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ORIGINAL PAPER

Inhibition of hydrogen production by endogenous microorganisms from food waste

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Abstract

Food waste can be used as substrate in the dark fermentation to produce value-added products such as hydrogen, a future renewable energy supply. However, biological reactor unstable conditions might affect its potential use as green energy by low production rates. This study examined the instability of hydrogen production by dark fermentation of food waste in an anaerobic sequential biological reactor through a microbial community analysis. Hydrogen production varied significantly with a maximum of 25.74 mL H_2/g VS_{added} to low production as 1.29–3.18 mL H_2/g VS_{added} until the end of the experiment. Microbial community analysis showed that the unstable stage was related to the displacement of hydrogen-producing bacteria as *Clostridium*, *Prevotella*, *Caloramator*, and *Bacteroides* by a predominant abundance of *Bifidobacterium*, a lactic-acid bacteria. Furthermore, microbial analysis of food waste revealed the endogenous abundance of lactic-acid bacteria as *Latilactobacillus* (43.73%), *Leuconostoc* (12.1%), *Lactiplantibacillus* (1.84%), *Lactococcus* (1.37%), *Lactobacillus* (0.43%), *Streptococcus* (0.39%) and *Bifidobacterium* (0.19%). Thus, the inhibition of hydrogen production could be caused by the incoming of *Bifidobacterium* from food waste, which could compete for the substrate changing the acetic/butyric fermentation to a possible lactic acid fermentation.

Keywords Unstable H_2 production · Microbial community structure · Hydrogen-producing bacteria · Lactic acid bacteria · Displacement · *Bifidobacterium*

Introduction

Global access to renewable energy is not enough to ensure universal access to affordable, reliable, sustainable, and modern energy by 2030 (United Nations 2021). To achieve Sustainable Development Goals (SDG), particularly SDG 7, promising technologies such as wind power, solar photovoltaic, and biomass are analysed to be implemented in a circular economy through prevention, reduction, recycling, and reuse of wastes. Organic wastes are potential resources that can supply chemicals, nutrients, and fuels needed by industries (Wainaina et al. 2020). However, poor organic waste management could lead to greenhouse gases during putrefaction in waste collection, transportation, and disposal. In particular, food waste (FW), an organic compound of municipal solid waste, is commonly disposed through open dumped, landfills, composting, and incineration (Ayilara et al. 2020). According to Cecchi and Cavinato (2019), FW disposal should first reduce all the negative environmental impacts related to waste management and apply technologies related to energy recovery through anaerobic digestion (AD).

Recently studies have proposed the valorisation of FW into a circular economy, using AD as a medium to produce value-added products from FW. It is possible to obtain chemical, pharmaceutical, cosmetic, food, and other valuable products using AD (EPRS 2017; World-Bank 2018). AD also has the advantage of generating biofuels as methane (CH_4) and hydrogen (H_2) in two-state systems through dark fermentation (DF) in the first step and using

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the digestate of DF during the methanogenesis in a second step (Borin et al. 2019).

DF is a biological process of degradation of organic substrates in anaerobic conditions where a wide variety of bacteria ferments carbohydrates to generate organic acids, CO_2, and H_2 (Ruggeri et al. 2015). Hydrogen production from FW has been studied in both batch, continuous and semi-continuous systems, focused on improved yield and productivity evaluating variables such as hydraulic retention time, organic loading, characteristics of substrates, and source of inoculum (Ferraz Júnior et al. 2020; Moreno-Andrade et al. 2015). The investigation over DF has been increasing in the last ten years due to operational modes and conditions that are not yet economically viable for an industrial scale (Lovato et al. 2021).

To achieve the development of a full-scale reactor, understand and reverse the low rates of hydrogen causes for inhibition by soluble metabolites, inhibitors from the substrate (as metal ions), and inhibition by mixed microflora (Bundhoo and Mohee 2016; Castelló et al. 2020). Therefore, it is essential to recognize the causes of instability during the process. Unfortunately, some events and conditions outside of the experiment can trigger an unstable hydrogen production. One cause of the unstable performance of the process is the change into community microbial composition, where homoacetogenic bacteria displace hydrogen-producing microorganisms (as *Clostridium*) are, sulphate-reducing bacteria, propionic fermenters, and lactic acid bacteria (Bundhoo and Mohee 2016; Castelló et al. 2020). However, the role of some bacteria in the fermentation process and their dynamic in the change of metabolic pathways is not yet understood. For example, lactic acid bacteria (LAB) has been associated with negative and positive roles in DF, where some reports mention that the presence of LAB could produce inhibition of DF, and the other hand, lactic-acid bacteria has been identified into mixed communities with high hydrogen production rates (Castelló et al. 2018, 2020; Lim 2016).

Therefore, this work aimed to evaluate how microbial community structure and reactor performance impact hydrogen production through DF of FW. The microbial community structure during the unstable operation was characterized by phylogenetic analysis.

Materials and methods

In this section, the experimental procedure will be detailed: (i) substrate characterization and inoculum source, (ii) reactor setup and operational conditions, (iii) microbial community analysis, and (iv) analytical methods.

Food waste substrate

The FW used in this study was recollected in a local central market of Queretaro City, Mexico. Bones and inert materials were discarded: The raw waste was crushed using a blender, homogenized, and sieved to obtain a substrate with a particle size smaller than 0.5 mm. The waste was divided into sub-lots of around 2 kg, and they were frozen at − 20 ºC. Before use, each sub-lot of FW was unfrozen at room temperature and stored at 4 °C until further use. The substrate was employed without any micronutrient supplementation. The characteristics of FW are shown in Table 1.

Inoculum source

Anaerobic granular sludge from a full-scale upflow anaerobic sludge blanket (UASB) reactor treating brewery wastewater was used as inoculum. The raw sludge was stored at room temperature for less than 3 days prior to the start of the experiments. The sludge was sieved with a #20 mesh to recover anaerobic granules. According to Carrillo-Reyes et al. (2020), to inactivate methanogens and select the hydrogen-producing microorganisms, the granules of the inoculum were heated at 105 ºC for 24 h in a laboratory convection oven. After the thermal treatment, the dry granules were broken up in a mortar, sieved with a # 20 mesh, and stored in a sealed bag container at room temperature until use.

Reactor system and operation

The biohydrogen production was evaluated in an ASBR with a working volume of 1 L and headspace of 0.6 L. The ASBR system was operated with an organic loading rate of 5 g·VS/L·day. During the operation, the following parameters were used: reaction time, 23 h; settle time, 1 h; fill and discharge time, instantaneous. Each cycle was 24 h. The exchange volume was 50% of total liquid volume per cycle, resulting in a hydraulic retention time (HRT) of 48 h. Temperature (37

Table 1 Characteristics of FW used into experiment

Parameter	Unit	Value
Moisture	%	62.7
Dry matter	%	38.3
Total volatile solids (VS)	% VS/TS	95.0
Total COD (TCOD)	mg O_2/g TS	860.0
Soluble COD (SCOD)	mg O_2/g TS	460.0
Total carbohydrates (TCARB)	mg Carb/g TS	230.9
Soluble carbohydrates (SCARB)	mg Carb/g TS	140.2
Soluble ammonia nitrogen (SN-NH_3)	mg N-NH_3/g TS	1.0
pH		5.1

ºC) and stirring (150 rpm) were controlled with a hot plate stirrer. A range pH of 5–5.5 was maintained intro the reactor with 1 M NaOH solution. Nitrogen gas was used for flushing the medium before activating the reactor. The schematic of the ASBR system is shown in Fig. 1.

Microbial community analysis

A total of seven biomass samples were extracted for bacterial community analysis. Five of them were obtained from 50 mL of digestate during the ASBR operation on days 7, 13, 19, 27, and 39. Inoculum DNA sample was collected from 50 mL of raw sludge, and food waste DNA sample was extracted from 5 g fresh weight. All biomass samples were centrifugated at 3500 rpm for 15 min. The supernatant was removed, and the biomass was frozen and stored at − 4 ºC. DNA was extracted using the DNeasy PowerSoil Kit in accordance with the manufacturer's instructions (Qiagen Inc., Ca, USA). The DNA concentration was quantified by spectrophotometry using a NANO Drop 2000c spectrophotometer (Thermo Scientific, USA). DNA analysis was performed by RTL Genomics (Lubbock, USA) by Illumina MiSeq sequencing using the universal primers 515F (GTG CCAGCMGCCGCGGTAA) and 806R (GGAC-TACHVGGGTWTCTAAT) of the 16S rDNA gene sequence. The sequences were processed in R (version 4.1) using the DADA2 package (version 1.20) (Callahan et al. 2016). A custom script from the DADA2 pipeline (available at https://benjjneb.github.io/dada2/tutorial.html) was used to process the data. Sequence reads were filtered using an expected error threshold of 2 with trimming of 250 and 230 bases for forward and reverse, respectively. Denoised sequences were merged, and chimeric sequences were removed. Taxonomic assignment of amplicon sequence variants (ASVs) was determined using Genome Taxonomy Database (GTDB; release 06-RS202, 27/04/2021). Phyloseq package (version 1.36) was used to generate an object of ASVs tables and calculate diversities of samples (Shannon index, Simpson index, and Chao-1).

Analytical techniques

Total solids (TS), total volatile solids (VS), chemical oxygen demand (COD), and pH were measured according to Standard Methods (APHA-AWWA-WEF 2012). Total and soluble sugar concentrations were measured using the phenol–sulfuric acid method (DuBois et al. 1956). Soluble ammonia nitrogen was determined by the salicylate method (Hach Method 8155).

The volume of biogas was measured by the liquid displacement method at room temperature (25–30 ºC). H_2, CH_4, and CO_2 contents of the biogas were determined using gas chromatography (GC-SRI 8610C) equipped with a thermal conductivity detector (TCD) and two packed columns (6′ × 1/8″ silica gel packed column and 6′ × 1/8″ molecular sieve 13 × packed column). The injector and detector temperatures were 90 °C and 150 °C, respectively. The initial column temperature was 40 °C, which was held for 4 min and then gradually increased to 110 °C at a rate of 20 °C·min^{-1}. The final column temperature was held for 3 min. Nitrogen was used as a carrier gas at a flow rate of 20 mL·min^{-1}.

Volatile fatty acids, including acetic (HAc), propionic (HPr), isobutyric + butyric (HBu), isovaleric + valeric (HVal), caproic (HCap) acids; and solvents as ethanol and acetone were determined by GC (Agilent 7890b) with a flame ionization detector (FID). The temperature of the

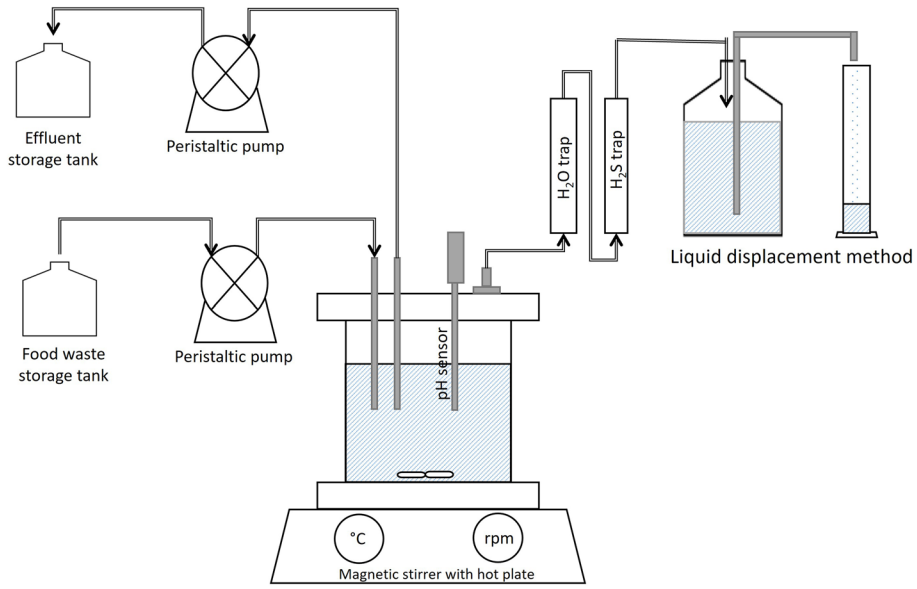

Fig. 1 A schematic representation of the biohydrogen ASBR system

injection port and the FID were 190 and 210 ºC, respectively. The temperature of the column was maintained at 60 °C for 1.5 min; then, it was increased to 90 ºC at a rate of 15 ºC·min^{-1}; later, the temperature increased to 170 ºC at a rate of 25 ºC·min^{-1}, and it was maintained for 4 min. The flow rate of nitrogen as a carrier gas was 2.5 mL·min^{-1}.

Based on the total acid concentration measured in the reactor contents by gas chromatography, the undissociated acid concentration was calculated using Eq. 1 (Van Ginkel and Logan 2005). The total acid concentration ($HA + A^-$) was determined by GC analysis. The pKa is 4.76 for acetic acid, 4.81 for butyric acid, and 4.87 for propionic acid.

$$pH = pK_a + \log \frac{A^-}{HA} \quad (1)$$

Results and discussion

Hydrogen ASBR performance

The ASBR-system was characterized in terms of volumetric hydrogen production rate (QH_2), hydrogen yield (YH_2), organic acid production, and other parameters through different states of performance (Table 2). The gas composition of biogas and performance of YH_2 during 43 days of operation are shown in Fig. 2A, B. During the first 8 days, the ASBR presented a start-up period with irregular QH_2 and YH_2. In days 9–18, QH_2, YH_2, and percentage of H_2 increased rapidly, resulting in maximum values of 257.42 mL H_2 $L_{reactor}$ day^{-1}, 25.74 mL H_2/g VS_{added}, and 43%, respectively. After day 19, the QH_2 and YH_2 decreased sharply. The reactor stabilized with a low YH_2 of 1.29–3.18 mL H_2/g VS_{added} until the end of the experiment.

According to other studies, an irregular variation of hydrogen production rates has been reported when the reactor presents instability. Jo et al. (2007) operated a continuous anaerobic reactor using FW for 72 d, presenting instability when the hydrogen production rate (HPR) dropped gradually from a maximum value of 2909 mL H_2 day^{-1} (17 days) to zero in the 55 days. Castelló et al. (2018) observed a drop in the HPR in a continuous stirred tank reactor (CSTR) fed with cheese whey, where the HPR decreased of 0.9 L H_2 day^{-1} to less than 0.1 L H_2 day^{-1}.

A low hydrogen production yield during system start-up was reported by Villanueva-Galindo and Moreno-Andrade (2020) and Jiménez-Ocampo et al. (2021), whose used a

Table 2 Summary of parameters and metabolites during different states of performance of ASBR-system

Parameters	Day 1–8	Day 9–18	Day 19–43
QH_2 (mL H_2/$L_{reactor}$/d)	15.6 ± 13.3	179.4 ± 65.2	14.4 ± 8.8
YH_2 (mL H_2/g VS FW_{added})	2.6 ± 2.2	19.1 ± 6.8	1.4 ± 0.9
YH_2 (mol H_2/mol $Hexose_{added}$)	0.05 ± 0.04	0.54 ± 0.20	0.04 ± 0.03
TCOD removal (%)	36.3 ± 6.5	24.5 ± 7.6	18.0 ± 17.8
SCOD removal (%)	– 5.7 ± 8.8	– 19.2 ± 4.0	– 9.3 ± 35.2
TCARB removal (%)	88.8 ± 4.5	90.8 ± 1.2	68.5 ± 26.5
SCARB removal (%)	88.8 ± 3.5	93.5 ± 2.9	58.6 ± 29.9
TS removal (%)	50.2 ± 8.4	54.3 ± 1.4	41.5 ± 9.8
VS removal (%)	67.5 ± 4.2	68.5 ± 6.2	61.0 ± 10.8
HAc (mM)	27.4 ± 7.0	32.9 ± 4.0	34.9 ± 6.9
Undiss-HAc (mM)	2.2 ± 1.6	7.2 ± 1.3	8.6 ± 2.8
HBu (mM)	5.7 ± 3.8	10.7 ± 3.8	8.1 ± 2.1
Undiss-HBu (mM)	0.6 ± 0.6	2.5 ± 0.7	2.2 ± 0.8
HPr (mM)	7.0 ± 3.9	5.2 ± 1.0	10.5 ± 4.1
Undiss-HPr (mM)	0.9 ± 0.7	1.5 ± 0.2	3.3 ± 1.6
HVal (mM)	1.0 ± 0.2	1.6 ± 0.3	2.7 ± 1.2
HCap (mM)	0.0 ± 0.1	1.9 ± 0.4	1.5 ± 0.4
Ethanol (mM)	4.3 ± 4.7	3.4 ± 6.7	0.5 ± 1.6
Acetate (mM)	0.2 ± 0.2	2.9 ± 2.9	0.0 ± 0.0
pH	6.0 ± 0.3	5.4 ± 0.1	5.4 ± 0.1

Note: Undiss = undissociated; *1 mM* = 1 mmol/L

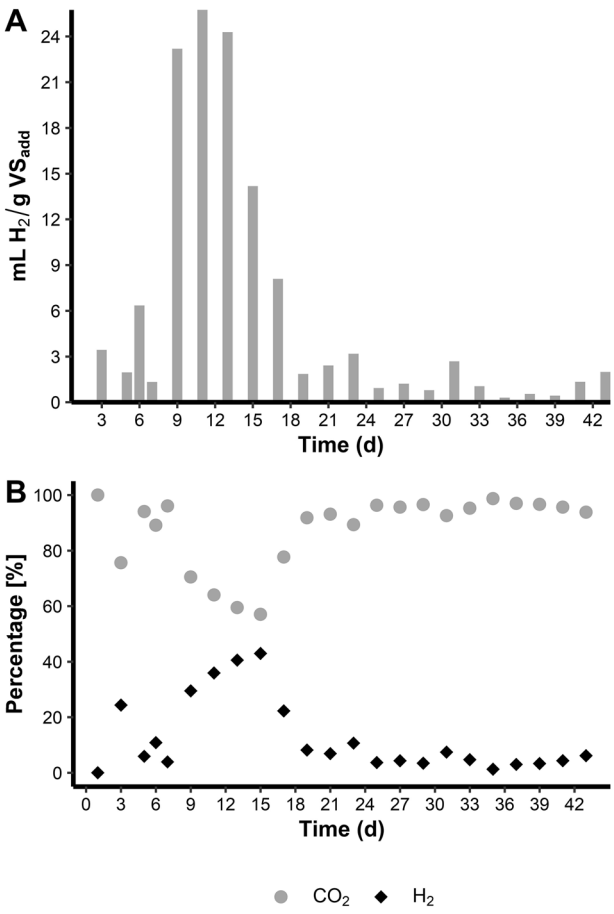

Fig. 2 Hydrogen yield (**A**) and gas composition (**B**) in ASBR system

similar ASBR configuration with pH of 5.5, HRT of 48 and 24 h, and FW as feedstock with an organic load rate of 5 g VS/L day. Villanueva-Galindo and Moreno-Andrade (2020) reported a low YH_2 of 4.4 mL H_2/g VS_{added} during the first stage of operation, from cycles 1 to 24. To revert this situation, they applied two bioaugmentation with *Bacillus subtillis*, which reached maximum values of 17.5 and 19.7 mL H_2/g VS_{added}. Jiménez-Ocampo et al. (2021) reported a low volumetric production of 50–170 mL H_2 $L_{reactor}$ day^{-1} with a content of 2–13% of H_2 into biogas in the start-up reactor, using an HRT of 24 h. They evaluated a feedback control strategy for optimizing the H_2 production, where the control strategy maintained short HRTs of 4–8 h with a stable H_2 production. In contrast to our results, our ASBR obtained the best percentage and yield previous the time of instability. The differences in performance at the beginning of the start-up reactor might be associated with the FW characteristics, due to our feedstock containing major total and soluble carbohydrates that the FW of the previous two studies.

DF is affected by different factors that can decrease the hydrogen production rates and cause instability problems during reactor operation. These factors could have an origin biotic due to the presence of hydrogen-consuming bacteria (HCB) that displacement hydrogen-producing microorganisms (HPB) by competition of substrate or release of toxins that affects the HPB (Bundhoo and Mohee 2016; Castelló et al. 2020). Abiotic factors as inhibitor compounds by metal ions, furan derivates, and undissociated acids could suppress hydrogen production when their concentrations affect bacterial metabolism (Bundhoo and Mohee 2016; Maddox et al. 2000).

The presence of methane has been reported as a factor that negatively impacts H_2 production. However, in this study, consumption of H_2 by methanogenic activity was discarded since CH_4 was not observed in the biogas, associated with the heat-shock pre-treatment of the inoculum. Similar to other studies, methanogenic archaea activity was supressed when thermal pre-treatment was used on inoculum (Bansal, et al. 2013; Cai and Wang 2016; Chen et al. 2001; Taherdanak et al. 2017; Wang et al. 2011). This implies that hydrogen-consuming microorganisms might utilize the H_2 or, a switch in the fermentation pathway might cause the consumption of H_2 during the conversion of organic acids.

Metabolic products

Figure 3 shows the VFA composition into the ASBR system. The predominant components of VFA in the liquid effluent were HAc, HPr, and HBu. The production of HAc and HBu is favourable for hydrogen production, according to Eqs. (2) and (3). HAc and HBu have been reported as main soluble metabolites under the mesophilic condition in continuous and discontinuous hydrogen reactors; both acids are associated with high HPR (Castillo-Hernández et al. 2015; Hernández-Mendoza et al. 2014; Santiago et al. 2020; Slezak et al. 2019). Moreover, the production of HPr, HVal, and HCap affect the consumption of hydrogen, according to Eqs. (4)–(6).

		$\Delta G^{0'}$ (kJ)	Equations
Acetic acid:	$C_6H_{12}O_6 + 2H_2O = 2CH_3COOH + 2CO_2 + 4H_2$	− 206.0	(2)
Butyric acid:	$C_6H_{12}O_6 = CH_3(CH_2)_2COOH + 2CO_2 + 2H_2$	− 254.0	(3)
Propionic acid:	$C_6H_{12}O_6 + 2H_2 = 2CH_3CH_2COOH + 2H_2O$	− 279.4	(4)
Valeric acid:	$CH_3CH_2COOH + 2CO_2 + 6H_2 = CH_3(CH_2)_3COOH + 4H_2O$	− 143.3	(5)
Caproic acid:	$CH_3(CH_2)_2COOH + 2CO_2 + 6H_2 = CH_3(CH_2)_4COOH + 4H_2O$	− 143.3	(6)

Bioreactions according to Saady (2013)

As previously mentioned, a type of instability is caused by a high concentration of undissociated acids due to the conversion of easily digestible biomass to organic acids (self-produced acids). This process is stressed by HPB, whose shift the organic acid production to solvents (as acetone, butanol, and ethanol) in a pathway known as solventogenesis (Bundhoo and Mohee 2016; Ward 2015). The inhibition of hydrogen production by solventogenesis depends on the concentration of undissociated acid, initial pH, the concentration of substrate in the influent, and metabolite inhibitor.

A switch to solventogenesis has been reported at a concentration of undissociated acids of 30–60 mM (Amador-Noguez et al. 2011; Infantes et al. 2012; Maddox et al. 2000). Van Ginkel and Logan (2005) reported a complete hydrogen production inhibition when the concentration of 50 mM of acetic and butyric acids were added in a continuous flow reactor operated at 30 °C, an HRT of 10 h, pH of 5.5 and fed with glucose. Also, these authors tested the formation of undissociated acids within a pH range from 4.5 to 6. A switch to solventogenesis was not observed in the pH range of 4.5–6.0 even though the total concentration of undissociated acids reached 15 mM at pH 4.5.

According to our results (Table 2), there was no evidence for the inhibitory effect of undissociated acids on hydrogen yield in this study. The total concentration of undissociated acids was 11.2 mM (day 9–18 of operation) and 14.1 mM (day 19–43 of operation), at a stable pH of 5.4. These concentrations of undissociated acids were lower than the reported by Infantes et al. (2012), Van Gilkel and Logan (2005), and Wang et al. (2008); who stated at least a concentration of 50 -100 mM to inhibit the hydrogen production.

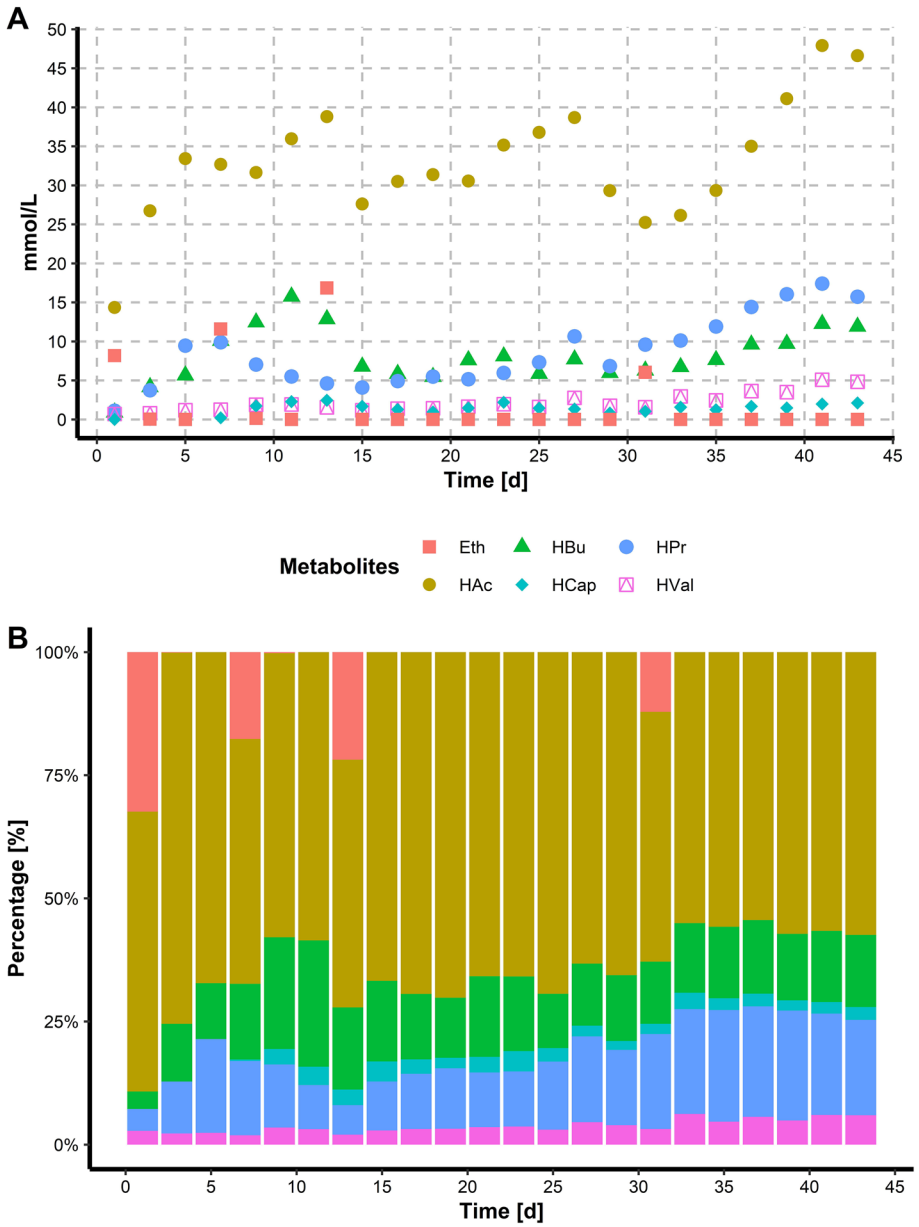

Fig. 3 Composition of metabolites across the time (**A**), and percentage of metabolites (**B**) in the effluent of the hydrogen ASBR system

Another factor of instability on hydrogen production is the accumulation of HPr due to the shock loading or overloading, or in the start-up stage (Sivagurunathan et al. 2014; Wang et al. 2006a, b). Some studies indicated that higher hydrogen partial pressure or higher hydrogen production rate promotes NADH accumulation, affecting the equilibrium of NADH/NAD^+ ratio inside the cell (Wang et al. 2006a, b). The intracellular redox state of NADH/NAD + is important to maintain the flow of electrons, H^+, NADH-shuttled H^+, into the H_2 production (Eq. 7) (Wu et al. 2017). To maintain the NADH/NAD^+ ratio, HPr fermentation will spontaneously replace HBu fermentation to reduce NADH concentration and maintain an appropriate NADH/NAD^+ ratio (Sivagurunathan et al. 2014; Wang et al. 2006a, b). However, Inanc et al. (1996) reported that the hydrogen partial pressure had no direct relationship with the accumulation of HPr and demonstrated that the shift from butyric to propionic acid fermentation was the result of changes into the population dynamics in acidogenic phase.

	ΔG^0 (kJ/mol)	Equation
$2NADH + H^+ \rightarrow H_2 + 2NAD^+$	+81	(7)

Bioreaction accoding to Balachandar et al. (2013)

Figure 3A, B illustrate the concentrations and percentage of organic acids during the operation of the H_2-ASBR.

In this study, during the start-up stage (1–8 days), the HPr concentration was 7.0 ± 3.9 mmol L^{-1}, but it dropped to 5.2 ± 1.0 mmol L^{-1} in the stage of adaptation (9–18 days). In the state of instability (19–43 days), a gradual accumulation of HPr was observed with a range of 5–17 mmol L^{-1}. As result of the increase in HPr concentration, the H_2 yield is reduced due to the consumption of H_2 during the formation of HPr from glucose, according to the theoretical stoichiometric equation (Eq. 4).

A similar accumulation of HPr was described by Sivagurunathan et al. (2014), who studied the effect of the temperature shift and the dynamic changes in the microbial community composition in a CSTR fed on beverage industry wastewater. They reported a HPr accumulation period (days 36–49) of 28–35 mmol L^{-1} dropping from 53 to 39% in the H_2 content. Koskinen et al. (2007) reported elevated concentrations of HPr in a fluidized-bed bioreactor fed with glucose, resulting in a shift from acetate/butyrate to acetate/propionate production with a decrease and instability in H_2 production by adhesion of H_2 consumer in the biofilm of the reactor.

The present experimental results indicate that the concentration of undissociated acids was insufficient to trigger a metabolic pathway shift. However, the increase of HPr concentration could suggest a change in microbial dynamic, according to Sivagurunathan et al. (2014) and Koskinen et al. (2007). The microbial community analysis can explain the depletion of hydrogen production through the presence of H_2-consuming microorganisms and by the formation of metabolites associated with the microorganisms.

Changes in microbial community

The characterization of the microbial community of the ASBR throughout the operating period is shown in Fig. 4, considering a sample of inoculum, a composite sample from FW, and five samples during the operation process. The most representative genera (relative abundance > 4%)

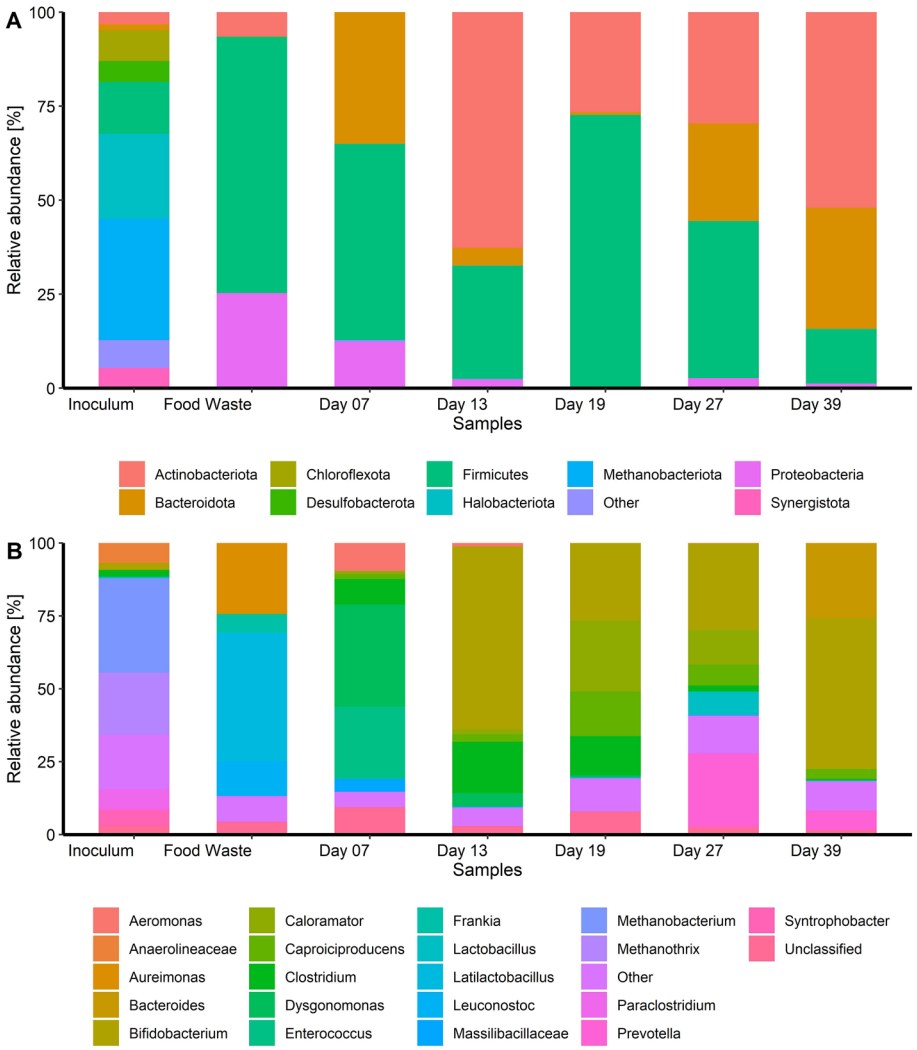

Fig. 4 The main microbial community structure at the level of phylum (**A**) and genus (**B**) in different samples

Table 3 Sequence reads and alpha diversity of each sample

Sample	Total reads	Filtered reads	Denoised Filters	Denoised Reads	Merged	Nonchim	ASV	Shannon	Simpson	Chao-1
Inoculum	50,497	47,093	46,413	46,523	40,936	23,298	366	4.9860	0.9902	442.24
FW	42,884	38,637	37,364	36,976	30,693	19,071	573	5.9076	0.9969	636.55
Day 7	42,990	39,988	39,460	39,524	35,473	20,506	363	4.9391	0.9896	451.14
Day 13	51,072	46,799	46,253	46,409	41,589	30,028	545	4.9290	0.9878	610.70
Day 19	47,682	44,448	43,843	43,803	35,505	20,235	515	5.4464	0.9941	562.88
Day 27	47,715	43,699	43,094	43,181	38,986	22,118	575	5.4904	0.9943	611.00
Day 39	54,644	50,539	49,862	49,926	46,104	28,301	393	4.9035	0.9892	477.29

are indicated in Fig. 4. The sequences analysis and index of diversity are depicted in Table 3.

The number of observed ASVs for the sample ranged from 363 to 575, with 1269 ASVs identified. The GTDB (release 06-RS202) database revealed 99.05% of variants assigned to the class, 97.32% to order, 96.69% to family, and 90.78% to genus. We detected 21 bacterial phyla, where three of them (*Firmicutes*, *Actinobacteriota*, and *Bacteroidota*) accounted the 84% of the total abundance (40.5, 29.1, and 14.6%, respectively). Overall, 121 genera were identified for all samples. Eight of them (*Bifidobacterium*, *Clostridium*, *Latilactobacillus*, *Dysgonomonas*, *Caloramator*, *Prevotella*, *Bacteroides,* and *Caproiciproducens*) represented 64% of total abundance (28.1, 6.6, 6.3, 5.3, 5.1, 4.6, 4.5 and 4.1%, respectively).

During the start-up process (sample of day 7) the microbial community was dominated by *Dysgonomonas*, *Enterococcus*, *Aeromonas,* and *Clostridium* with 34.95%, 24.79%, 9.62%, and 8.79%, respectively. Although the hydrogen production rate was low at the start-up stage, this phase is crucial for establishing of the mesophilic anaerobic conditions inside the reactor. Cabrol et al. (2017) suggested that the presence of not able, or less efficient, microorganisms to produce H_2 can positively contribute to enhance H_2 production through different mechanisms, such as cometabolism, granulation, oxygen consumption, or hydrolysis. For example, in the solubilization of macromolecular complex organic matter, facultative acidogenic microorganisms have been reported to use extracellular enzymes for breaking down into soluble molecules and to contribute to the consumption of oxygen (Botheju and Bakke 2011).

Even though *Dysgonomonas* (order *Bacteroidales*, phylum *Bacteroidetes*) and *Aeromonas* (order *Aeromonadales*, phylum *Proteobacteria*) are facultative genera that non-producing hydrogen (Gonçalves Pessoa et al. 2019; Hofstad et al. 2000), they have an important role in the hydrolysis and fermentation of organic matter resistant to biodegradation. Kim et al. (2011), during the start-up of three lab-scale anaerobic reactors treating with different wastewaters, reported that *Aeromonas* spp. and *Clostridium sticklandii* emerged as common and prominent acidogens in all reactors, where *Aeromonas* was related for the rapid initial utilization of carbohydrate with the accumulation of acidogenic products. Xiong et al. (2019) investigated the food waste fermentation in a leach bed reactor, with the high abundance of *Bacteroides* and *Dysgonomonas* at pH 7 and 8. They suggest that *Dysgonomonas* was able to ferment lignocellulosic fibers into organic acids.

In the case of *Enterococcus* genus (orden *Lactobacillales*, phylum *Firmicutes*), it is dynamic microbial unclear. Some studies have reported *Enterococcus* as a minor H_2-producing genus (Davila-Vazquez et al. 2009; Yang et al. 2019); whereas in other studies, it is reported as a lactic-acid bacteria that can produce acetate, ethanol, and lactate as end products of carbohydrate metabolism (Doi 2015). In this study, *Enterococcus* could have presented a role as a fermenter of carbohydrates due to the predominance of the hydrolytic genus as *Dysgonomonas* and *Aeromonas*.

Following the main changes in the microbial communities in the state of adaptation (sample of day 13), genera as *Dysgonomonas*, *Aeromonas,* and *Enterococcus*, which were dominated during start-up, decreased strongly to abundances of 4.66%, 1.18%, and 0.05%, respectively. They were replaced by *Bifidobacterium* (abundance of 62.69%), which dominated until the end of the experiment. In this period, the H_2-ASBR presented its best HPR with a high abundance of *Clostridium*. Genus *Clostridium* (order *Clostridiales*, phylum *Firmicutes*) is reported as a desired genus in the biological production of hydrogen by their high production rate and ability to use a wide range of carbohydrates (Chong et al. 2009; Ruggeri et al. 2015).

Previous studies revealed that the hydrogen production reactor performance is closely correlated with the predominant hydrogen-producer community structure. Alexandropoulou et al. (2018) investigated the influence of the HRT and the pH on the fermentative hydrogen production from a food industry waste in a CSTR. In that study, the highest hydrogen rate (10.79 ± 0.21 L H_2/L $_{reactor}$/day at HRT of 4 h) was related to a microbial diversity dominated by members of the *Clostridiaceae* family with an 87% of abundance. Yang and Wang (2018) reported a high abundance of *Clostridium* (79%) with Fe^{2+} addition of 400 mg/L during

the study of the enhancement of H_2 production from grass by addition of Fe^{2+}, obtaining a production of 72.8 mL H_2/g dry grass.

According to Hung et al. (2011) the selection of the type of substrates, ecological dynamics, and reactor operation parameters could determinate the evolution of characteristic hydrogen-producing communities. This means that the high presence of non-hydrogen producing bacteria, coupled with the low abundance of HPB, can cause irregular hydrogen production rates if the operation conditions limit the spread of HPB. This situation was observed during the instability stage of H_2-ASBR, where the establishment of HPB inside the reactor was not possible.

According to Fig. 4, HPB *as Clostridium*, *Caloramator*, *Prevotella*, and *Bacteroides* were identified during the instability stage; (Ciranna et al. 2014; Emerson and Weimer 2017; Ren et al. 2007; Shu-Yii et al. 2005); however, these genera presented an irregular abundance. The proportion of *Clostridium* in the medium decreased of 13.47% (day19), to 1.97% (day 27), and 0.44% (day 39). *Caloramator* (order *Clostridiales*, phylum *Firmicutes*) presented a similar situation, decreasing of 24.31% (day 19), to 11.76% (day 27) and 0.02% (day 39). *Prevotella* and *Bacteroides* (both order *Bacteroidales*, phylum *Bacteroidetes*) had an irregular abundance during day 7–13–19 with less de 0.02% of abundance. However, *Prevotella* had a spread during day 27 with 25.14% of abundance but decayed to 6.69% (day 39); meanwhile, *Bacteroides* presented a high abundance of 25.49% at the end of the experiment.

Cabrol et al. (2017) suggested that under specific operating conditions, *Clostridium* might not be the adequate genus to lead the H_2 production and other non-spore-forming HPB have been reported as significant and minor HPB, with specific metabolisms which enable to maintain acceptable H_2 performance when *Clostridium* is inactive. In this study, *Caloramator*, *Prevotella*, and *Bacteroides* might play the role of maintaining H_2 production when *Clostridium* abundance decreased into the reactor.

C*aloramator* is identified as forming-spore, Gram-variable, thermophilic bacterium, which produces minor quantities of H_2 and CO_2; also generates ethanol, acetate, lactate, and propionate as end products of glucose fermentation (Crespo et al. 2012; Fuess et al. 2018; Ogg and Patel 2009; Rubiano-Labrador et al. 2013). *Prevotella* has been reported as an abundance genus into ASBR feeding with FW when the reactor presented regular production of H_2 (Jiménez-Ocampo et al. 2021; Villanueva-Galindo and Moreno-Andrade 2020). In contrast, Castelló et al. (2009) suggested that *Prevotella* competes for substrate (as pyruvate) with HPB. Its role inside the reactor is uncertain because to it could play a part in the acetic-butyric acid fermentation and facilitated hemicellulose degradation from FW (Emerson and Weimer 2017). Finally, *Bacteroides* have been described with positive and negative roles in hydrogen fermentation. Some isolated strains of *Bacteroides* have shown hydrogen production (Fernández-Calleja et al. 2018) and it has been identified in continuous and discontinuous reactors with high hydrogen production (Ren et al. 2007; Santiago et al. 2020). Moreover, Saady et al. (2012) found H_2 consumption by homoacetogenic activity in the granular culture dominated by *Bacteroides*.

Although the microbial diversity during DF depends on the inoculum source, pre-treatment on inoculum, the type of substrate, and the operating conditions, these operating characteristics do not imply that only beneficial microorganisms will be selected to enhance hydrogen production. A disadvantage of using mixed inoculum and non-sterile feedstocks is the presence of microorganisms such as HCB and others that compete with HPB for substrate or inhibit HPB through their metabolites, resulting in a possible decrease of the hydrogen yield. This negative interaction between HPB and HCB could be assigned to methanogens, homoacetogens, sulphate-reducing bacteria, nitrate-reducing bacteria, propionate producers, iron-reducing bacteria and lactic-acid bacteria (LAB) (Bundhoo and Mohee 2016; Cabrol et al. 2017).

In the instability stage of H_2-ASBR, LAB as *Bifidobacterium* and *Lactobacillus* were identified with an abundance of 26.6–62.7% and 0.001–7.97%, respectively. These two genera are characterized by generating a higher proportion of lactic acid as the final product of their fermentation (Ibrahim 2016; Lim 2016; Pokusaeva et al. 2011). Consistent with other studies, we observed a negative interaction of HPB with LAB. Noike et al. (2002) found that hydrogen fermentation was replaced by lactic acid fermentation caused by *Lactobacillus paracasei* and *Enterococcus durans*, in the bean curd manufacturing waste. Jo et al. (2007) reported the change in microbial community from H_2-producing *Clostridium* spp. to lactic acid-producing *Lactobacillus* spp that caused the inhibition of hydrogen production from food waste of kimchi in continuous culture. Ren et al. (2007) observed an inhibitory effect of ethanol on hydrogen production associated with *Lactococcus*. Castelló et al. (2018) studied the causes of instability in the hydrogen production from cheese whey, concluding that high fluctuation in the hydrogen production might be caused by the high abundance of LAB.

Although the lactic acid could not be measured, we inferred that a lactic acid fermentation replaced the mixed acetic-butyric fermentation. Villanueva-Galindo and Moreno-Andrade (2020) mentioned a similar problem whose reported a high abundance of *Bifidobacterium* and *Lactobacillus*. In addition, Castelló et al. (2018) proposed that an increase in the relative abundance of *Bifidobacterium* could decrease hydrogen yield by substrate consumption. They

suggested a displacement of *Megasphaera* (an HPB) by LAB, resulting in a decrease in hydrogen production. Overall, we hypothesize that *Bifidobacterium* was the cause of instability in hydrogen production by displacement of HPB.

On the one hand, it has been reported that the presence of LAB can cause reactor instability and low hydrogen production (Bundhoo and Mohee 2016; Castelló et al. 2020). LAB can decrease hydrogen production by consuming pyruvate towards the generation of lactic acid instead of using it to form acetyl-CoA and posterior transformation to acetate or butyrate (Saady 2013). Another way to decrease hydrogen production is to release antimicrobial peptides (known as bacteriocins) by LAB. These toxins act on the cytoplasmic membrane, permeabilizing it through the formation of ionic pores that will release compounds such as potassium and amino acids, resulting in the destabilization of the membrane and consequently cell death (Mokoena 2017; Prudêncio et al. 2015).

On the other hand, other studies indicate that the presence of LAB is not entirely negative since there are not yet understood interactions between these and HPB. One of these interactions could be the formation of butyric acid (Eq. 8) from lactate and acetate by a group of *Clostridium* (Castelló et al. 2020; Matsumoto and Nishimura 2007). Also, the presence of LAB, both homolactic and heterolactic, has been reported inside the reactor, but without effects on hydrogen production (Chojnacka et al. 2011).

	ΔG^0 (kJ/mol)	Equation
$Acetate + 2Lactate \rightarrow H_2 + 2CO_2 + \frac{2}{3}Butyrate + H_2O$	− 156.6	(8)

Davila-Vazquez et al. (2009) identified the microbial community in a CSTR fed with cheese whey as a substrate for hydrogen production. They reported LAB as *Enterococcus faecium* and *Streptococcus* sp with a *Clostridium* dominance using a low HRT of 6 h. Moreno-Andrade et al. (2015) analyzed and correlated the microbial community with the H_2-ASBR reactor performance, finding that *Megasphaera* was the dominant genus over *Veillonella*, *Olsenella*, *Bifidobacterium,* and *Pelosinus*, when an HRT of 24 h was used.

The contamination of LAB into the reactor could have two sources: inoculation and feeding. Theoretically, heat-shock pretreatment inactivated non-spore-forming bacteria (as LAB) in the inoculum. On the other hand, the substrate was not sterilized, being applied daily in the ASBR feed. Thus, the microbial community of FW was analysed, being detected LAB genus as *Latilactobacillus* (43.73%), *Leuconostoc* (12.1%), *Lactiplantibacillus* (1.84%), *Lactococcus* (1.37%), *Lactobacillus* (0.43%), *Streptococcus* (0.39%) and *Bifidobacterium* (0.19%). Besides *Bifidobacterium* was the predominant LAB genus into ASBR, its relative abundance from FW was poor. The anaerobic condition, temperature, the interaction between species, availability of nutrients, and acid pH of the medium into ASBR could promote its rapid spread. This suggests that regardless of the heat-shock pretreatment on inoculum inactive the HCB (Bansal et al. 2013; Cai et al. 2009; Wang and Wan 2008), the constant incoming of indigenous LAB from substrate overcame the inoculated reactor biomass. The substrate as a source of contamination of LAB into the reactor has been reported by Jo et al. (2007) and Castelló et al. (2018), who worked with substrates rich in LAB.

Apart from LAB, *Caproiciproducens* (order *Clostridiales*, phylum *Firmicutes*) was identified as an H_2-consumer through the fatty acid chain elongation pathway by converting HBu to HCap (Garrett and Onderdonk 2015; Wang and Wan 2008). This genus close to *Clostridium* and *Ruminococcus*, was proposed as a novel taxon of the *Ruminococcaceae* family by the study of Kim et al. (2015). This genus stands out for its ability to produce caproic acid as a final product of its fermentation and generate acetic, butyric acid, and ethanol, using various carbon sources such as galactose, xylose, glucose, arabinose, glycerol, ribose, fructose, mannose, and sucrose. In this work, we discard the fatty acid chain elongation pathway as a significant source of H_2-consuming. We observed that HVal and HCap represented a mean of 5.8% and 4% of TVFA, respectively. Furthermore, Greses et al. (2021) reported HVal and HCap concentrations of 1.8% and 32.6–35.2%, resulting in a higher prevalence of medium-chain fatty acids with concomitant high hydrogen yields 395.5 and 62.7 mL H_2/g VS for melon and watermelon fermentation.

Some strategies reported in the literature to overcome the inhibition by LAB and enhance hydrogen production performances are summarised below. A preliminary strategy is the acclimatization of the microbial community of inoculum in batch to prevent substrate inhibitory effects or to control competition between different genus of bacteria (Bakonyi et al. 2014). A second strategy is storing the feedstock with low temperature or applying high temperature to feedstock could reduce the proliferation of LAB and limit the concentration of lactic acid in the feed solution (Jo et al. 2007; Noike et al. 2002). On the one hand, this may increase the operational cost in full-scale reactors. On the other hand, genera as *Bacillus* and *Bifidobacterium* have been reported as spore-forming bacteria with the ability to survive freezing, drying, thawing, and rehydration (Popov et al. 2021). So, this strategy should be complemented with others to avoid the spread of LAB into the reactor. A third strategy, bioaugmentation has been studied as a tool to improve hydrolytic activity and promote the increase of genera of interest like HPB (Villanueva-Galindo and Moreno-Andrade 2020). This could have a short effect, and

new bioaugmentation may be required. In laboratory and pilot-scale reactors, the re-inoculation could minimize the displacement of HPB, whereas the experimental conditions and productivities can be make-up by the regular adding of HPB. Finally, if the reactor presents a high abundance of LAB and is detected bacteriocins, trypsin in the combination of heating for 10 min at 80 °C can destroy this inhibitory compound (Lim 2016; Noike et al. 2002). Considering our results, the presence of LAB in the organic waste cannot be avoided because they are indigenous microbiota, but they can be suppressed using some strategy depending on the origin or characteristics of feedstock. In future studies, it will be critical to find that conditions or ecological interaction promote a negative effect of LAB on hydrogen production.

Conclusions

A constant decline in the yield and composition of H_2 into biogas was observed in the long-term operation of an ASBR system. This instability state caused an irreversible process of low hydrogen yield related to changes in metabolic pathways. The microbial community analysis showed that HPB as *Clostridium*, *Prevotella*, *Caloramator*, and *Bacteroides* were washed out from the reactor while *Bifidobacterium*, a lactic acid producer, predominate instead. This resulted in the switch of hydrogen fermentation to a possible lactic acid fermentation. LAB was incoming with the feedstock during the feed of the reactor. The operational conditions promoted the increase of *Bifidobacterium,* causing competition for substrate between HPB and LAB. The presence of endogenous microorganisms in the substrate can lead to changes in the microbial composition inside the reactor. To overcome instability and to promote high H_2 production, the factors involved in the steady-state and instability of dark fermentation process, should be further investigated.

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Declarations

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