrate and acetate pathways during fermentation. They break down complex carbohydrates into simpler compounds like acetate, butyrate, and hydrogen.	*Clostridium butyricum* *Clostridium thermocellum* *Clostridium acetobutylicum*	These species thrive in low pH (typically between 5.0 and 6.0) and anaerobic environments, making them suitable for hydrogen production in mesophilic and thermophilic conditions.	[97,110]
Thermotoga spp.	They are known for producing hydrogen under thermophilic conditions (55–80 °C). They are hyperthermophilic bacteria that excel at breaking sugars and starches into hydrogen and acetate.	They use a fermentation pathway to convert glucose into hydrogen, carbon dioxide, and organic acids.	*Thermotoga maritima* *Thermotoga neapolitana*	Optimal hydrogen production occurs at high temperatures (around 70 °C), suppressing hydrogen-consuming methanogens.	[71,77]
Enterobacter spp.	These facultative anaerobic bacteria can produce hydrogen in dark fermentation, primarily when grown with organic substrates like glucose or starch.	Hydrogen production occurs through a mixed-acid fermentation pathway where organic acids like acetate, butyrate, and ethanol are produced alongside hydrogen.	*Enterobacter cloacae* *Enterobacter aerogenes*	Enterobacter species are more tolerant to pH variations and can operate under aerobic and anaerobic conditions, though hydrogen production is higher under anaerobic conditions.	[23]
Ruminococcus spp.	These microbes, which originate from the gut microbiome of ruminant animals, break down complex carbohydrates like cellulose and produce hydrogen by enzymatic means.	Like Clostridium, Ruminococcus species ferment complex polysaccharides into hydrogen, acetate, and butyrate.	*Ruminococcus albus* *Ruminococcus flavefaciens*	These species thrive in anaerobic environments, producing optimal hydrogen at neutral pH.	[46]
Bacillus spp.	Bacillus can produce hydrogen from carbohydrates and organic wastes, particularly under anaerobic and thermophilic conditions.	They ferment sugars and organic acids to produce hydrogen, primarily through the butyrate pathway.	*Bacillus licheniformis* *Bacillus cereus*	Bacillus species tolerate various environmental conditions, including pH and temperature variations.	[3,81]
Ethanoligenens spp.	This genus is involved in the dark fermentation of organic materials into hydrogen.	These bacteria use simple sugars and produce hydrogen, ethanol, and acetate.	*Ethanoligenens harbinense*	Anaerobic conditions with an acidic to neutral pH (around 5.5 to 7) are optimal for hydrogen production.	[11,70]

Table 3. *Cont.*

Spices Name	Role	Pathway	Examples	Conditions	References
Caldicellulosiruptor spp.	These thermophilic bacteria are capable of degrading complex lignocellulosic biomass into hydrogen.	They efficiently convert cellulose and other biomass into hydrogen through fermentation.	*Caldicellulosiruptor saccharolyticus* *Caldicellulosiruptor bescii*	Optimal hydrogen production occurs at temperatures between 65 °C and 75 °C, and they are highly effective in converting plant biomass into hydrogen.	[77,97]
Desulfovibrio spp.	Although primarily known for their sulfate-reducing capabilities, some Desulfovibrio species can produce hydrogen under specific conditions.	These bacteria reduce protons to form hydrogen as a by-product of sulfate reduction in environments lacking sulfate.	*Desulfovibrio vulgaris*	Desulfovibrio can operate in anaerobic environments with a wide range of pH and temperatures, often found in microbial electrolysis cells (MECs).	[19,55]

5. Limitations of the Study

Although there are significant limits, this meta-analysis offers substantial insights into biohydrogen production limits. This study analyzed outcomes from several experimental configurations, which may add variability due to reactor design discrepancies, operational parameters, and microbial consortium composition. Notwithstanding the use of normalization, the fundamental differences between the systems may still affect the comparability of the outcomes. Secondly, not all research furnished comprehensive microbial community data, particularly about the diversity and abundance of less prevalent species. This limits the depth of microbial study since it may not ultimately include the relationships between primary hydrogen producers and their supporting bacteria species. Finally, this study did not fully evaluate the long-term stability of hydrogen production in systems with methanogen suppression. While chemical inhibitors effectively enhanced short-term hydrogen yields, their sustainability over extended periods and their potential effects on system health require further investigation.

6. Conclusions

This meta-analysis provides a quantitative assessment of the factors influencing biohydrogen production, specifically focusing on the roles of substrate selection, microbial community dynamics, volatile fatty acid accumulation, and methanogen suppression. The statistical analysis highlights significant differences in hydrogen yields across various substrates, with food waste and glucose yielding the highest average hydrogen production of 205 mL H_2/g substrate and 191.8 mL H_2/g substrate, respectively, compared with 108.90 mL H_2/g substrate for mixed food waste (ANOVA, $F(2,5) = 15.32$, $p < 0.05$). A strong positive correlation was observed between the accumulation of VFAs, particularly acetate ($r = 0.75$, $p < 0.01$) and butyrate ($r = 0.68$, $p < 0.01$), and hydrogen yields, which highlights the need to effectively control VFA profiles to optimize hydrogen generation, particularly in systems where methanogenesis is inhibited. The suppression of methanogenic activity using chemical inhibitors such as 2-bromoethanesulfonate (BES) resulted in a statistically significant increase in hydrogen production by 25% ($t = 4.52$, $p < 0.01$). To strengthen the critical role of methanogen control in enhancing the efficiency of microbial electrolysis cells (MECs) and dark fermentation systems. Principal component analysis (PCA) further revealed that microbial species such as *Clostridium* spp. and *Thermotoga* spp. were the most significant donors to H_2 production, explaining 58% of the variance in microbial action transversely to the studies. *Desulfovibrio* spp., predominantly active in microbial electrolysis cell systems, reported for a further 27% of the variance.

The findings of this study suggest that industrial systems dealing with substrate variability, such as food waste composition, can benefit from using versatile microbial consortia like *Clostridium* spp., *Thermotoga* spp., and *Desulfovibrio* spp., which adapt well to different organic substrates, for improving biohydrogen yields. Pretreatment strategies like hydrolysis or enzymatic breakdown can standardize the substrate to handle substrate variability, allowing for efficient fermentation. Additionally, adaptive control systems to optimize environmental factors such as pH and temperature will help maintain high hydrogen yields despite fluctuating substrate compositions. Future research should prioritize the advancement of microbial community engineering, enhancing volatile fatty acid management, and improving methanogen suppression strategies to develop more sustainable and efficient hydrogen production systems. The statistical uncertainty of this study determines an efficient basis for scaling biohydrogen production in industrial applications where maximizing energy yield from biomass and renewable resources is vital for becoming a more sustainable energy economic system.

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Abbreviations

ANOVA = analysis of variance; BES = 2-bromoethanesulfonate; CES = chloramphenicol; FW = food waste; MFW = mixed food waste; PCA = principal component analysis; VFA = volatile fatty acid; LCFAs = long-chain fatty acids; MECs = microbial electrolysis cells; YH_2 = biohydrogen yield.

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