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Factors that affect bacterial ecology in hydrogen-producing anaerobic reactors

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Abstract Hydrogen has been studied as an alternative to traditional energy sources; it is a clean and renewable fuel that on combustion generates only water as a by-product. Biological production of hydrogen can occur either via photosynthesis or fermentation. The latter is technically simple and can convert substrates like organic matter present in wastewater into a renewable energy source. Microorganisms belonging to the domains Archaea and Bacteria are responsible for the conversion of various carbon sources to biogas, including hydrogen and methane. It is important to determine the microorganisms responsible for such transformations, as they are the major players of the process. Studying the bacterial diversity, population structure, and processes that modify these communities leads to a better understanding of their ecological functions and productivity. The environmental conditions within an anaerobic hydrogen reactor can exert a selective pressure on the community, thereby affecting the population structure, diversity, and heterogeneity. Combination of appropriate operational parameters and ecological factors could lead to the development of effective bioprocesses to maximize hydrogen yield. Therefore, the objective of this paper is to present a review on bacterial ecology in anaerobic hydrogen reactors and the factors that can affect bacterial diversity.

Keywords Bacteria · Dark fermentation · Bacterial diversity · Renewable energy

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Introduction

Majority of the energy that is currently produced and consumed worldwide comes from non-renewable sources such as oil, gas, or coal with increasing contributions from renewable sources [1–3]. The intensification of industrial and technological development has encouraged the expansion of renewable energy sources to gradually replace fossil fuels along with increasing discussions on climate change due to concerns regarding global emissions, reduction of oil and gas reserves around the world, and the difficulty of finding and accessing new oil sources in deeper layers [1, 4].

Hydrogen, a clean and renewable fuel, has been studied as a possible alternative to traditional energy sources because on combustion it only generates water as a by-product and presents more energetic capabilities than fossil fuels [5]. It is 50 % more efficient than gasoline, and its abundance is 2.75-fold greater when compared with hydrocarbon fuel sources [6]. Hydrogen has a high-energy content per unit of weight (142 kJ/g) and no greenhouse gases are produced as a result of combustion, making it an environmentally friendly alternative to fossil fuels [7].

Hydrogen can be produced through biological and physicochemical methods [8, 9]. Biological production of hydrogen is a low-cost technology that requires low energy for the process of gas generation and occurs mainly via three processes [9]: photosynthesis, photofermentation, and dark fermentation. Both photofermentation and dark fermentation are

technically simpler processes that can convert substrates like organic matter present in wastewater into a renewable energy source. However, to achieve this goal, it is necessary to understand the ecology and bacterial community functions to refine the biological processes and improve the biotechnological applications such as the treatment of wastewater, anaerobic digestion of organic co-products, and the production of biogas [10, 11].

The study of ecological interactions in anaerobic reactors can provide information regarding how the bacterial community develops, changes, and degrades the substrate along the stages of reactor operation. Changes in the reactor functioning can be associated with shifts in the genetic pool of bacterial communities. The development and stability of bacterial activity are linked to the efficiency of anaerobic hydrogen reactors [12]. Therefore, a better understanding of the factors that affect the diversity and bacterial ecology in anaerobic hydrogen reactors could lead to increased efficiency of anaerobic treatments and biogas production.

Interactions between hydrogen- and non-hydrogen-producing microorganisms

The structure of bacterial communities can be manipulated to achieve specific goals such as hydrogen production, which requires the appropriate design and operation of bioreactors. Anaerobic digestion is a four-stage process divided into hydrolysis, acidogenesis, acetogenesis, and methanogenesis. Hydrogen is produced in both the second and third steps and is consumed at the fourth step when methanogenic archaea use carbon dioxide and hydrogen to produce methane [13].

Methanogens are the main consumers of hydrogen in anaerobic environments [14]. To achieve hydrogen production through the final step of this method, methanogenesis must be avoided in order to prevent hydrogen consumption. To inhibit methanogenic activity, it is necessary to control certain operational parameters such as pH [15], organic loading rate (OLR) [16], and pre-treatment of the inoculum [10, 17]. Another option to inhibit methanogenesis is by using chemicals like 2-bromoethanesulfonic acid (BESA) or chloroform [17]. A few studies indicate that BESA is not able to eliminate hydrogen consumers [18, 19]; additionally, BESA can reduce *Clostridia* diversity [20] and inhibit hydrogen production [18]. Furthermore, these treatments are not environmentally friendly and are too costly for large-scale operations [21].

Other groups of non-hydrogen-producing microorganisms that play a strong role in anaerobic production of hydrogen are homoacetogenic bacteria [22], sulfate-reducing bacteria (SRB) [23], and lactic acid bacteria (LAB) [24].

Homoacetogenic bacteria are strictly anaerobic microorganisms that harbor special enzymes with the ability to catalyze the formation of acetyl-CoA, which subsequently converts acetate from hydrogen and carbon dioxide [22], consuming hydrogen in the process. Siriwongrungson et al. [25] operated a Continuous Stirred Tank Reactor CSTR under thermophilic temperatures (between 45 and 60 °C), using digested dairy manure as the inoculum, and reported that almost no hydrogen was produced from the oxidation of butyrate, indicating that the hydrogen produced from butyrate was consumed in a subsequent step. They found that the hydrogen produced from butyrate degradation promptly reacted with carbon dioxide to form acetate by homoacetogenesis.

The SRB consume hydrogen as they use sulfate as a terminal electron acceptor. This group of bacteria consumes hydrogen at a fast rate in the presence of sulfate or nitrate, even under low hydrogen concentrations [23]. The SRB are known competitors of acetogens and methanogens in anaerobic digestion for a variety of substrates such as propionate, butyrate, ethanol, and acetate [26].

The LAB are known to inhibit hydrogen production through the secretion of bacteriocins, antibiotic polypeptides [24] that inhibit *Clostridia*, thus affecting hydrogen production. Noike et al. [24] studied the inhibition of hydrogen production by LAB and observed that hydrogen fermentation was replaced by lactic acid fermentation when two LAB strains were cultivated together with two hydrogen-producing strains. Under mesophilic conditions, LAB growth increased and the accumulation of lactic acid led to instability in the fermentation process. Wang and Zhao [27] operated a continuous system using food waste as substrate and observed that LAB promoted a decrease in hydrogen yields, from 71 to 49 mL $H_2\,g^{-1}$ VS, while lactic acid increased from 2.3 to 4.4 g L^{-1}. Furthermore, an increase in OLR favored LAB indigenous to the inoculum, which increased lactic acid concentrations and led to instability of the system.

Some authors have observed cooperation between species, such as facilitation [28], an ecological interaction in which at least one species benefits, causing no harm to any other participant of the relationship [29]. In anaerobic digestion, facilitation can cause a positive impact on hydrogen production. For example, *Klebsiella* sp. can consume low levels of oxygen in the environment thus, favoring the growth of strict or facultative anaerobes such as *Clostridium* species that produce hydrogen [28]. The same interaction was noted by Huang et al. [30]; during the lag phase, the dominant genus was *Bacillus* sp., a facultative anaerobe. Such dominance can be attributed to the fact that the authors did not sparge oxygen with nitrogen in the beginning of the experiment. As the community became established, the species of this genus consumed the remaining oxygen within the reactor. This allowed some strict anaerobes, such *Clostridium beijerinckii* and *Clostridum perfringens*, to become the newly dominant species during the exponential phase and after the steady-state was reached. According to the authors, this change in the microbiota ultimately resulted in increased hydrogen production.

Bacterial diversity and stability in anaerobic hydrogenogenic reactors

Changes to the operational conditions of the reactor can promote changes in the bacterial community structure because it affects the anaerobic process and dominance between the established species [31, 32]. After a disturbance, such as significant changes in one or more operational parameters, there will be a period where the microorganisms will readapt until a new community with a different organization from the previous stage is fully established. At that point, the reactor reaches the steady-state stage. This system's steady state is much simpler than what is described in Ecology as the "climax" community, a point of maximum biomass and development. However, for this set of conditions, including the functional stability promoted by a stable community, the steady state can be considered analog to the ecological climax state in a system [33, 34].

Some species that are inoculated into the reactor may disappear and previously undetected species can arise [35]. Because different species have different metabolic responses, each particular adaptation to the environment promotes different ecological interactions such as competition [35] and/or facilitation [35]. Therefore, the start-up period in a reactor, together with the operating conditions, will establish a new climax community based on the genetic pool of the various microbial species found in the different types of inocula. Some of these species can be used for the production of hydrogen by anaerobic fermentation [36, 37].

The ecological interactions can directly affect stability and/or function. Koskinen et al. [38] monitored bacterial community dynamics inside a dark fermentation fluidized-bed bioreactor to identify the cause of the instability in hydrogen production. The authors concluded that the instability in the production was due to changes in the microbial community structure, which were caused by rapid enrichment. This led to a change in the bacterial community structure and its metabolism from acetate–butyrate to acetate–propionate production, consequently resulting in a decrease in hydrogen production.

In dark fermentation, pyruvate can be converted to formate [39], which in turn can be converted to hydrogen and carbon dioxide by some hydrogen-producing bacteria such as *Escherichia coli* and *Enterobacter aerogenes*. Although hydrogen was neither produced nor consumed by *Desulfovibrio desulfuricans* in this study, it is known that this species can ferment pyruvate in the absence of sulfate or nitrate [40], thus becoming a competitor in hydrogen evolution.

The results of Koskinen et al. [38] detail a community with increasing diversity, along with environmental changes. These findings bring an important question to light; what is the relationship between bacterial diversity and ecosystem stability? This subject has very divergent approaches in bacterial ecology, because the stability in a system can limit the capacity of a diversity change by minimizing the possible alterations in the established community through resistance or resilience and functional redundancy [41].

Resistance is defined as the ability to withstand perturbation, expressed as the degree to which the system (structure or characteristics) remains unchanged when affected by a disturbance [42]. Resilience is defined as the ability to recover after the perturbation, expressed as the rate at which the system returns to its original state after a disturbance [42]. In hydrogen reactors, resilience plays a major role in productivity, especially in mixed cultures. The nature of the microbial communities to function synergistically increases the resilience, when compared with pure cultures, recovering hydrogen producers after significant changes in environmental conditions [43]. Functional redundancy implies that some members can act as "substitutes" for other members' functions in the community; this is expressed as the ability to carry out a biological process at the same rate as another taxon, if the same environmental conditions are applied. Thus, the ecosystem functionality and process rates are not altered despite the changes to the population structure [42]. The *Clostridia* genus, which contains many hydrogen-producing species, relies on redundancy to maintain the overall community function in anaerobic reactors [44]. Furthermore, resilience plays an important role in maintaining stable *Clostridium* populations in these conditions, as observed by Werner et al. [44], who monitored digester performance coupled to microbial community composition.

Resistance and resilience play a role in diversity and stability, the greater range of species that are able to respond differently to diverse environmental perturbations (either by resisting the disturbance or being able to recover from it), the more likely the ecosystem will stabilize in response to the applied disturbance [45]. However, even if the community can be recovered, the system function can be highly affected, thus altering the original function. The hydrogen reactor operated by Koskinen et al. [38] did not recover hydrogen production, while the bacterial community diversity increased after the disturbance, resulting in significant changes in the bacterial community. The community showed low resilience, it recovered slowly and not to the previous structure. It also had an average resistance, since the disturbances did not have to be intense in order to disrupt the community structure, and no detectable functional redundancy, the function was altered despite the recovered community. The community showed a very unspecific recovery, and after the reactor configuration changed, the production was momentarily reestablished, decreasing afterwards. Both the structure changes and the function decrease must be considered together with the ecological aspects: no functional redundancy and low resilience of the community promoted high instability, despite recovery.

Several studies agree that a large number of species can sustain functioning ecosystems [46, 47], which are based on two main components [46]: selection, based in individual

differences (e.g., metabolism, morphology) between species; and complementarity, which states that species discriminate between resources (niche diversification occurs). Based on probability, the richer a community is, the more likely this community contains one or more species that represents a significant effect on ecosystem functioning; thus, it can become more productive, because the range of resources they are able to use is larger and the system resilience tends to be higher [46, 47]. Some studies observed that thermophilic communities presented higher resilience and productivity when diversity was higher. Furthermore, the communities were able to recover growth and hydrogen production faster when compared with other temperature ranges, because of its higher resilience [48–50].

Xing et al. [51] operated a hydrogenogenic reactor and observed that the diversity quickly increased and then gradually decreased. It can be inferred that the community shifts gradually selecting the most productive and stable communities, and that the hydrogen production increased while diversity decreased. Koskinen et al. [38] demonstrated that a community increased in diversity during the fermentation process; however, they also demonstrated increased instability and decreased hydrogen production. The inoculum used by Koskinen et al. [38] was enriched in a series of batch incubations, in order to select the desirable species of hydrogen producers. The changes in diversity were due to new microorganisms that were gradually enriched, others that were likely already present in the inoculum, and some that may have entered with the unsterilized feed. Despite the selection of the most productive communities in the inoculum, the increasing presence of competitors changed the diversity and the function of the system. Thus, the higher the diversity, the more likely the system will be stable; however, if the initial diversity of the inoculum is lower than the final diversity, it indicates the growth of previously undetected species, or allochthonous microorganisms that further competed with or inhibited hydrogen producers, which possibly lead to system instability.

Molecular techniques for the characterization of mixed cultures in hydrogen reactors

Cultivation techniques, despite their value in microbiology, are very limited, especially considering that only a small fraction of the bacterial diversity can be cultivated. Although these techniques can be successfully applied in certain situations to study the microbial diversity and ecosystem functioning [51], it remains a poor solution because the richness can only be manipulated at low levels. Therefore, identification and assessment tools that do not require cultivation have received attention as a possible strategy to acknowledge the microbiological diversity in an uncultured environment [52].

Major fingerprinting techniques that have been used to characterize bacterial communities in hydrogen production fermentation processes include denaturing gradient gel electrophoresis (DGGE) [53] and terminal restriction fragment length polymorphism analysis (TRFLP) [54]. These techniques are used to investigate structure and characteristics of microbial communities such as differences or changes in diversity and temporal changes in structure [54–58]. A major quantitative technique is fluorescent in situ hybridization (FISH), a polymerase chain reaction (PCR)-independent technique that allows fast identification and quantification of bacterial cells by hybridizing target 16S rRNA molecules with fluorescently labeled oligonucleotide probes [59–61].

In hydrogen production, these techniques play a significant role because they can be used to analyze the inoculum, to evaluate the effects of the applied pre-treatment to determine the best pre-treatment used for each inoculum and carbon source, and to monitor the community structure along the reactor operation [52, 53, 58–62]. Thus, during a hydrogen reactor operation, it is possible to rapidly determine variations in different samples by investigating the effects of the operational parameters on the hydrogen producing community, providing a reliable strategy to analyze and predict system disturbances [52, 53, 58–62].

Operational parameters that affect the community structure in hydrogenogenic reactors

Anaerobic digestion occurs in four distinct stages: hydrolysis, acidogenesis, acetogenesis, and methanogenesis. Hydrogen consumption occurs in the third stage by homoacetogenic bacteria that produce acetate and in the fourth stage by hydrogenotrophic methanogenic archaea that produce methane [13]. Some operational parameters are designed to avoid hydrogen consumption, mainly by inhibition of methanogenesis, thus affecting the original bacterial community structure.

Several factors can affect bacterial diversity in an anaerobic hydrogenogenic reactor (Table 1), including pH [51], OLR [16, 64], carbon source [63], inoculum source [37], and pretreatment of the inoculum [10]. Furthermore, to understand the causes of unstable operations, the relationship between stability and diversity in anaerobic reactors was investigated. The stability of a bacterial community depends on its structure, which can change due to environmental disturbances such as changes in operational conditions or ecological interactions [42] such as competition.

OLR, pH and temperature

Some operational parameters, such as pH and OLR, can be changed to increase hydrogen yields and/or production. Mariakakis et al. [16] noticed shifts in the bacterial community structure after increasing the OLR to up to 34 kg COD m^{-3} day^{-1}. They observed that the dominant population

Table 1 Main factors affecting microbial diversity in anaerobic hydrogen reactors

Reference	Factors affecting dynamic	Carbon source	Inoculum	Inoculum source	Dominant genera	Pre-treatment	pH
Temudo et al. [63]	Carbon source, pH	Glucose, glycerol, xylose	*Clostridium*, *Klebsiella*, *Pectinatus*, *Enterobacter*, *Bacteroides*, uncultured bacteria	Sludge from a distillery wastewater treatment plant and sludge from a potato starch processing acidification tank	*Clostridium*, *Klebsiella*	N/A	4.0–8.5
Xing et al. [51]	OLR, pH	Molasses from a beet sugar refinery	*Clostridium*, *Acidovorax*, *Kluyvera*	Silt of domestic sewage drainage	*Clostridium*, *Ethanologenbacterium*, *Acidovorax*, *Kluyvera*, *Bacteroides*, *Spirochaetes*	N/A	4.0–4.5
Mariakakis et al. [16]	OLR	Sucrose	*Bacteriodetes*, *Firmicutes*, *Tetrasphaera*, *Olsenella*, *Clostridium*	Anaerobic digester of a sewage treatment plant	*Ethanoligenes*, *Prevotella*, *Selonomonas*	N/A	5.5
Ferraz Júnior et al. [64]	OLR	Raw sugarcane vinasse	*Lactobacillus*, *Megasphaera*	Fermentation of vinasse (autochthonous microorganism growth)	*Megasphaera*, *Sutterella*, *Lactobacillus*, *Thermoanaerobacterium*, *Clostridium*	N/A	6.5–5.5
Pendyala et al. [37]	Pre-treatment, inocula source	Glucose	Granular sludge: *Clostridium*, *Bacillus*, *Enterococcus*, *Bacteroides*, *Eubacterium*, *methanogens* (*Methylophilus*) Flocculent sludge: *Clostridium*, *Bacillus*, *Enterococcus*, *Propionobacterium*, *Brevibacillus*, *Bacteroides*, *Lactobacillus*	Granular and flocculent sludge from wastewater facilities treating industrial and municipal effluents	Granular: *Clostridium*, *Enterococcus*, *Bacteroides* (heat, loading shock, LA, BESA, acid) Flocculent: *Clostridium*, *Enterococcus* (heat, loading shock, LA, BESA, acid) Granular/ flocculent: *Clostridum*, *Enterococcus*, *Bacillus*, *Clostridium*, *Fusobacterium* (alkali)	Heat, shock loading, acid, alkali, linoleic acid, 2-bromoethane sulphonic acid (BESA)	6.0
Maintinguer et al. [10]	Pre-treatment, inocula source	Xylose	(Phyla) *Proteobacteria*, *Firmicutes*, *Chloroflexi*, *Actinobacteria*, *Cyanobacteria*, *Fusobacteria*, *Deferribacteres*, uncultured bacteria	Sediment taken from reservoir	*Firmicutes*	Heat shock	5.5
Koskinen et al. [38]	Stability	Glucose	*Clostridium*, *Desulfovibrio*, *Escherichia*, *Schwartzia*, *Acidaminococcus*, *Anaerofilum*	Anaerobic digester treating municipal wastewater sludge	*Clostridium*, *Escherichia*, *Desulfovibrio*, *Megasphaera*, *Bacteroidetes*, *Lachnospiraceae*	N/A	6.0–4.6

N/A not applicable

during the start-up period consisted of homoacetogenic bacteria, which were subsequently replaced by acidogenic species belonging to the *Selenomonas* genus at the steady state, with minor presence of *Ethanoligenes* and *Prevotella* being detected. The dominance of the acidogenic over the homoacetogenic species likely occurred due to an accumulation of by-products generated by the former group, which inhibited the latter. Additionally, an increase in OLR increases the amount of organic matter, favoring the development of acidogenesis, which provides an energetic advantage when compared with that of the homoacetogens. Moreover, species from the *Clostridium* genus were predominant when the OLR reached 22 kg COD m^{-3} day^{-1}, but they were no longer observed when the OLR was further increased. The species used in this study were probably strains sensitive to substrate concentration, where an OLR increase resulted in inhibition.

Mariakakis et al. [16] also observed that along the reactor operation, the number of detected *Clostridium* spp. dramatically decreased during phases 5 and 6, the most productive stages in terms of hydrogen production. Subsequently, the number of detected *Clostridium* spp. increased again in phase 8, defined by poor reactor performance. When the number of species further increased in phase 9, hydrogen production ceased. These results imply that the amount of bacterial species adversely influenced reactor performance, an effect that was also suggested by Koskinen et al. [38], Hafez et al. [65], and Kim et al. [66]. These results showed higher microbial diversities with increasing OLR, due to higher substrate availability.

The pH is an environmental factor that is crucial to anaerobic microorganisms due to its effects on hydrogenase enzymes; it is essential for the growth of hydrogen-producing bacteria and, consequently, on metabolic pathways and bacterial community structure [67]. Hydrogenase enzymes can be regulated by changes in extracellular pH [68]; this affects hydrogen production by altering the activity of the enzyme through a reduction in the amino acid potential at the active sites [69]. Substrate hydrolysis is also affected by changes in the external pH. Membrane-bound pumps extrude protons from the cell producing a gradient that allows for solute translocation [70]. This affects the hydrolysis of carbon sources and nutrient influx, which occurs by a pH gradient across the membrane [68]. Thus, pH can directly affect the reaction rate of hydrogen production, the resource management of the bacterial community, and the survival of the most adapted microorganisms. The pH is also a factor that prevents methanogenic activity since the range in which most methanogens can grow is very limited (pH 6–8) [71].

Xing et al. [51] assessed the effects of both OLR and pH changes on the bacterial structure within a hydrogenogenic reactor and noted that the diversity increased on the first weeks of the experiment, reached its highest level, and then gradually decreased. This may have occurred due to increases in the OLR, which consequently increased the amount of organic matter, changing nutrient availability and ecological niches. These changes favor the development of acidogenesis, favoring the acidogenic population. The adapted species belonging to the *Clostridium*, *Acidovorax*, and *Kluyvera* genera were dominant and prevailed over other species; therefore, these community shifts could be explained by competition between the species. Both studies [16, 51] show that diversity inside a reactor can constantly shift due to population changes through competition for resources; this is also indicated by changes in their metabolic by-products.

Liu, Chan, and Fang [72] studied the start-up period of two acidogenic reactors. They monitored the microbial community dynamics and found that when the pH decreased, the communities of both Bacteria and Archaea domains changed, followed by a decrease in methane formation and an increase in hydrogen and volatile acid production. Further analysis showed that the bacterial population in the acidogenic reactor increased from 63.1 to 90.3 %, while the archaeal population decreased from 34.1 to 4.3 %, within the first 13 days. The study revealed that it is possible to establish a suitable microbial population in the acidogenic reactors in less than 2 weeks, but in order to obtain stable metabolic activity, a longer period (up to 71 days) is necessary.

The operation temperature of the reactor also affects the microbiota and, therefore, hydrogen production. Hydrogen can be produced at two major temperature ranges: in mesophilic conditions, between 20 °C and 45 °C [89, 90], and in thermophilic conditions, between 45 °C and 60 °C [91, 92]. Some bacteria have high activity under mesophilic conditions, such as *Bacillus coagulans* and *Clostridium acetobutylicum* [93, 94]. However, some thermophilic bacteria have even higher hydrogenogenic activity, as can be seen on the most common thermophilic hydrogen-producing species, which belong to the *Thermoanaerobacterium*, *Thermotoga*, *Thermoanaerobacter*, and *Caldoanaerobacter* genera [95]. This characteristic is due to the [Fe]hydrogenase enzyme [96]; the hydrogen production process is dependent on this enzyme, which is directly affected by temperature. Several studies have tested a wide range of temperatures for hydrogen production, between 25 °C and 75 °C [75, 97–100]. The highest hydrogen yield (2.73 mol hydrogen/mol substrate) was found under thermophilic conditions (75 °C) [75], showing that this enzyme is more efficient at thermophilic, rather than mesophilic conditions.

Inocula

In hydrogen bioreactors, it is possible to use both pure [73–75] and mixed cultures [76, 77]. Viable hydrogen yields can be obtained using pure cultures. Ngo et al. [75] achieved up to 2.73 mol hydrogen/mol substrate by utilizing glycerol as the carbon source and *Thermotoga neapolitana* as the selected

thermophilic hydrogen-producing species. On the other hand, the use of a community eliminates the need for isolation or purification of any particular strain, which reduces the costs and complexity in full-scale reactors [78].

Many of the hydrogen-producing microorganisms belong to the *Clostridium* genus, which are strict anaerobes and spore-forming bacteria [79–81], *Enterobacter* [82–84], and other phylogenetically related microorganisms. Some of these microorganisms can be found in different sources including wastewater treatment systems [85–87], rumen fluid [88], and sediment [10].

Maintinguer et al. [10] studied the diversity of anaerobic bacteria in the sediment of a reservoir to evaluate the application of this inoculum in biohydrogen production. They observed a highly diverse source of microorganisms belonging to many phyla such as *Proteobacteria*, *Firmicutes*, *Chloroflexi*, *Actinobacteria*, *Cyanobacteria*, *Fusobacteria*, *Deferribacteres*, and uncultured bacteria. The dominant phylum in the final stage was *Firmicutes* and the production of hydrogen increased, confirming the efficiency of this community in hydrogen production. In this study, the community likely involves members with probable functional redundancy and facilitation.

Pre-treatments

Some species need to be eliminated or inhibited in order to induce the community to select the desirable genera. A pre-treatment may be required to eliminate certain potential hydrogen consumers like methanogens [17, 101], which are also present in this environment. However, if inappropriately applied, pre-treatment of the seed sludge can also suppress the activity of hydrogen-producing bacteria [102].

Some of the hydrogen-producing bacteria, except methanogens and some homoacetogens [21], are able to sporulate, a natural process that occurs when these microorganisms are in adverse conditions [103, 104]. Bacteria that can produce hydrogen during fermentation of glucose are mainly *Clostridium* and *Enterobacter*. As previously stated, *Clostridium* can form protective spores when they are under harsh conditions like heat shock and pH pre-treatments, which are the most commonly used conditions to eliminate non-spore-forming microorganisms that do not survive these processes [103, 104]. However, *Enterobacter* are not spore-forming, despite being hydrogen producers, which implies that many of these non-spore hydrogen producers will likely be destroyed after the pre-treatment, possibly affecting hydrogen production [104].

Clostridium and *Enterobacter* comprehend strict and facultative bacteria. The latter can also survive in the presence of low oxygen levels [21, 102], while the homoacetogens are strict anaerobes, the presence of oxygen causes them to die. The aeration parameters vary [102, 104, 105], resulting in different hydrogen production yields due to different aeration times. Therefore, an appropriate aeration pre-treatment could ensure hydrogen-producing diversity while avoiding homoacetogens by raising the oxidation–reduction potential [104].

Another common type is a chemical pre-treatment that uses specific inhibitors such as chloroform [106], nitrapyrin [107], or BESA [108], to prevent the proliferation of methanogens. These chemicals are competitive inhibitors of the coenzyme M-reductase, causing inhibition of the enzymatic activity that catalyzes the final step in the formation of methane, thus blocking the methanogenesis that is essential to their metabolism [109–111].

There are other methods of sludge pre-treatment such as hydraulic or organic shock loading and heating [105], acid/alkali, and freezing/thawing [17]. Pendyala et al. [37] studied mixed anaerobic cultures under the influence of various pre-treatments (heat, shock loading, acid, alkali, linoleic acid, and BESA). The authors claimed that thermal pre-treatment was the most efficient and that it also increased the diversity of hydrogen-producing bacteria, with dominance of the *Clostridium* genus. The thermal pre-treatment stimulated spore production and, therefore, promoted an increase in the diversity index, specifically for hydrogen-producing bacteria. However, even by eliminating methanogens, hydrogen consumption can persist because homoacetogenic bacteria can consume hydrogen. Heat or acid pre-treatments induce the formation of spores by some hydrogen-producing bacteria [17]; thus, these treatments may not be enough to improve hydrogen production because some of the homoacetogenic bacteria are also spore-forming and some hydrogen-producers are not spore-forming. Furthermore, sulfate-reducing bacteria are hydrogen consuming and can tolerate high temperatures; therefore, this treatment is not effective.

Using a different approach, Ning et al. [112] was able to inhibit methanogenic activity and obtain a stable hydrogen production using inocula treated with chloroform at different concentrations. The authors observed that the species changed as the chloroform concentration increased, promoting the selective inhibition of methanogens. Additionally, the appropriate concentration of chloroform was determined to enhance anaerobic hydrogen by 0.050 %.

Carbon source

The carbon source directly affects the bacterial dynamics in a community. Temudo et al. [63] investigated how different carbon sources (glucose, glycerol, and xylose) affected the bacterial community structure. The authors found that the use of glycerol resulted in increased bacterial diversity when compared with the inoculum, which was withdrawn from a distillery wastewater treatment plant. Furthermore, after reaching a steady-state/climax condition, the observed

dominant species were *Clostridium intestinale* and *Klebsiella oxytoca*, both of which are able to convert glycerol into hydrogen [113].

The carbon source can also be changed during the operation, as determined by Jo et al. [114]. These authors observed changes to the bacterial community in a hydrogenogenic reactor where the *Clostridium* genus predominated, which was caused by a change to the initial substrate, food waste. Afterwards, the carbon source was changed to fermented vegetable waste, causing rapid growth of lactic acid bacteria. Additionally, a decrease in hydrogen-production due to an accumulation of lactic acid was observed. The dominance shifted, with the *Lactobacillus* genus dominating in this new stage. These were probably allochthonous species that entered the system along with the unsterilized influent. Incoming microorganisms from the carbon source can influence the community composition in a reactor if it has not been sterilized or pre-treated to avoid allochthonous microorganisms.

Bacterial ecology and system functioning of hydrogen reactors

Bacterial communities are vital for the adequate functioning of all ecosystems, including those of artificial origin, which emphasizes the need to understand bacterial processes and interactions [115]. A lab-scale anaerobic reactor is a controlled system; thus, it is a more manageable system for studying processes than a full-scale ecosystem. This system allows for an in-depth study of bacterial diversity, population structure, and the processes that modify these communities.

To improve hydrogen production and overcome the possibility of instability, the ecological processes must be investigated using methods capable of detecting and identifying microorganisms that exist in the community of a dark fermentation reactor. Some of these microorganisms have unclear ecological or productive roles, because they do not directly interact with the substrate nor produce hydrogen, but interact with the producing microorganisms [14, 25].

The stability and productivity of a diverse bacterial community depends on other species and on operational parameters, which contribute to community promoting interactions and functional characteristics that are important at every stage of the reactor operation, even if these secondary species are not directly related to the production. Ecological interactions such as competition and/or facilitation between bacterial populations can favor or hinder certain bacterial groups so that hydrogen-producing bacteria can act cooperatively with non-hydrogen-producing bacteria in the final stable community. Allochthonous or indigenous microorganisms could compete for the available resources with the hydrogen producers and other co-existing genera that provide beneficial interactions with the hydrogenogenic microorganisms. The operational parameters or the addition of specific inhibitors can be used to prevent the possible proliferation of undesired microorganism genera [14].

As previously stated, the relationship between community diversity and ecosystem stability is still a matter of debate. Stability directly refers to the ability of the ecosystem to minimize fluctuations through resistance or resilience, defying or avoiding changes after disturbances [25]. Resistance and resilience can be specially noted on environments with high functional redundancy states, which by definition are highly diverse communities with different microorganisms capable of maintaining some of the system's function [42].

Diversity alone cannot explain function stability since system stability is the outcome of functional redundancy, resistance, and resilience but is a strong indicative of an environment with higher probability of developing a successful community, depending on the disturbances applied to it [25, 42, 51]. A highly diverse bacterial community is more likely to possess higher functional redundancy, in which case, it could confer functional resilience to the community, even in major disturbances, maintaining a stable and functional community [42].

The point at which the community structure changes is also unclear. The community diversity must be at least partially sensitive (not highly resistant) to disturbances, not highly resilient, and the microorganisms have to be functionally dissimilar in order for changes to occur in the community, thus allowing the community to change [67].

The operating parameters in an anaerobic reactor can act as selective pressure on the community affecting population structure, diversity, and heterogeneity, as seen by Koskinen et al. [38]. The performance of bioreactors depends on the bacterial activity in the system. Thus, understanding bacterial community structures could lead to higher hydrogen yields through selection of the most adequate genera by manipulation of the environmental conditions imposed to the community. Combining operational parameters with ecological factors could lead to maximizing the development of effective bioprocesses by assessing the differences and synergies of bacterial ecology [51, 116].

Conclusion

A successful operation in a hydrogen bioreactor can be achieved through the correct control of the operational parameters. The community's structure in mixed cultures is influenced by incoming microorganisms, by operating conditions, and by interactions among microorganisms. If the hydrogen production is based on an unsterilized carbon source, the inoculum has to be properly analyzed so the diversity shifts during reactor operation, from start-up to steady-state, may become more comprehensible. Utilizing the interplay between ecological factors and operational parameters to induce hydrogen production might result in a stable community with partial

selection to hydrogen-producing bacteria to increase hydrogen yields.

Pre-treatments of the inoculum are widely used in improving hydrogen production; however, there is no consensus on the treatment that best selects for hydrogen-producing microorganisms. Negative and positive interactions between hydrogen-producing and other microorganisms must be considered when choosing the inoculum and the form of pre-treatment that will be applied because it will have great impact on the community structure. In order to avoid hydrogen-consuming microorganisms such as methanogens, homoacetogenic bacteria, SRB, and LAB, many pre-treatment such as heat- and shock-loading are used. However, every pre-treatment based on the induction of spore formation can negatively affect hydrogen production by reducing non-spore-forming hydrogen producers. Treatment by aeration can inhibit these hydrogen consumers; however, this treatment can also inhibit some strict anaerobic hydrogen producers like *Clostridium butyricum*. One of the alternatives to select only the desired hydrogen-producing species would be chemical treatment with specific inhibitors. On the other hand, these solutions are not environmentally friendly. Furthermore, the addition of chloroform to the reactor influent, which is an efficient approach to avoid methanogens in lab-scale reactors, is an expensive and ecologically inadequate option when applied to real-scale anaerobic bioreactors.

Controlling the operational parameters presents the best approach to inhibit methanogenic and homoacetogenic activity, while maintaining a community with potential hydrogen-producing bacteria. Therefore, correct manipulation and selection of the community could be achieved through the control of the OLR and pH. The operating temperature also plays a major role in hydrogen production, since hydrogen is more efficiently produced in thermophilic conditions, thus increasing hydrogen yields. However, if the temperature increases too much, enzymes will become inactivated, decreasing hydrogen production. The main disadvantage of thermophilic conditions is that more energy is used for heating the reactors, making mesophilic reactors the reasonable choice.

Mesophilic reactors, operating at high OLR and an acid pH range (4.0 to 6.0) could strongly favor hydrogen-producing bacteria, depending on the substrate and inoculum. Controlling these parameters is a preferable option as they are inexpensive and safe approaches to avoid methanogens. This leads to a natural induction of the sludge to shift its community into adapted hydrogen-producing bacteria.

The functional characteristics and interactions among species strongly influence ecosystem properties. Additionally, species loss or changes in composition can produce different results, depending on the functional redundancy. Furthermore, some species may not contribute significantly, or not contribute at all to ecosystem properties; however, the higher the diversity, the more likely it will be for a system to maintain its stability.

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