

Review

# Seed Pretreatment for Increased Hydrogen Production Using Mixed-Culture Systems with Advantages over Pure-Culture Systems

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**Abstract:** Hydrogen is an important source of energy and is considered as the future energy carrier post-petroleum era. Nowadays hydrogen production through various methods is being explored and developed to minimize the production costs. Biological hydrogen production has remained an attractive option, highly economical despite low yields. The mixed-culture systems use undefined microbial consortia unlike pure-cultures that use defined microbial species for hydrogen production. This review summarizes mixed-culture system pretreatments such as heat, chemical (acid, alkali), microwave, ultrasound, aeration, and electric current, amongst others, and their combinations to improve the hydrogen yields. The literature representation of pretreatments in mixed-culture systems is as follows: 45–50% heat-treatment, 15–20% chemical, 5–10% microwave, 10–15% combined and 10–15% other treatment. In comparison to pure-culture mixed-culture offers several advantages, such as technical feasibility, minimum inoculum steps, minimum media supplements, ease of operation, and the fact it works on a wide spectrum of low-cost easily available organic wastes for valorization in hydrogen production. In comparison to pure-culture, mixed-culture can eliminate media sterilization (4 h), incubation step (18–36 h), media supplements cost (\$4–6 for bioconversion of 1 kg crude glycerol (CG)) and around 10–15 Millijoule (MJ) of energy can be decreased for the single run.

**Keywords:** chemical; fermentation; hydrogen; heat; microwave; mixed-culture; nanoparticle; organic-wastes; ultrasound

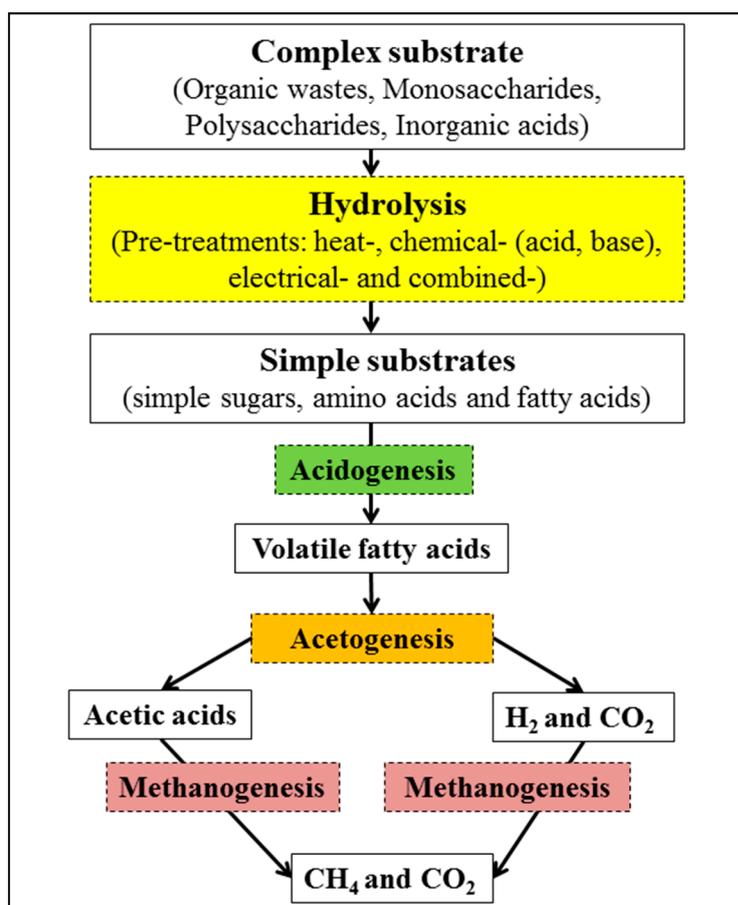
## 1. Introduction

Hydrogen can be used as a clean source of energy [1] that on combustion produces water vapor with non-greenhouse gas as the by-product [2]. Hydrogen combustion produces large amounts of energy (143 MJ/kg), being 50% more efficient in comparison to using gasoline (43–57 MJ/kg) [3]. Reports of easy hydrogen storage [4] along with the efficient transmission of hydrogen through natural gas pipelines [5] makes way for hydrogen as the fuel of the future.

Hydrogen production is carried out using chemical/physical methods such as electrolysis, steam reforming, and thermal processing that require energy-intensive steps, is very expensive and are exhaustive as they are dependent on fossil fuels [6]. In contrast, the biological method of hydrogen production requires less energy input, can be carried out at ambient conditions and is less expensive [7]. Biological hydrogen production can utilize different low-cost substrates such as agro-industry wastes like apple pomace, crude glycerol, food waste, rice straw; and use wastewater sources from molasses, food and municipal solid wastes and other lignocellulosic biomass [8,9].

Mixed-culture systems are emerging as an attractive alternative to pure/co-culture culture systems for the utilization of organic wastes along with the production of hydrogen [10,11]. Based on the natural selection process, mixed-culture systems depend on seed inoculum treatment to utilize a wide range of substrates to produce a narrow spectrum of by-products [10]. Mixed-cultures possess a high rate of hydrogen evolution, giving thermodynamically favorable yields with simple fermentation setups. Mixed-culture performs two major functions: transmitting molecular signals and communicating with each other using metabolites. These signals contribute to the division of labor for easy and rapid degradation of the complex substrate. Thus the use of mixed-culture systems helps in substrate utilization and degradation efficiency at a faster rate in comparison to pure-/co-culture systems [12].

Anaerobic digestion is a valuable energy resource, however, conventional anaerobic digestion is not an efficient process, requiring long hydraulic retention time with very low biogas recovery [13]. The metabolic pathway of anaerobic fermentation is represented in Figure 1. Mixed-culture of hydrogen production follows anaerobic fermentation that comprises four steps: hydrolysis, acidogenesis, acetogenesis, and methanogenesis. However, by blocking the methanogenesis step hydrogen production using mixed-culture systems is more feasible [14,15].



**Figure 1.** Anaerobic digestion of complex substrates using biochemical conversion pathways.

The mixed-culture system depends on the pretreatment step used for the seed inoculum, such as heat-, chemical-(acid, base), aeration-, electrical- and combined-pretreatments to block the methanogens and to increase hydrogen production [16].

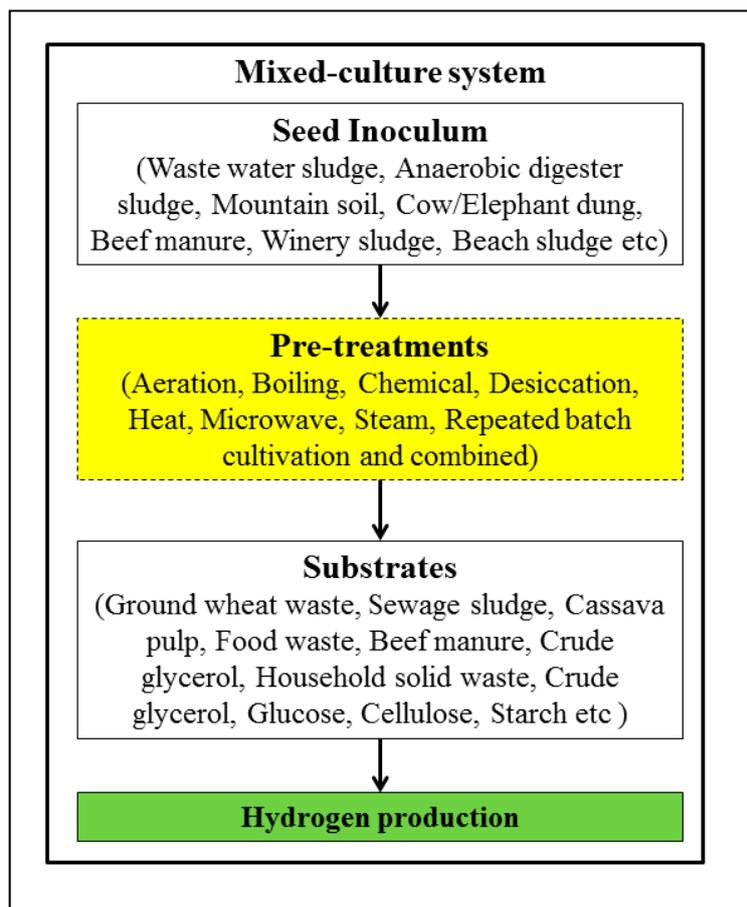
Seed inoculum pretreatment results in a 1.9–9.8 fold increase of hydrogen production in comparison to without pretreatment [17,18]. The approach of mixed-culture using industrial waste as seed inoculum, benefits industries with an effluent treatment at a decreased cost and provides an in-house energy source [11]. Efficient, low-cost improved pretreatment methods, limitation of waste release and waste recycle will be mandatory for industries in the future. The information about the selection of the most efficient pretreatment method for increased hydrogen production depending on the type of the seed inoculum is limited. Researchers nowadays are focused on developing and optimizing various pretreatment methods to streamline hydrogen production using mixed-culture systems.

### *Methodology*

The aim is to develop an approach for the utilization of organic wastes using simple pretreatment of a mixed-culture system for improved hydrogen production. The objective of this review is to elaborate the collective results on mixed-culture systems using different seed inocula; discuss the pretreatment conditions used and suggest strategies for increased hydrogen production. Initially, we gathered the articles published using the keywords: “mixed-culture system” “hydrogen production” and “seed inoculum” by searching different databases (ScienceDirect, Scopus, etc.). The published articles on mixed-culture systems were screened with objective of segregating according to the various pretreatment methods used, such as heat, chemical, microwave, others and combined treatments, depending upon the hydrogen production. Here, we highlight the importance of the mixed-culture systems, summarize different pretreatment methods and analyze the effects of pretreatment on the seed inoculum for hydrogen production. The necessary information from each of the articles published (inoculum source, the substrate used, details of pretreatment and production of hydrogen) is presented. In addition, information on the substrate pretreatment, molecular techniques used, challenges and advantages for mixed-cultures, approaches and future perspectives are also summarized in this review.

## **2. Mixed-Culture System: Pretreatment of Seed Inoculum**

The seed inocula used during mixed-culture system generally come from complex sources, such as wastewater sludge, soyabean waste, cellulose waste, crude glycerol and so on, as represented in Figure 2. Mixed-cultures are more suited for industrial purposes due to their lower operating costs, the convenience of microbial diversity and the fact fermentation helps to reduce the organic loads of effluent treatments [10]. During fermentation, steps of acidogens with acetogens are necessary for hydrogen production, however, hydrogenotrophic methanogens and acetoclastic methanogens should be minimized, as they consume hydrogen along with the produced CO<sub>2</sub> to release methane [16,19]. To overcome this hurdle, steps such as aeration of sludge, high dilution rates in the case of continuous fermentation and different pretreatment methods have been utilized. However, pretreatment methods deliver several advantages and are more effective in comparison to other steps [20]. Each species have specific characteristics and the reaction parameters involved during the fermentation can be manipulated to obtain higher hydrogen yields. The rate of hydrogen production depends on a few parameters, such as hydraulic retention time (HRT), pH, loading rate, high dilution rates and biogas circulation [10,21–23]. These parameters are varied and optimized to block the methanogenesis for increased hydrogen production. Despite maintaining these conditions, methanogenic archaea are still able to proliferate [24] and require an additional pretreatment step before the fermentation for the suppression of the hydrogen-consuming microbes [24,25].



**Figure 2.** Different types of pretreatment used for the selection of hydrogen producers from seed inocula.

Pretreatments of a seed inoculum before fermentation in the form of heat, alkali or acid, microwave, chemical or microwave or combined treatments are represented in Figure 2. The pretreatment step is carried out to inhibit the hydrogen-consuming bacteria and selectively favor hydrogen-producing microflora [26]. The basis for the development of a pretreatment step depends on the physiological differences between the hydrogen-producing and hydrogen consuming bacteria. Once subjected to pretreatment, hydrogen-producing microflora are capable of forming endospores considered as “survival structures” [27–29]. When the conditions turn favorable, they return to their vegetative state of hydrogen production. In contrast, methanogens (hydrogen-consuming) are not capable of forming endospores and are consequently growth-inhibited [30]. In addition, pretreatment steps help to accelerate hydrolysis of the seed inoculum and decrease the impact of the rate-limiting step, resulting in enhanced hydrogen production [29,31]. Pretreatment steps vary depending on the seed inoculum, and are carried out under different conditions, possess unique properties and have a significant effect on hydrogen yield. Therefore, the comparison between different pretreatment methods to optimize and obtain the optimal yield for increased hydrogen production is discussed in the following sections.

### 2.1. Heat-Treatment

Heat-treatment is the most common, simple, effective and inexpensive pretreatment method used for elevating hydrogen-producing bacteria and hydrogen production incorporating mixed-cultures [26,29,32–35]. Across the studies summarized in Table 1, heat treatment is used in multiple articles for hydrogen production. In addition to hydrogen production, heat-treatment inhibits the hydrogen-consuming *Archae* and non-sporulating methanogens, enhances the activity

of hydrogen-producing bacteria, mainly from the *Clostridium*, *Enterobacter* family [36] and assist in sludge solubilization [37,38]. The combination of seed inoculum and heat-treatment can completely eliminate methanogenic activity and produce only hydrogen and carbon dioxide. Methanogen inhibition along with activation of spore germination is advantageous, as sporulation during fermentation leads to decreased hydrogen production [39,40]. In the absence of heat-treatment on the mesophilic sludges, fermentation results in methane and carbon dioxide production with no hydrogen production [41]. The heat pretreatment method on the mesophilic sludge inactivates the hydrogenotrophic methanogens, improves the availability of hydrogen producing microorganisms and produces more hydrogen in comparison to no pretreatment (control production) [41].

**Table 1.** Hydrogen yield by mixed-culture system using heat pretreatment.

Inoculum Source	Substrate Used	Method Description	Control Production	Hydrogen Production	Ref.
Mesophilic sludge	Primary sewage sludge	110 °C for 20 min	No hydrogen production	18.14 mL H <sub>2</sub> /g of solid or 0.37 mol H <sub>2</sub> /mol hexose	[41]
Sewage Sludge	Sewage sludge	121 °C for 30 min	0.35 mL H <sub>2</sub> /g of VS	16.26 mL H <sub>2</sub> /g of volatile solid (VS)	[29]
Anaerobic digester sludge	Sewage sludge	100 °C for 15 min	21 mL H <sub>2</sub> /g of VS	25.5 mL H <sub>2</sub> /g of VS	[42]
Anaerobic wastewater sludge	Ground wheat waste	90 °C for 10 h	NA	200.47 mL H <sub>2</sub> /g of solid	[43]
Swine wastewater UASB	Sucrose	90 °C for 15 min	No hydrogen production	3.2 mL H <sub>2</sub> /g of total solid (TS) or 1.6 mol H <sub>2</sub> /mol	[44]
Anaerobic sewage sludge	Glucose	85 °C for 1 h	2112 mL H <sub>2</sub> /g of TS	3780 mL H <sub>2</sub> /g of TS or 1.67 mol H <sub>2</sub> /mol glucose	[45]
UASB granules of a local brewery company	Cassava pulp	105 °C for 2 h	1.7 mL H <sub>2</sub> /g of VS	343 mL H <sub>2</sub> /g of VS	[40]
Mountain soil	Glucose	95–105 °C for 1 h	40–60 mL H <sub>2</sub> /g of solid	318 mL H <sub>2</sub> /g of solid or 1.93 mol H <sub>2</sub> /mol glucose	[46]
UASB anaerobic brewery sludge	Sweet sorghum	105 °C for 2 h	NA	925.76 mL H <sub>2</sub> /L of substrate or 0.68 mol H <sub>2</sub> /mol hexose	[47]
Elephant dung	Cellulose fraction of sugarcane bagasse	105 °C for 2 h	NA	46.15 mL H <sub>2</sub> /g of TS or 7.1 mmol/g cellulose	[48]
Cow dung, sewage sludge, and pig slurry	Glucose	80 °C for 30 min	NA	2330 mL H <sub>2</sub> /L of substrate or 2 mol H <sub>2</sub> /mol glucose	[34]
UASB brewery sludge	Food waste	105 °C for 3 h	NA	104.79 mL H <sub>2</sub> /g of VS	[49]
Digested sewage Sludge	Glucose	100 °C for 15 min	65 mL H <sub>2</sub> /g of glucose	221 mL H <sub>2</sub> /g of glucose or 1.8 mol H <sub>2</sub> /mol glucose	[26]
Wastewater sludge	Corn Stover	105 °C for 2 h	NA	1196 mL H <sub>2</sub> /g of TS or 3.0 mol H <sub>2</sub> /mol glucose	[50]
Anaerobic winery Sludge	Corn Stover	100 °C for 30 min	102 mL H <sub>2</sub> /g of VS	182 mL H <sub>2</sub> /g of TS or 1.53 mol H <sub>2</sub> /mol glucose	[51]
Beef Manure	Beef Manure	90 °C for 3 h	NA	65 L H <sub>2</sub> /kg TS	[52]
Anaerobic digester sludge	Food waste and sewage sludge	90 °C for 10 min	NA	11 mL H <sub>2</sub> /g of VS	[53]
Municipal sewage plant sludge	Cassava Starch	95–100 °C for 1 h	9.36 mL H <sub>2</sub> /g of VS	62 mL H <sub>2</sub> /g of VS	[54]
Mesophilic sludge from UASB	Cassava stillage	90 °C for 1 h	17.8 mL H <sub>2</sub> /g VS	53.8 mL H <sub>2</sub> /g of VS	[55]
Tomato and wheat soil, compost, sludge	Glycerol	121 °C for 30 min	NA	131 mL H <sub>2</sub> /g of TS or 0.28 mol-H <sub>2</sub> /mol-glycerol	[35]

NA = Not Available.

The heat-treatment temperatures use varied from 65 °C to 121 °C and the duration varied from 10 min to 24 h [29,34,35,46]. Statistical models to determine the optimum temperature and heating time for seed inoculum such as mountain soil have been developed [46]. The optimized conditions of 105 °C

for 30 min resulted in an increased hydrogen yield of 318 mL H<sub>2</sub>/g of solid or 1.93 mol H<sub>2</sub>/mol glucose in comparison to untreated soil (control production) of 40–60 mL H<sub>2</sub>/g of solid [46] as summarized in Table 1. The heat-treatment plays a very important role in the metabolic pathway, with an increase in temperature from 65 to 120 °C resulting in a pathway shift from ethanol-type to acetate-formate type for hydrogen production. During low pre-treatment temperature of 65 °C ethanol-type fermentation was dominant, with a contribution of 24% among metabolite distribution which decreased to 14% with a pretreatment temperature of 80 °C. However, an increase to higher pre-treatment temperatures of around 95–105 °C resulted in acetate-type fermentation with the contribution rising from 7% to 21% [46]. The amount of hydrogen and by-products produced during the fermentation depends on the thermodynamics, Gibbs free energy change of the process and mass/energy balance between substrate and product [3]. Heat-treatment of soil inoculum successfully enhanced potential hydrogen-producing *Clostridium* strains and eliminated non-spore-forming bacteria in comparison to an untreated soil inoculum [46]. The heat-treatment at 80 °C for 30 min was carried out on three different seed inocula, such as cow dung, sewage sludge, and pig slurry. The study combined different seed cultures using the heat-treatment method to determine the metabolic response resulting from inter-relationship of different hydrogen-producing bacteria [34].

In the presence of digested sludge, pretreatment was carried out using heat (boiling at 100 °C for 15 min) resulting in 1.78 mol H<sub>2</sub>/mol glucose in comparison to pretreatment at pH 3/10 for 24 h that resulted in 0.80/1.09, while pretreatment with 2% chloroform resulted in 0.69 and aeration with air for 24 h resulted in 0.86 mol H<sub>2</sub>/mol glucose [26]. In comparison to acid, and alkali treatments, heat-shock was the most efficient one for hydrogen production. A hydrogen yield of 221.5 mL/g of glucose with substrate degradation efficiency of 97.2% was achieved using heat-treatment in comparison to acid (100 mL/g of glucose), alkali (135 mL/g of glucose), aeration (110 mL/g of glucose), and chloroform (80 mL/g of glucose) [26].

Even after the heat-treatment of the seed inoculum, it is necessary to maintain the fermentation temperature and pH during the hydrogen production. The difference in the initial temperature and pH regenerates the possibility of homoacetogenesis and methanogenesis activity of seed inocula resulting in decreased hydrogen production [55]. Production of hydrogen at higher temperatures such as thermophilic conditions is also effective in inhibiting methanogens and enhancing the reaction kinetics towards hydrogen production [56]. However, heat treatment has a disadvantage of lower net energy yield due to a higher energy demand [57].

Heat-treatment is specific to the type of inoculum and more specifically to the type of substrate used for hydrogen production. Heat-treatment varies with substrates; in the case of carbohydrates, there is an improvement in hydrogen production, however, in presence of hemicellulose, lignin and cellulose, the mixed-culture consumed the produced hydrogen. The complexity of substrate and non-availability of simple sugars diverts the metabolic pathway of mixed-culture towards solvent production resulting in decreased hydrogen production [58]. Similarly, seed inocula such as cow dung, sewage sludge, and solid waste require additional pretreatment steps to improve their hydrogen production. Few of the Homoacetogenic bacteria tend to survive heat treatment and carry out acetogenesis to produce acetate resulting in decreased hydrogen production [35]. The complexity of substrate, the composition of seed inoculum and diversity of microorganism present altogether nullify the heat-treatment method, so researchers have developed additional treatments and also carry out combined treatment methods to improve hydrogen production.

## 2.2. Chemical-Treatment

A major factor that influences the rate of hydrogen production is the pH, which plays an extensive role in the inhibition of hydrogen-consuming methanogens [22,23]. Methanogens grow across a narrow pH range of 6.8–7.2 [59] while major hydrogen producers such as *Clostridium* and *Enterobacter* can grow in a broad pH range of 5–9 [60]. Hence controlling fermentation pH conditions in either a low (<6.8) or high pH (>7.2) region will improve the specific growth of hydrogen-producing species but alter and

repress the metabolic pathways of methane-producing bacteria [61]. Chemical-treatments at different pH values 3 to 5 for acid-treatment and 10–12 for alkaline-treatment were carried out, respectively. The acid-treatment at pH 3 resulted in a 333-fold increase (from 0.24 mL to 80 mL H<sub>2</sub>/g of VS) in the hydrogen yield in comparison to alkali-treatment at pH 10 with a 200-fold increase (from 0.24 mL to 46.3 mL H<sub>2</sub>/g of VS) [62]. During pH adjustment, generally HCl or orthophosphoric acid for acid treatment and NaOH for alkali treatment are employed [16]. The acid-treatment at pH 3 results in an enrichment of hydrogen-producing organisms, eliminating the growth of methanogens and improving hydrogen production in comparison to alkali-treatment at pH 10 [62]. The prolonged treatment condition of 24 h at pH 3 resulted in the elimination of methanogens and a later fermentation pH set between 5.0–7.0 resulted in increased hydrogen production [63]. The acid-treatment on the sludge was carried out by adjusting the pH to 3.0 using 1 mol/L HCl for 10 min over a magnetic stirrer followed by 12 h acclimation at room temperature. Acid-treated sludge resulted in highest hydrogen yield of 0.86 mol H<sub>2</sub>/mol of glucose and molecular analysis revealed the presence of hydrogen-producing *Clostridium*, *Enterococcus*, and *Bacillus* sp. The hydrogen yield (0.86 mol H<sub>2</sub>/mol of glucose) was highest in comparison to other treatments (base (0.11), heat (0.41), freezing and thawing (0.17)) and molecular analysis identified *Lactobacillus* sp., suggesting a reason for the decreased hydrogen production in other treatments [64]. The pH treatment in addition to inhibition of methanogens also contributes to regulating the buffer conditions during fermentation. Table 2 shows that glucose and sucrose seem to be common substrates across chemical-treatments and the highest hydrogen yield of 6.12 mol/mol sucrose was obtained by using alkali treatment (pH: 10, 2 N NaOH).

**Table 2.** Hydrogen yield by mixed culture using chemical methods.

Inoculum Source	Substrate	Pretreatment Method of Inoculum	Description (Optimum Method)	Control Production	Hydrogen Production	Ref.
Sewage sludge	Glucose	Acid and Alkali	Acid: 1 N hydrochloric acid and Alkali: 1 N sodium hydroxide	No hydrogen production	80 mL H <sub>2</sub> /g of TS	[62]
Anaerobic mixed wastewater culture	Sugar-beet pulp	Alkali	Alkali-pH 12, 2 M NaOH for 30 min, Heat-121 °C, 1.5 bar, autoclave 30 min, Microwave-700 W, 170 °C, 30 min	10 mL H <sub>2</sub> /g of COD	115.6 mL H <sub>2</sub> /g of COD	[65]
Digested wastewater sludge	Sucrose	Alkali	Acid-pH 3 using 1 N HCl 30 min, Base: 10 with 2 N NaOH, 2-bromoethane sulphonic acid (BESA)/ Iodo propane-10 mmol for 30 min	22 mL H <sub>2</sub> /g of TS	32 mL H <sub>2</sub> /g of TS or 6.12 mol/mol sucrose	[63]
Beach sludge	Glucose	Acid	Acid-1 M HCl to pH 3 Alkali-1 M NaOH to pH 4	0.20 mol H <sub>2</sub> /mol glucose	0.86 mol H <sub>2</sub> /mol glucose	[64]
UASB Anaerobic chemical waste water sludge	Dairy waste water	Sodium BESA	Chemical-BESA at 0.2 g/L for 24 h	0.01 mL H <sub>2</sub> /g of COD	0.20 mL H <sub>2</sub> /g of COD	[66]
Methanogenic granules	Glucose	Chloroform	Chloroform-0.05 to 0.1% added Acid-pH 3 using 0.1 N HCl	124.99 mL H <sub>2</sub> /g of TS	145.32 mL H <sub>2</sub> /g of TS	[15]
Sewage sludge	Sucrose-based synthetic wastewater	Acid and Alkali	Acid: ~3.0 by 0.1 N HCl for 24 h and restored to 7.0 by 0.1 N NaOH	NA	Batch: 3.03 Continuous: 1.5 mol/mol sucrose	[67]
Sewage sludge	Starch	Acid and Alkali	Acid: ~3.0 by 0.1 N HCl for 24 h and restored to 7.0 by 0.1 N NaOH	0.0018 mmol H <sub>2</sub> /g	0.22 mL H <sub>2</sub> /g of COD or 0.0317 mmol H <sub>2</sub> /g COD	[68]

Compared to heat-treatment, chemical-treatment has gained popularity due to its effectiveness and ease of implementation [69]. Hydrogen-consuming methanogens can be selectively and efficiently removed by the use of reagents such as 2-bromoethane sulphonate (BES), 2-bromoethane sulphonic acid (BESA), iodopropane, acetylene, fluoroacetate, and chloroform [63,70,71]. BESA is a structural analog of coenzyme-M which is specifically present only in the methanogens. Treatment using BESA follows competitive inhibition blocking the substrate uptake and promoting the effective removal of methanogens [63,71]. Moreover, BESA was also found to inhibit the acetate utilization step by

methanogens [72]. Small quantities of BESA (25–100 mmol) have proved effective in killing the methanogenic bacteria in sludge [73,74]. Iodopropane functions as a corrinoid antagonist, capable of denaturing the activity of B12 enzymes and acting as a methyl carrier for methanogens [75]. Chloroform is used for inhibiting methanogenesis [76,77] and blocking corrinoid enzymes such as coenzyme-M reductase required by sulfate reducers for acetate production [71]. Acetylene and fluoroacetate are used for inhibiting the acetate production step [78]. During chloroform treatment, the synthesis of acetate from glucose by *Clostridium thermoacetium* was repressed [79] which inhibited the hydrogen-producing acetate [78]. Production of acetate shifts the metabolic pathway towards solventogenesis, which decreases the optimum pH and further decreases hydrogen production.

Six pretreatments methods (acid, alkali, aeration, BESA, heat and iodopropane) were applied to digested wastewater sludge. Heat and acid-treatment repressed methanogenic activity along with hydrogen production and BESA and iodopropane specifically inhibited methanogens with no hydrogen production. The base-treatment was unable to suppress methanogens and affected hydrogen production. In the case of aeration it was unable to repress methanogens and slightly affected hydrogen production. However, in a subsequent second-step batch, base-treatment resulted in increased hydrogen production and delivered the best hydrogen yield of 6.12 mol/mol sucrose in comparison to other treatments [63]. The pH of the subsequent second-step batch for base treatment was around 5.5, which repressed the activity of methanogens and was optimum for hydrogen production activity, resulting in increased yield [63]. In another report, Hu et al. [15] compared heat, acid, and chloroform treatments and concluded that chloroform improved hydrogen production (145.32 mL H<sub>2</sub>/g of TS) in comparison to the others (124.99 mL H<sub>2</sub>/g of TS) [15].

The presence of acetylene in the headspace was as effective as BESA in producing hydrogen using anaerobic digester sludge. Moreover, it has been found that chemical treatments were not sustainable over continuous-run and were found to possess BESA-resistant mutants causing side effects on the hydrogen-producing organisms [73,75]. Also, it has been reported that acid treatment of sludge resulted in low substrate removal and decreased the hydrogen production [66]. Chemical-treatment is still being explored and incorporation at a large-scale will be an expensive approach. In addition, increased concentration of acid and alkali in the fermentation medium will cause equipment material corrosion and increasing the maintenance cost, requiring alternative treatment options for mixed-culture.

### 2.3. Microwave-Treatment

Microwave irradiation (MW) is an alternative to conventional thermal methods to terminate microbes and also to conserve energy [80]. MW focuses direct heat irradiation by reducing the energy losses during energy transmission. Irradiation causes the kinetic energy of the water dipoles to increase to the boiling point, consequently bursting the cells of the microorganism and releasing the water inside [81,82]. MW-treatment is used effectively in sludge solubilization, reduction of fecal coliforms count and dewatering depending on the type of sludge used [83]. The thermal effects of MW can be channeled along a relation between temperature and intensity to destroy the hydrogen-consuming bacterial species. At temperatures between 110–175 °C, the solubilization is improved with an increase in temperature and decrease in MW intensity indicating that temperature is a more important criterion than MW intensity [84,85]. Song et al. [86] studied the variation of the effects irradiation at higher MW (2450 W) power with the time period (1.5 min) and observed improved hydrogen yield of 144.3 mL H<sub>2</sub>/g-corn stalk with 1 min of microwave irradiation (2450 W) [86]. Hydrogen yields by mixed culture systems using microwave methods are presented in Table 3.

Cow dung used as inoculum was treated using the microwave method at different maximum powers (160, 320, 480, 640 and 800 W) for 5 min. Increased hydrogen yield of 90–100 mL of H<sub>2</sub>/g or 14 mmol per mol of sugar was obtained under treatment conditions of 320 W for 5 min. The high power of microwave irradiation (above 320 W) on the inoculum, partially damaged the microbial community and bacterial cell walls, which resulted in a decreased hydrogen production [87].

**Table 3.** Hydrogen yield by mixed culture using microwave methods.

Inoculum Source	Substrate	Description (Optimum Method)	Control Production	Hydrogen Production	Ref.
local municipal wastewater sludge	Organic substrate from sludge	Heating temperature 80–170 °C and time 1 to 30 min	540–560 mL of H <sub>2</sub> /g of VSS	720–740 mL of H <sub>2</sub> /g of VSS	[13]
Cow dung	Sweet solid waste	320 W for 5 min at 95 °C	NA	90–100 mL of H <sub>2</sub> /g of VSS or 14 mmol H <sub>2</sub> /mol sugar	[87]
Cow dung	Corn stalk	Microwave 1.5 min at 2450 W	71.1 mL H <sub>2</sub> /g of corn stalk	144.3 mL H <sub>2</sub> /g of corn stalk	[86]

MW-treatment has a slight advantage over the other treatments due to its compactness, reduced time of extraction, ease of control and rapid heating [88,89]. MW-treatment is also replacing traditional pretreatment methods such as heat and pH, however insufficient information about the dielectric properties of the organic wastes, difficulties associated with scale-up and equipment cost prevent MW from being widely used. [90]. However, microwave systems are efficient only with a working volume of 50–100 mL. In addition to solubilization of hemicellulose using the microwave, it also includes the risk of solubilization of phenolic compounds releasing acids, which inhibits bacterial growth [65]. At a larger scale, reproducibility is difficult, requiring higher energy consumption and longer process time, creating a problem for larger reactors [91] suggesting alternative treatment options for mixed-culture.

#### 2.4. Other Treatments

All the previously discussed treatments have advantages and disadvantages in one way or another; researchers are also exploring new improved techniques such as aerobic stress, electric current, and freezing with thawing. Aerobic stress is used to repress hydrogen consumers, and can be easily implemented due to the availability of air blowers in large-scale setups [92]. Researchers have reported the preparation of sludge inoculum by forcing in compressed air for an hour in an anaerobic reactor and found that methanogens were efficiently inactivated [93]. Advantages of aerobic stress, selects stable inoculum with no hydrogenotrophic-methanogenic activity, ensuring the highest microbial diversity, on-site availability of blowers/diffusers for aeration and hydrogen production is easier to control [92,94]. Although aerobic stress is an energy consuming process [92], granular sludge sparging with air for 30 min was unsuccessful, resulting in 3–4 days of lag phase for hydrogen production [63]. Roychowdhary et al. [95] used electric current to select hydrogen-producing organisms from anaerobic sludge. The applied low voltage of 3–4.5 V resulted in suppression of methanogenesis and hydrogen production from cellulosic landfill [95]. The sludge with repeated freezing and thawing cycle can change the floc structure into a more compact form [96] and increase the bound sludge availability for microbes during hydrogen production [97,98]. Cheong et al. [18] freeze-dried a sludge for 24 h at 10 °C and then thawed it for another 6 h at 30 °C, to obtain a hydrogen yield of 90 mL/g hexose consumed. The application of infra-red irradiation has also been used to treat the seed inoculum [99]. The cow dung compost was baked using an infrared oven for 2 h to get an increased hydrogen yield of 290.8 mL/L-culture and the hydrogen content was around 52% with no methane production [99,100]. Reports found that ultrasonic treatment at 20 W, 9 kHz for 30 min gave higher hydrogen production than other pretreatments, such as autoclaving at 121 °C for 30 min, heating in hot water at 60 °C and freezing at –10 °C for 15 h [74]. The adaptation of seed inoculum with household solid waste as substrate and xylose as co-substrate at 70 °C for hydrogen production was conducted using repeated batch experiments. The batch experiment producing the highest hydrogen were used as inoculum for the second batch cultivation; and this repeated transfer of inoculum was carried out to enrich the highest producing inoculum to obtain 1.62 mol H<sub>2</sub>/mol of xylose [101]. Hydrogen yields obtained by mixed culture systems using other methods such as aeration, boiling and batch cultivations are presented in Table 4.

**Table 4.** Hydrogen yield by mixed culture using other methods.

Inoculum Source	Substrate	Pretreatment Method of Inoculum	Description (Optimum Method)	Control Production	Hydrogen Production	Ref.
Cow dung Compost	Cornstalk wastes	Aeration	Using forced air for 3 h at 50 °C	NA	149.69 mL H <sub>2</sub> /g of VS	[102]
Waste water sludge	Molasses	Aeration	Settled for 10 days, aerated for 30 days	NA	848 mL H <sub>2</sub> /g of VS	[94]
Solid waste CSTR	Household solid waste and Xylose	Repeated batch cultivations	Repeated transfers: culture from the first generation with the highest hydrogen production was used as the inoculum for the second batch	NA	1.36 mol H <sub>2</sub> /mol xylose	[101]
Elephant dung	Acid hydrolyzed sugarcane bagasse	Boiling	Boiling water at 100 °C for 2 h	NA	109.5 mL of H <sub>2</sub> /g of VS or 2.49 mol H <sub>2</sub> /mol sugar	[103]
Intertidal Anaerobic Sludge	Sea water + butyrate + glutamate	Boiling	Boiling water at 100 °C for 90 m	84 mL of H <sub>2</sub> /L of substrate	1225 mL of H <sub>2</sub> /L of substrate	[104]
CSTR methanogenic reactor fed with manure Methanogenic reactor fed with manure	Household Solid Waste	Control pH	pH at 7.0 using 1 N NaOH	11 mL of H <sub>2</sub> /L of substrate	107 mL of H <sub>2</sub> /L of substrate	[105]

In addition, the inoculum obtained from a methanogenic reactor fed with manure and sludge from an intertidal zone was subjected with no pretreatment to enrich the bacterial consortium. No pretreatment steps to inhibit methanogens was carried out at extreme-thermophilic conditions (70 °C) using household waste and photofermentation using enriched sea water with acids for hydrogen production [104]. After repeated batch cultivation (six times) at controlled pH 7.0, the inoculum was ready for the hydrogen production resulting in 2.25 mol/mol yield in the presence of butyrate [104].

### 2.5. Combined Pretreatment

Each pretreatment method has its own efficacy and effect on the hydrogen yield in a unique way which is dependent on the type of inoculum and substrate used during the fermentation conditions [66]. It has been reported that among the various pretreatments as discussed above, heat treatment is the most preferred and found to maximize hydrogen production [26,70]. However heat-treated sludge produced higher hydrogen from glucose in comparison to alkali-treated sludge, but heat-treated sludge using dairy wastewater obtained a low hydrogen yield [62]. Thus the efficiency of the applied pretreatment method strongly depends on the type of seed inoculum, the nature of the substrate used and the operating conditions [106–109]. Hydrogen yields obtained by mixed-culture systems using combinations of various pretreatments for hydrogen production are presented in Table 5.

A combined heat shock at 98 °C for 2 h and acid-treatment at pH 2 for 24 h for sludge was applied to produce 1.9 mol H<sub>2</sub>/mol glucose. This study indicated that a combination of heat and acid-treatment provided a stronger reaction to degrade organic matter and resulted in a higher hydrogen yield [110]. Treatment of sludge included combinations of pH, heat and BESA treatments using dairy wastewater as substrate. The combination of pH and chemicals (0.029 mmol H<sub>2</sub>/g COD) gave a 16 fold enhancement in comparison to pH and heat (0.0207 mmol H<sub>2</sub>/g COD) with an 11.5-fold increase over untreated sludge (0.0018 mmol H<sub>2</sub>/g COD) [66]. A combination of microwave-alkali and heat-alkali was also investigated, with each yielding 108.2 ml H<sub>2</sub>/g COD and 66.7 ml H<sub>2</sub>/g COD of hydrogen, respectively [65].

The combined-treatments (dry heat, desiccation and heat shock) help to inhibit methanogens and selectively enrich hydrogen-producing consortia. Likewise, chemical-treatment provides buffering stability to fermentations and heat-treatment can inhibit methanogens to increase the hydrogen production. In addition to pretreatment of the inoculum, substrate pretreatment is an additional step carried out in mixed-culture systems for hydrogen production.

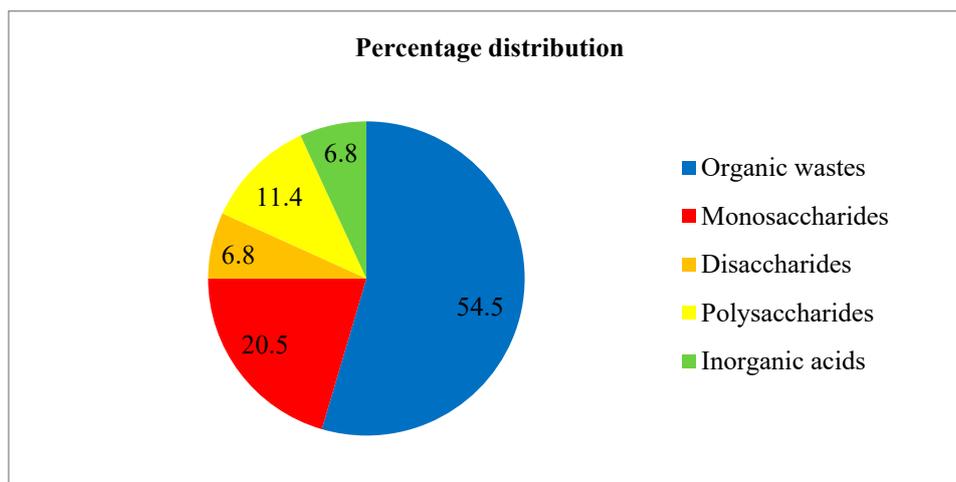
**Table 5.** Hydrogen yields produced by mixed cultures using combined methods.

Inoculum Source	Substrate	Pretreatment Method of Inoculum	Description (Optimum Method)	Control Production	Hydrogen Production	Ref.
Anaerobic granular sludge	Glucose and oat straw hydroxylase	Heat and Grinding	104 °C for 24 h Grinding with a mortar and sieved using 850 µm filter	NA	29.6 mL of H <sub>2</sub> /L of substrate or 0.81 mol H <sub>2</sub> /mol of glucose	[106]
UASB soya sludge	Raw glycerol	Dry heat and Desiccation	Heat-100 °C for 15 min, Acid-pH 3 using 1 M HCl, Alkali-pH 10 using 1 M NaOH Dry heat & Desiccation-2 h in a hot oven at 105 °C, and desiccating jar for 2 h, Freezing at -10 °C for 24 h, thawing at 30 °C for 6 h	NA	0.15 mL of H <sub>2</sub> /L of TS	[107]
Anaerobic mixed consortia	Distillery wastewater	Heat-shock and Acid treatment	heat-shock treatment (100 °C; 2 h) and acid treatment (adjusted to pH 3 with orthophosphoric acid; 24 h)	45.4 mL of H <sub>2</sub> /L of COD	169 mL of H <sub>2</sub> /L of COD	[108]
Cattle manure sludge	Glucose	Pre- and Post-Acidification	Pre-acidified to pH 3 with 10 N HCl, Chemical Acid- Perchloric acid for 10 min to bring pH to 2, BESA-0.5% for 10 min, Freezing and thawing-at-10 °C for 24 h, thawed for 6 h at 30 °C, Desiccation-2 h in oven at 105 °C, and desiccating jar for 2 h	NA	130 mL of H <sub>2</sub> /g of COD or 0.88–0.92 mol H <sub>2</sub> /mol of hexose	[18]
UASB Anaerobic chemical wastewater sludge	Dairy wastewater	Acid + Sodium BESA + Heating	Chemical- BESA at 0.2 g/L for 24 h, Acid-pH 5 orthophosphoric acid and heating at 100 °C for 1 h	NA	0.07 mL H <sub>2</sub> /g of COD	[66]
Activated anaerobic methane plant sludge	Water hyacinth	Steam heating, microwave heating/alkali and enzymatic hydrolysis	Steam heating: 112 °C with 15 min, microwave heating/alkali: 1.0% (w/v) NaOH solution for 24 h at 45 °C and heated for 1.0 min at 420 W and cellulase (5% of substrates) with 0.05 g CaCl <sub>2</sub> for 48 h at 45 °C in a shaker at 120 rpm	NA	76.7 ml H <sub>2</sub> /g TVS	[109]

### 3. Substrate Pretreatment

Substrate pretreatment is also quite common as most complex substrates are not ideal and require breaking down into simple structures for easy access during hydrogen fermentation [107]. After appropriate pretreatment, depending upon the composition of the substrate, the hydrogen-consuming bacteria can easily digest the simple substrates to improve the hydrogen yield. Zhang et al. [102] found that cornstalks after acid pretreatment at pH 7.0 produced more hydrogen (149.69 mL H<sub>2</sub>/g TVS) around 46-fold higher in comparison to untreated cornstalks (3.21 mL H<sub>2</sub>/g TVS). Figure 3 shows the frequency of nature of complex substrates used in the mixed-culture system during hydrogen production. Studies of lignocellulosic substrates with and without pretreatment were also performed, and pretreated substrate produced 3.17 mmol of H<sub>2</sub>/g approximately 50% more than untreated biomass. It was also suggested that biological pretreatment using cellulase enzyme gave the highest yield of 4.54 mmol of H<sub>2</sub>/g of the substrate in comparison to acid and alkali pretreatments [41,50].

Ultrasound is an emerging technique, energy effective using mechanical disruption [111] and commonly used for sludge degradation [112] to break down complex macromolecular flocs into simpler structures (efficiency > 95%). Ultrasonic hydrolysis forms cavitation bubbles, leading to a rise in temperature capable of disrupting cell walls and releasing a large amount of organic matter from the macromolecular flocs [38,113]. The high temperature generated inside the solution for longer durations has an advantage of killing pathogenic microbes and increasing the solubility of the organic matter in solution [113].



**Figure 3.** Percentage distribution of available literature reports on hydrogen production from different substrates using the mixed-culture system.

Guo et al. [38] showed that sonication power density of 2 W/mL applied for 5 min on a municipal sludge resulted in a hydrogen yield of 4.68 mL/g COD. Ultrasonication also assists to extract heavy metals such as Cu, Ni, and Zn from sludge to reduce the heavy metals toxicity on hydrogen production [38]. The organic matter released enhances the efficiency of the sludge digestion process and reduces the cost of final treatment of the sludge [114]. Ultrasound treatment in comparison to other pretreatments is highly efficient but consumes a lot of energy.

Cassava pulp, which on a dry basis contains 50–60% of starch, when subjected to acid-hydrolysis will release glucose and xylose, ideal for hydrogen production. Acid hydrolysis of cassava pulp was carried out at 121 °C for 60 min using 1:15 (solid: liquid ratio) by varying sulfuric acid concentration from 0.25 to 5% (*v/v*). The optimum concentration of 0.5% of acid resulted in 13.33 g/L of glucose and 11.66 g/L of xylose, and further increase in the acid concentration resulted in the production of microbial inhibitors such as organic acids and furan derivatives [40]. In order to determine the sugar substrate utilization by the mixed-culture system, hydrogen production using mannose, galactose, arabinose, and xylose was carried out. Using glucose the hydrogen yield was around 3.21, galactose was 3.0, xylose was 2.3, using mannose was 1.4 and using arabinose after a lag phase delay of 60 h resulted in 0.4 yields. The initial substrate study was carried out to evaluate the best hydrolysate obtained from neutral and acidic pretreatment of corn stover followed with steam-explosion hydrolysis at 190–220 °C for 3–5 min. The hemicellulose from corn stover after neutral (220 °C for 3 min) and acidic (190 °C for 2 min) steam-explosion hydrolysis resulted in mixed sugars substrate (glucose, xylose, galactose, mannose, and arabinose) to deliver hydrogen yield of 2.84 and 3.0 [50]. Likewise, thermal acid-pretreatment (TAP) of corn stover using 0.2 N H<sub>2</sub>SO<sub>4</sub> over 90 min resulted in 8.1 g/L of soluble sugar and using microwave-assisted acid pretreatment (MAP) with 0.2 N H<sub>2</sub>SO<sub>4</sub> at a power of 700 W for 90 min resulted with 12.3 g/L of soluble sugar in comparison to no pretreatment with 5.2 g/L. The combined pretreatment of microwave irradiation and acidification resulted in a high efficiency of lignocellulosic hydrolysis, increased the release of soluble sugars and improved the accessibility of microorganisms of corn stover as substrate [51].

Use of sewage sludge as fermentation substrate offers several advantages over synthetic substrates, as sewage sludge is abundant and available at low cost [41]. However, direct use of sewage sludge results in decreased soluble carbohydrate availability and decreased hydrogen production. Hence, pretreatment of sewage sludge by heating to 70 °C for 1 h followed by cellulase enzyme treatment, helps to increase the soluble carbohydrate level from 2.6 to 13.5% [41]. In addition to heat treatment resulting in the breakdown of the sludge gel structure [29], enzyme treatment is necessary to increase sugars substrate availability for increased hydrogen with no methane production [41]. Use of pretreatment methods (heat and enzyme) on the sewage sludge helped to increase total soluble carbohydrate,

overcome the laboratory scale work, continuously operated using 5 L reactor and produced hydrogen at 18.14 L/kg of dry solids [41].

Substrate pretreatment plays an important role in hydrogen production, influences substrate degradation, and sugar distribution to benefit bacterial growth. It is also necessary to carry out the fermentation at elevated operating temperatures (65–70 °C) to improve substrate degradation rate and increase hydrogen production [56]. However, a mesophilic range (30–37 °C) of operating conditions is found to be optimum, balances energy recovery and hydrogen production [45]. The mixed-culture system needs pretreatment of the seed inoculum and the substrate to improve the hydrogen productions, in addition to the use of the statistical models to optimize the fermentation parameters; use of co-substrate, combined dark-light system and use of immobilization will further increase hydrogen production.

#### 4. Improvements in Mixed-Culture

The maximum molar yield of hydrogen will be around 4 mol from 1 mol of glucose with complete conversion to acetate as the end-product [115]. The highest hydrogen yield reported was around 2.8 mol/mol of glucose in case of mixed-culture systems [116] and was around (4.5 and 5.77 mol/mol of glucose) using an engineered *Thermotoga maritima* (Tma100 and Tma200) strain [117]. The gene inactivation of the lactate pathway, insertion of a transporter subunit and the strain construction method together improved the fermentation efficiency, and energy management with an increase in hydrogen production. The fermentation parameters need to be optimized to block the unwanted pathway(s), minimize by-product formation, to improve mass and energy balance between substrate and product, in presence of hydrogen producing microorganisms to reach maximum molar hydrogen yield [117]. The gene engineering of unwanted-, inactivation- and blocking pathways can be carried out on the hydrogen-producing pure-culture [117], obtained only after carrying out a mixed-culture pre-treatment step. Use of statistical model with experimental design plays a vital role to screen and vary the parameters to obtain optimum conditions resulting in the enhancement of hydrogen yield. Central composite design (CCD), Taguchi analysis, Box-Behnken, and Plackett-Burman design are some of the common design models studied. Plackett-Burman is used to narrow down the screened parameters of the study, followed by CCD to determine the relationship between the variables and their response to the system to decide the optimum conditions [118].

Using CCD, the optimized conditions for crude glycerol, 20 g/L, pH 7 and inoculum size 20%, resulted in 29.43 mmol/L of hydrogen in comparison to 15.18 mmol/L of hydrogen with the non-CCD-optimized conditions [11]. Varonne et al. [119] used a Plackett-Burman screening design on parameters such as pH, temperature and glycerol concentration, and then used a Box-Behnken design for optimization. The optimized conditions at 37 °C, pH of 7.9 and glycerol concentration of 15 g/L resulted in a hydrogen yield around 0.96 mol H<sub>2</sub>/mol glycerol [119]. In the presence of heat-treated digested anaerobic granular sludge with CCD, glucose 14.01 g/L, pH 5.61 and nickel nanoparticles 14.01 g/L as co-supplement resulted in a hydrogen yield of 2.54 mol/mol of glucose. The study demonstrated a linear and interactive effect of glucose and nickel nanoparticle concentration to increase hydrogen production by 23% in comparison to the absence of nanoparticles [120].

The kinetics of hydrogen production such as the relation between the substrate, biomass and products formation can be evaluated by using the Michaelis–Menten equation and logistic model [37]. The modified Gompertz equation helps to determine the hydrogen production and microbial growth at the operating conditions [45]. The information from all these models and equations helps to determine the activation energy for hydrogen production (107.66 kJ/mol) and microbial growth (204.77 kJ/mol) and helps design a better mixed-culture system of hydrogen production [45].

Another emerging approach is the combination of dark and light fermentation, also called two-stage fermentation. It has three steps, pretreatment for hydrolysis, dark fermentation, and photofermentation. Dark-fermentation by mixed-culture uses the substrate hydrolysis to convert it into hydrogen, acetate, and butyrate. Light-fermentation by mixed-culture uses volatile organic

acids (acetate and butyrate) to produce hydrogen in the presence of light energy. The advantage of two-stage fermentation is the high conversion of substrate into hydrogen, which is not possible by dark-fermentation alone [121]. The combination of organic waste and photosynthetic bacteria like *Rhodobium sphaeroides* and *R. palustris* has yielded up to 7.2 mol H<sub>2</sub>/mol hexose [122], in comparison with the yield of 3.8 mol H<sub>2</sub>/mol hexose by dark-fermentation [123] and using the engineered strain *Thermotoga maritima* (Tma100 and Tma200) the yield was around 4.5 and 5.77 mol/mol of glucose [117]. The two-stage system of hydrogen production using engineered strains can achieve high hydrogen yield at the expense of requiring two separate reactors. Combined fermentation using heat-treated anaerobic sludge for dark and using *Rhodobacter* for light fermentation, has the advantage of reducing the fermentation time with a single reactor to produce hydrogen using the released volatile fatty acids (VFA) in the medium [43]. The combined dark-light fermentation reduces the fermentation time from 10 days to 6 days and decreases the maintenance and production cost of hydrogen [43].

There have been increased efforts, such as improving bioreactor design and employing statistical models to scale-up the operating conditions to enhance the hydrogen production. While most of the hydrogen production experiments have been carried in batch reactors due to their simplicity and ease of control [118], large-scale applications require continuous operation and make use of Continuous Stirred Tank Reactors (CSTRs) for hydrogen production. [124]. While conventional CSTRs may experience washout, even at lower HRT of 2.5 days, due to biomass having the same composition as effluent [125]. Using an immobilized mixed-culture set-up, a short HRT (12 to 2 h) without washout will maintain higher biomass concentration throughout the fermentation. It has been proved that increasing the HRT to optimum conditions increases the hydrogen production to 1.5 L/h/L of starch hydrolysate [68,118].

CSTR systems are time-consuming, and their intrinsic structures, operational instability, and continuous feeding cause degradation of microorganisms resulting in decreased hydrogen production [47]. In recent years, development of granule type and biofilm-based bioreactors has been gaining attention as they are effective in maintaining the biomass concentration as well as the useful microbes [124]. An alternative to CSTR systems is the anaerobic sequencing batch reactor (ASBR) with a high degree of process stability, working at high biomass concentration, process flexibility, eliminate the use of clarifier, efficient operating controls and high organic removal efficiency using simple operation [47]. ASBR works on various substrates, such as dairy wastewater, chemical wastewater and sucrose reported increased hydrogen yield by using acid-enriched sewage sludge microflora with a yield of 2.5 mol/mol of sucrose at 4–13 h HRT [47].

The improvements as discussed above help in developing mixed-culture systems to produce hydrogen at commercial scale and the hydrogen-producing bacteria need to be analyzed to identify the percentage similarities across the reference microorganisms.

## 5. Molecular Techniques Used in Mixed-Culture

Microbial identification and their community structures need to be determined to qualitatively define the pretreatment methods and conditions to increase hydrogen production [126]. Most of the microbial analysis are studied by collecting the seed inoculum samples, isolating the genomic DNA and by common freeze-thaw methods [58]. After DNA extraction, PCR amplification of the V3 region of 16sDNA was performed by using a universal primer set [127]. The PCR amplified DNA undergoes denaturing gradient gel electrophoresis (DGGE) to generate profiles and the sequence variations were also analyzed. The 16sDNA later cloned and sequenced to find the similar sequence to identify the closest reference organism using Basic Local Alignment Search Tool (BLAST) [128]. The changes in the microbial community caused due to the pretreatment conditions can be monitored by using the Fluorescence In-situ Hybridization (FISH) method. Suitable fluorescent probes are added depending on the genera and species of the microorganism present and details of species are confirmed using a laser scanning microscope [54,64]. The analysis of microbial structures was carried out using denaturing gradient gel electrophoresis (DGGE) followed by phylogenetic analysis to determine the

percentage similarities with the reference species. Use of heat-treated swine wastewater during the mixed-culture revealed the presence of *E. cloacae* (98%), *Clostridium* sp. (90%), and *C. acetobutyricum* (98%), as potential hydrogen producers [44]. The DGGE analysis also determines the pretreatment changes in the microbial community that accounts for the hydrogen production [64]. The DGGE analysis determined the change in the microbial community depending upon the type of pretreatment method carried out. Acid and heat-treatment favored enriching hydrogen-producing bacterium such as *Clostridium* sp., *Enterobacter* sp. and *Bacillus* sp., while on the contrary base, freezing and thawing treatment methods enriched non-hydrogen-producing bacterium, such as *Lactobacillus* sp. *Lactobacillus* sp follows a different pathway to produce CO<sub>2</sub> causing a decrease in hydrogen production and *Lactobacillus* released bacteriocins inhibits hydrogen-producing bacteria [64].

The Scanning Electron Microscope (SEM) image analysis illustrates the physical appearance of hydrogen-producing bacteria from the rod-shaped morphology in case of heat-pretreated and non-treated soil inocula [46]. SEM analysis examines the cellular level and can be performed for the qualitative assessment of the hydrogen-producing microorganisms [129]. In addition to the determination of molecular profiling, SEM can also be used to identify the cellular destruction of corn stover after hydrolysis. The effects of microwave hydrolysis on corn stover can be analyzed by determining the particle size and specific area of corn stover, and further SEM analysis elucidate the enchantment mechanism of hydrolysis [51].

The molecular analysis of the mixed-culture system using a variety of feedstock identifies around 60–70% hydrogen producers from genus *Clostridium*, followed by 10–20% from *Bacillus* and 5–10% from *Enterobacter* species [49]. The molecular techniques used in the mixed-culture system help identify the alterations in the microbial community caused by changes in the pretreatment methods. Any changes to the microbial community will give rise to accumulation of non-hydrogen-producing microorganisms resulting in decreased hydrogen production.

## 6. Challenges for a Mixed-Culture System of Hydrogen Production

Hydrogen production using defined substrates, such as glucose, sucrose, starch, and cellulose is very successful. However, using the same fermentation conditions on complex organic wastes, such as sewage sludge, crude glycerol and wastewater involves several difficulties [41]. The composition of sludge or organic wastes is not always constant and keeps varying due to the different sources, differences in environmental conditions and differences in the collection points, creating a challenge to obtain consistent hydrogen-producing microorganism seed samples [41,46]. Variation in hydrogen production was observed due to difference in collection points of the sludge sample and also the hydrogen production decreased during the reviving of the stored seed inoculum [41,46]. Unlike pure-cultures, the mixed-culture seed inoculum can only be stored/preserved after carrying out the identification of microorganisms by molecular techniques as discussed in the above section. In addition, seed inoculum preparation by conventional techniques is complex, time-consuming and in the end, will deliver a small fraction of active inoculum. Improvements in techniques with dilution to extinction, enrichment by acid incubation, concentration to extinction, sludge baking and sludge acidification helped to decrease the methane production but not methanogenic bacteria [46]. Hydrogen-consuming bacteria consume the substrate as well as utilize the hydrogen formed as an electron, thus significantly reducing the yield of hydrogen [130,131]. Metabolic dexterity of the microbes can result in the initiation of unwanted metabolic pathways, which can reduce the rate of hydrogen production [12].

Hydrogen production using mixed-culture depends on several factors such as substrate concentration, pH, temperature, inoculum size/biomass concentration, nutritional requirements, media supplements and oxidation-reduction potential [45]. The initial pH affects the activity of the iron-containing hydrogenase enzyme, extends lag phase, affects the rate of biochemical processes and also affects the metabolic pathways during the fermentation of hydrogen production [40,48]. Acidic pH of 5–6 is optimum for hydrogen production, a decrease and increase from the optimum pH results in a metabolic shift with the production of volatile fatty acids (VFA) [58,132]. Similarly,

low biomass concentration can cause a delayed lag time with slow fermentation rates and in case of high concentration will have adverse effects on rates and yields of by-products [40]. Change in substrate concentration affects microorganisms community structure, metabolic pathway, and excessive substrate concentration lead to the production of volatile fatty acids [133]. A temperature change of 4 °C increments up or down from optimum resulted in a shift of hydrogen production to metabolic flux with the production of VFA and other gaseous products (CO<sub>2</sub> and methane) [52]. The decrease in the hydrogen yield can be attributed to a un-optimized conditions resulting in the unwanted reactions such as homoacetogenesis and methanogenesis [55]. In order to attain increased hydrogen production using the mixed-culture system, there is a need to optimize the above parameters. To obtain increased hydrogen production from cellulase using elephant dung, required the parameter optimization for initial pH (6, 7 and 8), temperature (45, 50, 55 and 60 °C) and cellulose concentration (0.1, 0.25, 0.5 and 0.75 *w/v*). A different set of experiments for each parameter increases the number of experimental runs and is time-consuming with the addition of individual parameters [48,104].

Use of organic wastes for a mixed-culture system serves as a low-cost substrate, however additional media supplements increases the medium cost during the hydrogen production [54]. Additional costs, such as buffer addition, use of endo-nutrients [134], in the presence of nutrient solutions [35], using minimal medium [119,135] and use of complex modified HM 100 medium [136] has to be taken into account to enrich the mixed-culture system for hydrogen production.

## 7. Advantages of Mixed-Culture for Hydrogen Production

Anaerobic digestion is complex in comparison to a mixed-culture system with different operational and performance variables (pH, temperature, salts, alkalinity), and there is a lack of information on the physical, chemical and biochemical interactions during the digestion [137]. The success of anaerobic digestion depends on the composition of solid substrate (20–30%), external addition of complex media and nitrogen source, release of toxic compounds (hydrogen sulfide production), accumulation of metabolites decreases methane yield and use of conventional digestion (with limited scope for technology improvements) has very less economic viability [8,137,138]. In comparison to anaerobic digestion for methane production, mixed-culture system of hydrogen production is efficient (able to reach substrate degradation efficiency of 97.2%) [26], efficiently eliminated hydrogen-consuming bacteria's [69,93], requires short hydraulic retention time (12 to 2 h without washout) [125] and able to produce high biogas recovery (pure hydrogen: 80–90%) [13]. The produced hydrogen from mixed-culture system releases large amounts of energy (143 MJ/kg) with 50% more efficient in comparison to using methane (50–55 MJ/kg) and gasoline (43–57 MJ/kg) [3]. The life cycle assessment (LCA) in the case of anaerobic digestion is best suited for the treatment options of total CO<sub>2</sub> and SO<sub>2</sub> [139]. However, the LCA in case of mixed-culture system delivers advantageous results for sustainable hydrogen production from organic wastes [138]. The strategy to use organic wastes needs the first step of the mixed-culture system of hydrogen production followed by the second step of anaerobic digestion to utilize the spent media to complete the assessment.

Mixed-culture is preferred over pure culture systems mainly due to its technical feasibility, minimum inoculum steps and ease of operation despite lower yield than in pure cultures. Pure-/co-cultures generate hydrogen with very high efficiency, minimum by-product formation [140] and can be easily manipulated to achieve the required operating conditions. However pure cultures being sensitive to contamination, require sterile conditions throughout the fermentation, which is not feasible for large scale production. In some studies, mixed-culture did not require an aseptic environment for the cultivation of bacteria nor for the final production process [23,30,38]. Mixed-culture also offers the possibility to work on a wide spectrum of low cost, easily available substrates to be utilized for hydrogen fermentation and simultaneously valorizes industrial organic wastes [141]. In addition, single microorganisms have less hydrolytic capacity when compared to a consortium of microbes. Mixed-cultures derived from sludge [62], compost [142] and soil [143] are capable of using various substrates, overcome inhibitor concentrations, carry out complex reactions and are more

effective in complete degradation of substrates. The metabolic flexibility of these microorganisms allows them to develop a synergistic metabolic process which can result in better substrate utilization and elimination of feedback inhibition [144,145]. Mixed microbial consortia capable of metabolic flexibility can survive harsh environmental conditions, operating mishaps [56,146] and offers an economical, continuous production at industrial scale [10,49].

In the mixed-culture systems, the sewage sludge from wastewater containing a diverse population of microorganisms, also rich in proteins and carbohydrate is used as seed-inoculum and substrate for hydrogen production. Heat-treatment eliminated methanogens, improved the growth rate of hydrogen producing species and also helped to break-down sludge structure to release intracellular simple structures. The technique of simultaneous saccharification and fermentation (SSF) accelerates and increased hydrogen production from 0.35 mL using raw sludge to 16.26 mL H<sub>2</sub>/g of heat-treated sludge [29]. The mixed-culture from elephant dung possessed cellulolytic bacteria to degrade cellulose into glucose and non-cellulolytic bacteria to degrade generated glucose into hydrogen production [48]. Elephant dung is rich in cellulolytic bacteria, as an elephant feeds mainly on plant materials which are rich in lignocellulosic materials. The elephant dung as seed inoculum for mixed-culture system serves advantage of cellulose degradation and hydrogen production [48]. The advantages of the mixed-culture system have drawn researchers to use abundant waste products, work on a variety of feedstocks and continuously produce hydrogen in the absence of any external energy source.

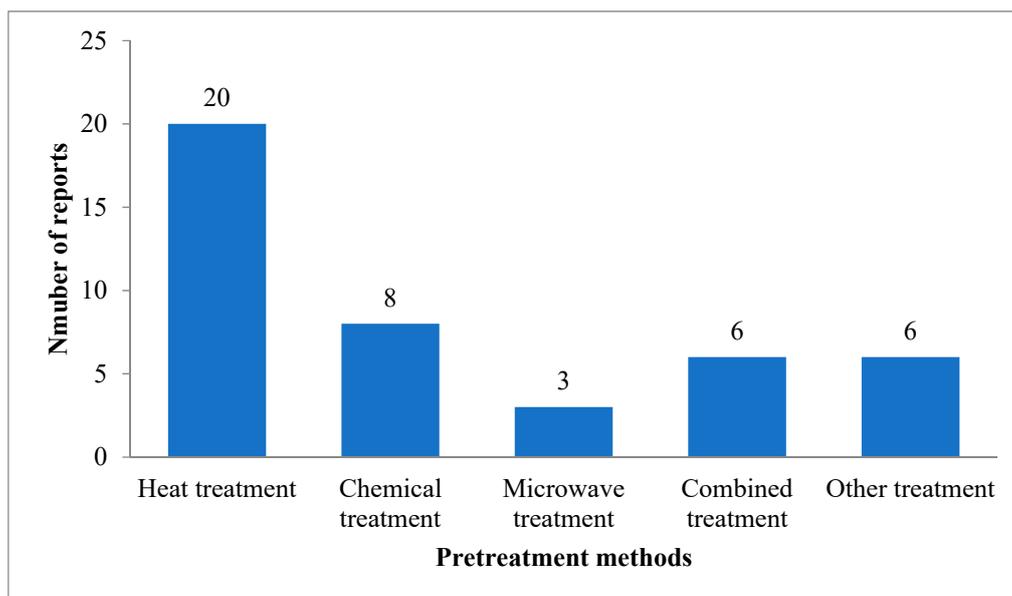
## 8. Approaches and Future Perspectives for Mixed-Culture in Hydrogen Production

Hydrogen anaerobic fermentation (HAF) has been extensively researched using pure cultures under sterile conditions [147,148], as well as mixed-cultures under non-sterile conditions [23,30,38]. Despite the fact pure cultures give much higher hydrogen yields, mixed-culture is preferred for its economical option due to its lower operating costs. The possibility of operational control is based on the differential kinetics of microbial subgroups and treatment of industrial organic wastes [32,141,149]. The synergistic effect of microorganisms during mixed-cultures are capable of forming desirable products at higher concentrations [100].

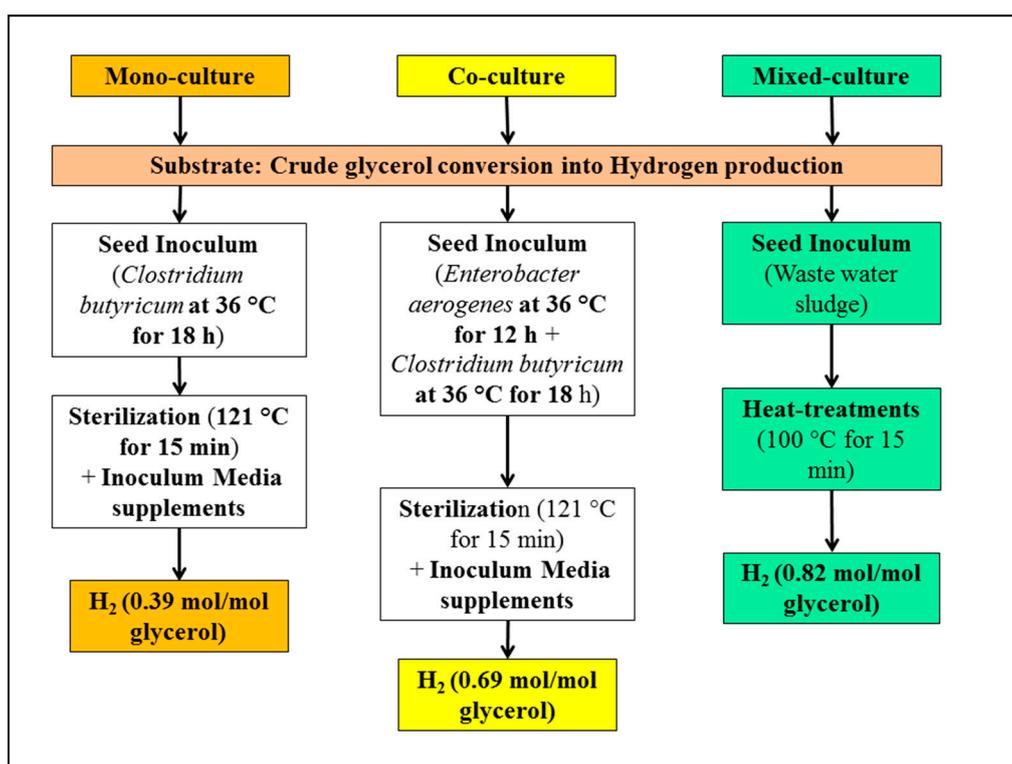
Of the various studies, the most recurrent seed inoculum employed is wastewater anaerobic sludge, with close to 10 reports, followed by municipal sewage sludge (used across four reports). Among the various organic matters used as seed inoculum for hydrogen production, wastewater sludge is preferred over the other inocula.

The choice of substrates for hydrogen production should come from non-food crops in order to avoid food insecurity. Organic wastes are promising substrates, abundant, low in cost, rich in polysaccharides and are easily hydrolyzed to produce fermentable sugars for hydrogen production [48]. Choice of the substrate is also particularly important, and a quick look at Figure 3 shows that the emphasis has been on organic wastes, such as dairy wastes, or primary sewage waste. Sludge is a large repository of different types of microorganisms and organic matter constitutes around 41% proteins, 25% lipid, and 14% carbohydrates [150,151]. Substrate pretreatment is thus a very common strategy to make them available for fermentation and to increase the hydrogen yield. Use of glucose, xylose, and sucrose also remain popular options, despite the high cost as they are established and efficient sources of carbon for the microorganisms.

Heat-pretreatment at 100 °C for 15–30 min proved effective in killing the methanogens and is able to maintain a high hydrogen yield irrespective of the nature of the seed inoculum and substrate. Figure 4 represents the frequency of the pretreatment methods used to select the hydrogen producing microorganisms. A common platform using crude glycerol (CG) was studied across mono-, co- and mixed-culture system for hydrogen production [11] as represented in Figure 5. The heat treatment can also be used in combination with other treatments, such as acid and BESA (2-bromoethane sulphonic acid) to obtain increased hydrogen yield.



**Figure 4.** The frequency of top few pretreatments methods used for selection of hydrogen-producing microorganisms.



**Figure 5.** Comparison of the mixed-culture system across mono- and co-culture system using a common substrate crude glycerol for hydrogen production, in reference to seed inoculum, sterilization time and cost of inoculum media supplements.

Therefore, it may be suggested that the technology that uses common primary sewage water as substrate and anaerobic wastewater sludge as seed inocula, coupled with heat-treatment at 100 °C for 15–30 min reported a higher yield of hydrogen. In addition to hydrogen production, the fermentation steps in mixed-culture are fewer in number in comparison to mono- and co-culture systems. In the case of a mixed-culture system, the inoculum media sterilization (121 °C for 4 h), incubation step of

microorganism growth (18–36 h) and inoculum media supplements addition cost (Canadian dollars \$4–6 for bioconversion of 1 kg CG into hydrogen) in the case of pure- and co-culture can be eliminated. Thus, by eliminating the incubation step, around 10–15 MJ of energy for a single run can be saved by carrying out a mixed-culture system of hydrogen production [152]. Studies involving photo fermentation using mixed substrate yielded high hydrogen production efficiency with propionate and butyrate as substrate. Reports indicate that photo-fermentation by mixed bacteria using these organic acids as substrates is likely to further enhance substrate conversion efficiency on large scale. In another approach, the spent media from mixed-culture system supplemented with fresh media can be used for hydrogen and lipid production using the closed system as discussed in [153].

The mixed-culture uses the interactions of microbes to widen the range of substrates and enhance the product conversion efficiency for higher yield. Presently, though the microbial fermentative hydrogen production is low, the scope of mixed-culture in the future is vast with a potential decrease in fermentation steps and media components cost and thus may replace pure culture systems in the future.

## 9. Conclusions

The study demonstrated that the different seeding inocula, ranging from mountain soil, elephant dung to wastewater, require a variety of treatment methods to utilize different substrate feedstocks ranging from pure to synthetic to organic substrates for hydrogen production. The pretreatment methods alter the composition of the microbial community; help to determine the efficiency of substrate utilization and increase the efficiency of hydrogen production. The use of different seed inocula and random selection of pretreatment method may not deliver the required hydrogen yield, and successful pretreatment methods deliver potent mixed-cultures that can increase the percentage of hydrogen while avoiding the formation of methane. The mixed-culture system of hydrogen production removes the inoculation time and saves on the input of electricity with energy savings of around 10–15 MJ/run. The mixed-culture system combines highly efficient waste reduction with continuous bioenergy production, together contributing to a clean environment and reducing the dependence on fossil fuels.

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