Waste and Biomass Valorization (2024) 15:2145–2158
https://doi.org/10.1007/s12649-023-02279-1

ORIGINAL PAPER

Effect of Inoculum Pretreatment and Operational Mode of Reactor on BioH_2 Production from Nixtamalization (Nejayote) and Abattoir Wastewater

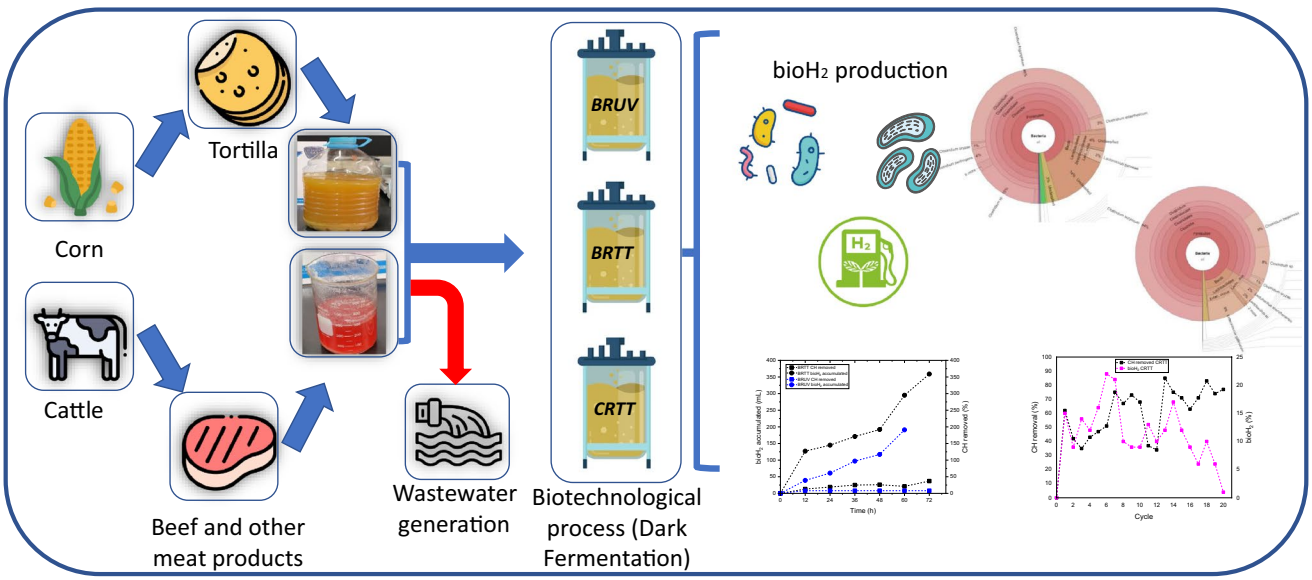
Roberto Carlos Campos-Flores[1] · Lirio María Reyna-Gómez[1] · Santiago Iván Suárez-Vázquez[1] · Armando Robledo-Olivo[2] · Arquímedes Cruz-López[1]

Received: 11 April 2023 / Accepted: 7 September 2023 / Published online: 26 September 2023

Abstract

Dark fermentation process appears to be one of the most promising techniques to obtain clean energy, however, there are some limitations to this process. In the present study, stirred tank reactors under different modes [batch (BR) and continuous (CR)] were used to determine the evolution of hydrogen-producing microbial communities when nixtamalization (nejayote) and abattoir wastewater were co-digested. The inoculum consisted of granular sludge subjected to (a) thermal treatment (TT); and (b) UV irradiation. When the inoculum underwent TT (15 min at 100 °C) in BR (40 RPM, 35 ± 1 °C, pH 5.50 ± 0.05), the highest cumulative biohydrogen (bioH_2) content was obtained (350 mL). In this case, the organic matter degradation reached 70%. In addition, the sequencing analysis showed *Clostridium butyricum* was the predominate strain (68–87%) between 48 and 72 h of the reaction. Conversely, when UV-pretreated inoculum was used, *Clostridium butyricum* only made up 1% after 60 h of reaction. The CR was operated for 20 cycles at a hydraulic retention time of 6 h. Volatile fatty acids confirmed butyrate acetate fermentation. Herein, the bioH_2 yield was generated at a fivefold rate compared to previous studies. Specifically, 223 mL H_2 g^{-1} total volatile solids and 8 mL H_2 L^{-1} h^{-1} were produced. Moreover, *Clostridium* was present at 59–62% after 14 cycles, confirming a better efficiency in bioH_2 production when the reactor works in continuous mode.

Graphical Abstract



Keywords Green hydrogen · Fermentation · Bioenergy sources · Food industry wastewater

Extended author information available on the last page of the article

Statement of Novelty

- The TT was the most effective treatment suppressing the methanogenic microorganisms
- *Clostridium* was the main genera present in 59–62% after 14 cycles of fermentation
- The maximum yield of bioH_2 was 223 mL H_2 g^{-1} TVS using a CRTT at HRT = 6 h
- The bioH_2 production was generated at a fivefold rate with CRTT in comparison to BRTT

Introduction

Population growth is closely related to increasing energy demands. Therefore, the consumption of fossil fuels, including coal and oil, and greenhouse gas emissions have increased substantially in recent years [1]. Hydrogen gas (H_2) does not generate CO_2 emissions and displays an energetic density of 120 kJ g^{-1} which is 2.5 times higher than that of gasoline [2]. It is considered an important candidate to replace fossil fuels. H_2 is an odorless, colorless, and tasteless gas that is nonpolluting, as it only produces water after combustion [3]. When H_2 is generated via a biological pathway, it is called biohydrogen (bioH_2). These pathways include water biophotolysis, indirect biophotolysis, photofermentation, microbial electrolysis cells (MECs), and dark fermentation (DF) by green algae, cyanobacteria, photosynthetic bacteria, electrogenic bacteria, and anaerobic bacteria, respectively [2]. Dark fermentation is considered a simple process that has several advantages over the other biological technologies. In addition to not requiring light, DF has high conversion rates and can be performed with a variety of substrates, including municipal, industrial, and agricultural wastes [4–9].

Substrates for bioH_2 production through DF must display a high carbohydrate content generated from sustainable and accessible resources, require minimal pretreatment, and, if possible, must be cost-effective [2]. According to previous studies, the efficiency of bioH_2 production can be improved when two different substrates are used (co-digestion) because, under these conditions, a better buffer effect can be achieved [8, 9]. In addition, a better carbon: nitrogen (C/N) ratio can be obtained using two or more substrates, owing to their physicochemical characteristics. In fact, a C/N ratio of 20–40 is widely used as a standard parameter as it is associated with the enhancement of bioH_2 production, owing to its buffering effect on the system [9, 10]. An optimal C/N ratio is required for the proper growth of microorganisms during this process. The optimization of the C/N ratio is one method used to reduce or avoid ammonia inhibition. When C is higher than the optimum N concentration in the C/N ratio, there is poor biogas production. However, when C is lower, this results in ammonia accumulation [11].

In addition to substrates, inoculum selection is extremely important in DF because it is the main component containing the microorganisms responsible for bioH_2 production. An appropriate amount of inoculum is essential to achieve effective DF [12]. *Enterobacter aerogenes*, *Clostridium butyricum,* and *Clostridium acetobutyricum* were used as pure cultures for bioH_2 production. However, mixed cultures of granular anaerobic sludge were also selected because they contained different microorganisms, including *Clostridium* spp., *Prevotella* spp., and *Megasphaera* spp. [9]. However, these mixed cultures require pretreatment before use in bioH_2 production. One of the most frequently reported pretreatments is thermal treatment (TT). However, there are other options, including UV irradiation, alkaline and acid treatments, and microaeration, which require less energy and processing time. More in-depth studies of these processes are necessary to determine their impact on bioH_2 production [12, 13].

Nejayote (NEJ) and abattoir wastewater (ABW) have high contents of biodegradable organic matter and are produced in large amounts in different countries. In Mexico, the annual generation of NEJ reaches 14,800 million liters [14], and the processing of cattle requires approximately 1,000 liters of water per animal. It is estimated that 95% of this water becomes wastewater [15]. Therefore, they can be used as substrates for DF [16]. Nixtamalization is a thermal-alkaline method applied to maize that allows the elimination of the pericarp from the kernel, generating two main products, nixtamal and NEJ. Nixtamal represents softened grains that serve as ingredients for the preparation of tortillas, flour, and other nixtamalized maize products, whereas NEJ is an alkaline wastewater resulting from cooking kernels [17, 18]. Previous studies have indicated that NEJ generation reaches 2.2–3.5 m^3 t^{-1} of processed corn annually [17, 19]. Calcium hydroxide is added during the nixtamalization process, therefore displaying an alkaline pH (12–14) [14] in addition to a chemical oxygen demand (COD) of approximately 25–40 g L^{-1} and a TS concentration of approximately 9–25 g L^{-1} [20, 21]. In contrast, ABW has a COD of 20–24 g L^{-1}, pH values between 6 and 7, and a TS concentration of 30–35 g L^{-1} [15].

García-Depraect et al., reported a bioH_2 production of 6400 mL when tequila vinasses were co-digested with NEJ (ratio 20:80) and used in DF for 72 h at a 5.8 pH and a temperature of 35 °C, NEJ was used as the main source of carbon [14, 22]. This process was performed in a 3 L batch bioreactor in the presence of an inoculum containing

Lactobacillus, *Clostridium*, *Acetobacter*, and *Sporobacillus* as the predominant species [14]. In addition, Maravilla et al., evaluated the effect of NEJ concentration on bioH_2 yield. For this purpose, these researchers selected 250 mL anaerobic reactors with glucose as substrate and inoculum and maintained it at 37 °C. According to their results, the highest bioH_2 composition (47.26 ppm) was obtained after 48 h, when NEJ was added at a rate of 40% [23]. Thus, NEJ has been used in mono-digestion and co-digestion processes to produce bioH_2 via DF. However, 80% of NEJ components correspond to complex sugars that are hardly degradable by microorganisms. These polysaccharides include arabinose, xylose, glucose, and D-glucuronic acid, among others [23, 24]. Different treatments including sonochemistry can be used to improve the hydrolysis of these sugars. Sonochemical methods are efficient for the hydrolysis of lignocellulose and complex sugars [25, 26]. In contrast, ABW has been used in co-digestion with oil palm trunk hydrolysate (OPT) for bioH_2 production as the main nitrogen source. Khamtib and Reungsang reported bioH_2 production of 2604 ± 86 mL H_2 L^{-1} substrate using ABW-OPT co-digestion in 120 mL batch reactors with an initial pH of 6.50 and at a temperature of 60 °C using *Thermoanaerobacterium thermosaccharolyticm* KKU19 as inoculum [27].

Accordingly, this study aimed to determine the effect of the type of pretreatment of granular sludge (GS) inoculum: TT (100 °C/30 min) and UV irradiation (254 nm/15 min), as well as the operational mode of reactors (batch (BR) and continuous (CR)) using co-digestion of NEJ-ABW previously treated with sonochemistry in 5 L reactors for bioH_2 production. In addition, the evolution of microbial communities during DF was studied.

Materials and Methods

Inoculum

In this study, GS inoculum obtained from an anaerobic reactor operating at the wastewater treatment plant of a brewery located in Monterrey, Mexico was used. Samples were collected according to NOM-004-SEMARNAT-2002 using random sampling [8]. For each sample, the temperature and pH were measured in situ before placing them in 1 L plastic bottles after rinsing 3 times with the same sludge and stored at 4 °C until required. In this study, two different pretreatments (TT and UV irradiation) were used to suppress the growth of methanogenic microorganisms and promote bioH_2 production [28]. In the thermal pretreatment, the inoculum was heated for 30 min at 100 °C using a hot plate (Cimarec, Thermo Scientific). For UV irradiation, a UV lamp (Coospider CUV-7/11, 7 W) was used at a wavelength of 254 nm. During the procedure, the UV lamp was placed inside a quartz-cooling jacket that was immersed in a 1 L beaker containing the inoculum. The solution was stirred continuously to ensure homogenization. The complete system was placed in a dark wooden box. The inoculum was then irradiated for 15 min.

Co-digestion Procedure and Characterization

In this study, DF was performed through the co-digestion of NEJ and ABW, which have been characterized before its use. The substrate mixture was adjusted to a (C/N) ratio of 30. Before DF, hydrolysis of the polysaccharides present in the NEJ-ABW co-digestion was performed through a sonochemical procedure at 20 kHz for 30 min using an ultrasonic processor (750 watts Cole-Parmer) [25]. Subsequently, pH was adjusted to 5.50 ± 0.05 to provide the optimal environment for bioH_2 production [28]. To adjust the pH, NaOH 1N and H_2SO_4 5N solutions were used.

Set Up of Bioreactors

In these experiments, 5 L bioreactors (Sartorius Stedim Biostat A) with a working volume of 3 L were used. The bioreactors were set up in batch and continuous modes. Two separate reactors were used in the BR experiments to determine the effect of inoculum pretreatment on bioH_2 production and one in the CR experiment to determine the impact of operational conditions. As previously indicated, the inoculum was subjected to (a) TT and (b) UV. In addition, the continuous reactor with thermal treatment (CRTT) displayed the same characteristics as the batch reactor thermal treatment (BRTT). The three bioreactors were continuously stirred at 40 RPM and maintained at a temperature of 35 °C. The CRTT was fed using an internal Biostat A peristaltic pump and an external peristaltic bomb (Diagger Thermo Scientific) was used to extract the bioreactor effluent to maintain a hydraulic retention time (HRT) of 6 h. The dissolved oxygen, pH, and temperature were automatically regulated with the bioreactor software. To maintain the proper pH, NaOH 1N and H_2SO_4 1N were added as required. The biogas produced during the DF was quantified using the water displacement method [30]. In the experiments, the inoculum and the reinforced clostridial medium (RCM) (10 g L^{-1} meat extract, 10 g L^{-1} peptone, 3 g L^{-1} yeast extract, 5 g L^{-1} D-glucose, 1 g L^{-1} starch, 5 g L^{-1} NaCl, 3 g L^{-1} $C_2H_3NaO_2$, 0.5 g L^{-1} L-cysteine chloride, and 0.5 g L^{-1} agar-agar) were added to the reactor at a 10% v/v ratio with respect to the working volume. The selected working volume was 3 L, and NEJ-ABW co-digestion was performed at a C/N ratio of 30 and a pH of 5.5. The BRTT and BRUV were operated for 72 h and 60 h with an initial total volatile solid (TVS) concentration of 11,326 and 14,307

mg L^{-1}, respectively. Whereas the CRTT was operated for 20 cycles at an HRT of 6 h with an organic loading rate (OLR) of 27 ± 8 g L^{-1} d along the process.

Analytical Methods

For substrate characterization, the following parameters were determined: (a) pH, using a pH meter (Thermo Scientific); (b) total carbohydrates (CH) using the Dubois method [29]; (c) chemical oxygen demand (COD), according to NMX-AA-030-SCFI-2001 using the methodology reported by Lirio María Reyna-Gómez et al., [9]; and (d) total nitrogen (TN) was quantified using the standardized Hach method (Hach kit 10–150 mg/L). A UV-Vis Perkin Elmer Lambda 365 spectrometer was used for the CH, COD, and TN measurements.

The composition of biogas and volatile fatty acids (VFAs) were determined by gas chromatography (7820A, Agilent Technologies), as previously reported [30]. The biogas composition was determined using a thermal conductivity detector, a silica capillary column (30 m × 0.53 mm, Supelco Carboxen 1006 plot), and argon as the carrier gas. In addition, VFAs were analyzed using a flame ionization detector (FID) with an HP-Inowax column (50 m × 0.20 mm) and argon as the carrier gas.

Samples from the BRTT and BRUV were analyzed every 12 h, whereas the CRTT samples were analyzed at the end of each cycle. Physicochemical analyses were performed as previously mentioned. The measured parameters included COD, pH, and CH. Total solids (TS) and TVS were determined using the methodologies reported in the "Standard Methods for the Examination of Water and Wastewater" [31].

The bioH_2 yield was calculated using equation 1 for BR and equation 2 for CR:

$$Y_{BioH_2} = \frac{V_{BioH_2}}{TVS_{cons}} \quad (1)$$

Where V_{BioH_2} is the volume of bioH_2 produced and TVS_{cons} is the concentration of TVS consumed during the DF process.

$$Y_{BioH_2} = \frac{BH_2PR}{TVS_{influent} \bullet F_{influent}} \quad (2)$$

where: Y_{BioH_2} is expressed as a function of the OLR for TVS in the influent (mL bioH_2 g^{-1} $TVS_{influent}$), BH_2PR is the bioH_2 production rate (mL_{H2} h^{-1}), $TVS_{influent}$ is the TVS concentration in the influent gTVS mL^{-1}, $F_{influent}$ is the daily volume of the influent (mL h^{-1}).

DNA Extraction and Analysis

The microbial compositions of the BR and CR were determined. For this purpose, the following samples were analyzed: (a) BRTT at 24 and 72 h, (b) BRUV at 60 h, and (c) cycles 7 and 14. DNA extraction was done in a 0.25 g sample using the QIAGEN DNeasy PowerSoil kit [8]. In this case, cell lysis was promoted through mechanical and chemical methods that caused bacterial cell walls to rupture [32], allowing for the release of genetic material. Nucleic acids were adsorbed using different salts, and genomic DNA was captured on a silica membrane. After centrifugation (30 s at 10,000×*g*), DNA was rinsed and eluted [33]. After DNA extraction, sequencing was performed to identify the microbial species present in the samples, as previously described. The DNA samples were sent to the RTL Genomics Laboratory in Texas, USA, where amplicon sequencing was performed by PCR of the 16s rRNA gene with the aid of a MiSeq Illumina platform.

Results and Discussion

Substrate Characterization

Substrate properties have been reported to substantially affect the bioH_2 yield and stability of co-digestion systems [34]. Thus, the physicochemical characterization of substrates is required to properly design bioreactors and processes (Table 1). The results indicated that in the case of NEJ, the substrate presented an alkaline pH (10.35 ± 1.97), TN of 179 ± 80 mg L^{-1}, high CH concentration (17,747 ± 1910 mg L^{-1}), and a COD of 35,212.0 ± 2,054.0 mg L^{-1} [14, 17, 19]. In addition, ABW displayed a pH of 5.69 ± 0.06, low CH concentration (1217 ± 358 mg L^{-1}), COD of 15,969.0 ± 4,936.0, and TN levels of 2588 ± 793 mg L^{-1} [16, 20, 21]. Which are similar to those reported by García-Depraect et al., as they used NEJ as a substrate in DF to produce bioH_2. These researchers reported that NEJ presented a pH of 12 ± 0.2, TN levels of 440 ± 21.2 mg L^{-1}, and a CH concentration of 16,015.8 ± 1649.7 mg L^{-1} [14]. The pH of NEJ varies because different corn grinders use different amounts of calcium hydroxide during nixtamalization. With

Table 1 Physicochemical characteristics of individual substrates

Substrate	pH	CH (mg L^{-1})	COD (mg L^{-1})	TN (mg L^{-1})	TS (mg L^{-1})	TVS (mg L^{-1})
NEJ	10.35 ± 1.97	17,747 ± 1910	35,212 ± 2054	179 ± 80	24.21 ± 5.24	18.13 ± 4.81
ABW	5.69 ± 0.06	1217 ± 358	15,969 ± 4936	2588 ± 793	18.22 ± 0.02	16.64 ± 0.01

respect to ABW, Khamtib, and Reungsang [27] reported pH values of 7.12 ± 0.14, which are different from those obtained herein (5.69 ± 0.06). It was also found that the system contained CH concentrations of 440 ± 80 mg L^{-1}, almost a third of the values (1217 ± 358 mg L^{-1}) observed in this study. The addition of ABW to the feedstock offers a valuable opportunity to enhance process stability and improve the carbon and nitrogen balance. The contribution of N by ABW can bolster the buffer capacity [35]. Furthermore, it resulted in a significant decrease in feedstock pH compared to that of the NEJ. According to these data, both substrates were successfully used in the co-digestion when the C/N ratio was adjusted to 30. This C/N ratio is extremely important because it directly affects $bioH_2$ production during DF by providing a balance of micro-and macronutrients and promoting the buffering effect [8, 9, 36].

However, given the alkaline pH of NEJ, the 5 N H_2SO_4 solution was added until a pH of 5.5 ± 0.05 was reached. According to the literature, this pH is within the reported range and can be used even when the inoculum is pretreated to suppress methanogens [37, 38].

Effect of Inoculum Pretreatment on NEJ-ABW Co-digestion Using Batch Bioreactors

Organic Matter Removal

Figure 1 shows the results of CH consumption in the BRTT and BRUV bioreactors. In the first experiment, NEJ-ABW co-digestion showed CH consumption between 10 and 35% from the beginning of the process and up to 72 h. In contrast, BRUV presented a CH consumption of 37% during the first 12 h, reaching 70% after 60 h. These differences may be due to the higher microbial diversity of BRUV. This probably occurred because, in batch reactors, UV treatment was less effective than TT in ensuring the abatement of methanogenic activity. The TT allowed the selection of species participating in $bioH_2$ generation. To the best of our knowledge, CH removal using NEJ-ABW co-digestion has not been previously reported. Previous studies have used thermally pretreated GS as an inoculum in co-digestion processes using BR [8, 9]. In other experiments, CH was removed in 30–80% and 25–70% after 30 h of co-digestion with cheese whey-brewery and -bakery wastewater, respectively. According to the authors, the balance between macro-and micronutrients (including CH) and a C/N ratio of 30 favor the metabolic pathways involved in $bioH_2$ generation. In addition, in mono-digestion processes, CH removal was up to 50% when the C/N ratio varied between 15 and 30 [8]. Moreover, Del Angel-Acosta et al., performed a co-digestion of NEJ brewery wastewater. Their data was similar to that of this study as they indicated a CH removal of 68% after 122 h [16].

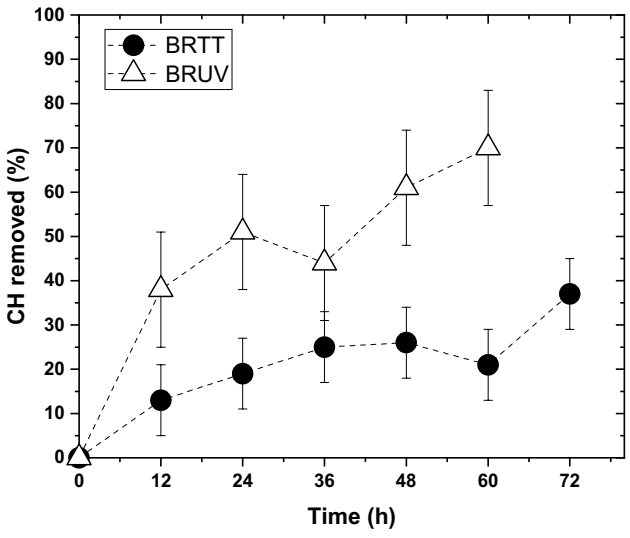


Fig. 1 CH removed during DF processes in batch reactors assay at 40 RPM, 35 ± 1 °C, and pH 5.50 ± 0.05)

Table 2 displays COD, TS, and TVS removal, as well as the percent of $bioH_2$ present in the biogas after NEJ-ABW co-digestion in batch reactors. In BRTT, the highest COD reduction was observed at 12 h. After this point, COD mostly showed a percent removal of 37 ± 10% and decreased to 19% at the end of the reaction. This behavior may be caused by the growth of $bioH_2$-producing microorganisms within the first few hours. The BRUV showed greater stability as compared to BRTT, as a COD removal of 30.0 ± 4% was achieved during the 60 h of reaction. Even when BRUV contained a higher microbial diversity (Fig. 7) metabolic pathways may present a limiting factor [37]. Thus, in our experiments, slightly higher COD removal was reached as compared to that reported by Garcia-Peña et al., who indicated a value of 25% when fruits and vegetable residues were used as substrates [39].

Table 2 Percentage of COD, TS, and TVS removed during DF in BRTT and BRUV bioreactors

Reactor	Time (h)	COD (%)	TS (%)	TVS (%)	$BioH_2$ (%)
BRTT	12	47 ± 10	4 ± 2	3 ± 2	66 ± 16
	24	25 ± 10	3 ± 2	4 ± 2	66 ± 16
	36	31 ± 10	15 ± 2	27 ± 2	11 ± 2
	48	37 ± 10	18 ± 2	31 ± 2	13 ± 2
	60	37 ± 10	17 ± 2	31 ± 2	66 ± 16
	72	19 ± 10	13 ± 2	27 ± 2	33 ± 16
BRUV	12	31 ± 4	4 ± 2	15 ± 3	16 ± 4
	24	31 ± 4	9 ± 2	21 ± 3	9 ± 4
	36	35 ± 4	15 ± 2	32 ± 2	8 ± 4
	48	30 ± 4	16 ± 2	34 ± 2	16 ± 4
	60	24 ± 4	18 ± 2	39 ± 2	17 ± 4

The data also indicated a final TS reduction of 16 ± 2 and 17 ± 2% for BRTT and BRUV, respectively. The reactor with the highest microbial diversity exhibited the greatest organic matter reduction. The same behavior was observed when TVS removal was quantified. In this case, 39% of TVS was reduced in BRUV after 60 h, whereas 27% was removed in BRTT after 72 h.

$BioH_2$ Production and Biogas Composition

Figure 2 and Table 2 show the $bioH_2$ production and COD, TS, and TVS removal in the BRTT and BRUV bioreactors, respectively. After 72 and 60 h, the accumulation of $bioH_2$ reached 359 and 191 mL, respectively. In addition, after DF in BRTT, 66 ± 27% of produced biogas corresponded to $bioH_2$ and the remainder to CO_2. Nevertheless, between 36 and 48 h, a reduction in $bioH_2$ generation occurred only in 13 ± 2% (Table 2). The absence of CH_4 in the biogas resulted from the efficiency of the inoculum pretreatment method. However, BRTT reactivation after 60 h probably occurred because of changes in the microbial consortium [40]. With respect to BRUV, the composition of the biogas corresponded to $bioH_2$ (15 ± 4%), methane (19 ± 6%), and the rest was CO_2 at 60 h. Thus, $bioH_2$ production was affected after 60 h of DF (Table 2) [28, 37].

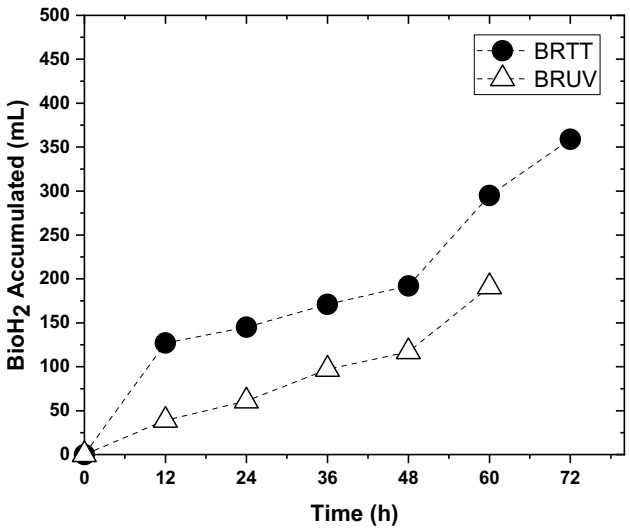


Fig. 2 $BioH_2$ accumulated during DF process in batch reactors (40 RPM, 35 ± 1 °C, pH 5.50 ± 0.05)

The results showed that the BRTT and BRUV yielded 112 and 38 mL H_2 g^{-1} TVS; and 1.96 and 0.94 mL H_2 L^{-1} h^{-1}, respectively. Accordingly, the performance of the bioreactors was completely different. Specifically, BRTT presented an efficiency and productivity of 81 and 57% higher, compared to BRUV.

Table 3 presents $bioH_2$ production when different substrates and GS were used in the DF process, as well as the results obtained in the present study. These data indicate that the yields obtained in our experiments were 8 times higher than those obtained in previous studies [39, 41, 42]. In contrast, $bioH_2$ production in the present study was slightly lower than that shown in Table 3. This behavior was attributed to the use of more complex substrates. In addition, microorganisms require more time to adapt to the carbon sources (NEJ) [17].

Volatile Fatty Acids

Figure 3a and b show the production of VFAs during DF in the co-digestion of NEJ-ABW in the BRTT (12, 24, 48, and 72 h) and BRUV (12 and 60 h). In BRTT (Fig. 3a), the predominant compound was acetic acid (24–18 mmol), followed by formic acid (5–2 mmol), propionic acid (~1 mmol), and butyric acid (<1 mmol). The highest VFA concentration (38 mmol) was observed after 12 h. At this point,

Table 3 Comparison of hydrogen yield and productivity in $bioH_2$ production in different studies

Operation mode	Codigestion	Inoculum and Pretreatment	Operation conditions	Hydrogen yield (mL h_2/gtvs)	Productivity (mLh_2/L h)	Reference
BATCH	Fruit peels + primary sludge	Granular sludge, TT	C/N = 30, pH 5.5, T = 37 °C, V = 0.1 L	25	2.6	[42]
BATCH	Fruit and vegetable wastes	Granular sludge, TT	C/N = NR, pH 5.5, T = 35 °C, V = 0.5 L	60	1.58	[40]
BATCH	Municipal wastes + food wastes	Granular sludge, TT	C/N = NR, pH 4.0, T = 30 °C, V = 0.125 L	14	NR	[41]
BATCH	BRTT	Granular sludge, TT	C/N = 30, pH 5.5, T = 35 °C, V = 3 L	112	1.96	This study
BATCH	BRUV	Granular sludge, UV	C/N = 30, pH 5.5, T = 35 °C, V = 3 L	38	0.94	This study

T temperature, *V* volume, *NR* not reported

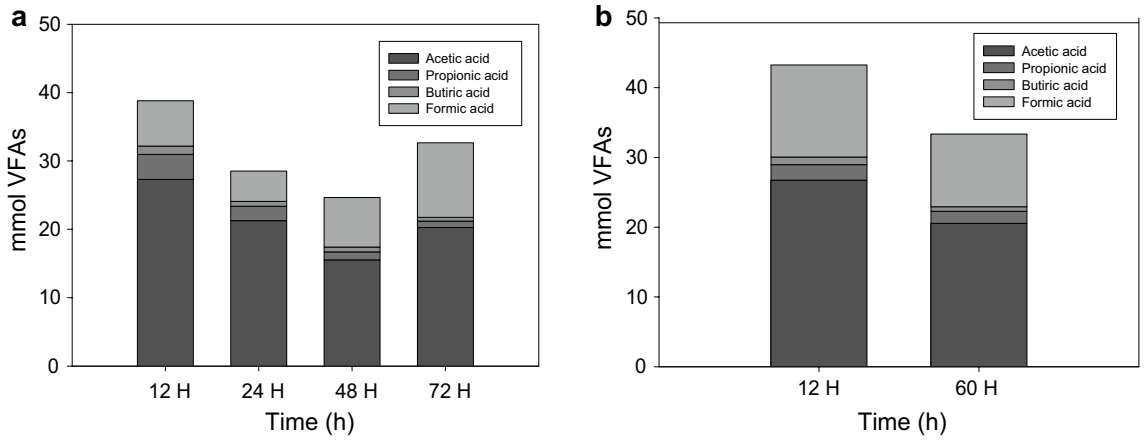


Fig. 3 VFAs production (mmol) during DF carried out in batch reactors: **a** BRTT, **b** BRUV (40 RPM, 35 ± 1 °C, pH 5.50 ± 0.05)

27 mmol acetic acid was generated. The maximum bioH_2 production occurred at this time, reaching 127 mL of bioH_2. This value represents 66% of bioH_2 present in the biogas. These metabolites indicate that butyrate-acetate fermentation occurred [43]. These data agree with those reported by Khamtib and Reungsang, who co-digested palm tree hydrolysates and ABW. They also found that the highest bioH_2 production during the DF process was reached when acetic acid and butyric acid presented the maximum concentrations [27].

The VFA concentration was slightly higher in BRUV than that of BRTT (Fig. 3b), as 43 mmol of these compounds was quantified after 13 h of DF. In addition, 13 mmol corresponded to formic acid. In addition, VFA concentrations decreased after 60 h. This was attributed to changes in the metabolic pathways associated with CH_4 production. The concentration of formic acid was maintained at a constant. According to Chong et al., VFA accumulation hinders the DF by increasing the ionic strength of the medium. However, this process did not completely inhibit the methanogenic hydrogen-consuming bacteria present in the inoculum [44].

Effect of Continuous Operation on NEJ-ABW Co-digestion

Organic Matter Removal

Figure 4 shows CH consumption when NEJ-ABW was digested for 20 cycles in CRTT operated at an HRT of 6 h. The highest variation in CH degradation was observed in the first cycle (60%). In the second cycle, degradation decreased to 40% and increased until reaching 70%, a potential cause of this variation can be the addition of RCM. It then decreased during cycles 11 and 12 before increasing again. This behavior could be explained by the adaptation of the microbial species involved in the co-digestion process.

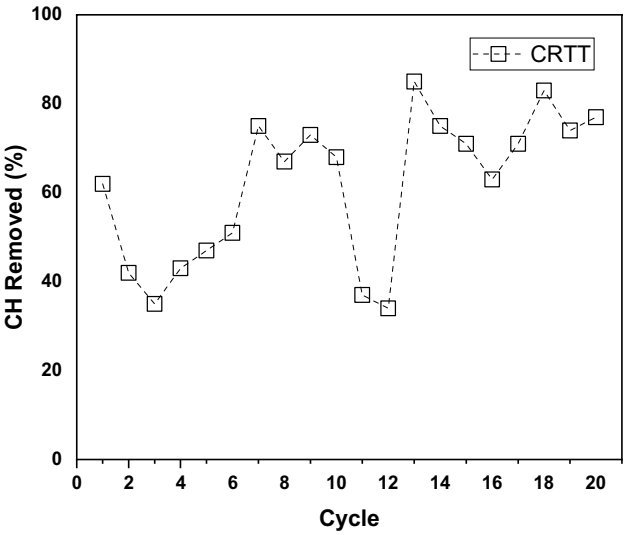


Fig. 4 CH removed when DF was performed in a CRTT. (40 RPM, 35 ± 1 °C, pH 5.50 ± 0.05, HRT 6 h)

According to the results, 85% of CH was removed in CRTT in cycle 13. In contrast, the BRTT showed a reduction of 37% CH. Thus, when DF was carried out in continuous mode, the microbial communities were able to adapt better because of the constant presence of nutrients in the substrate [39, 45]. These results are superior to those reported in previous studies in which NEJ was used as the main source of CH [16].

Table 4 shows the data for organic matter removal (COD, TS, and TVS) during NEJ-ABW co-digestion in CRTT with an OLR of 27 ± 8 g L^{-1} d. Results correspond to cycles 3, 7, 10, and 17 when the reactor was operated at an HRT of 6 h. The data indicated that 22 and 42% of COD removal

Table 4 Percentage of COD, TS, and TVS removed during DF in CRTS

Reactor	Cycle	COD removed (%)	TS removed (%)	TVS removed (%)
CRTT	3	22 ± 12	3 ± 2	20 ± 7
	7	32 ± 12	28 ± 2	49 ± 7
	10	37 ± 12	22 ± 2	43 ± 7
	17	42 ± 12	10 ± 2	37 ± 7

was achieved and there was no significant difference. These numbers were higher than those obtained with the BRTT. In the experiments, COD was maintained between 10 and 15 g L^{-1} and removal was 3–28%, which is in correspondence to CH reduction. Thus, CRTT showed higher consumption than BRTT because, in the former, more nutrients were available due to 6 h of HRT, which facilitated the adaptation of $bioH_2$ producing microorganisms [37]. The TVS were degraded at a rate between 20 and 49%. These data were similar to those published in [8]. These researchers reported a TS and TVS removal of 20 and 40%, respectively, when cheese whey and brewery wastewater were co-digested in the presence of GS using an up-flow anaerobic sludge blanket (UASB) reactor operated at an HRT of 9 h.

$BioH_2$ Production and Yield

Figure 5 shows cumulative $bioH_2$ production when NEJ-ABW was used as the substrate in the DF of the CRTT operated at an HRT of 6 h. After 20 cycles, the $bioH_2$ volume was 1805 mL, which is 5 times higher than that obtained using BRTT [46]. However, $bioH_2$ showed a concentration of 10–20% over the 20 cycles. It is likely that the operating conditions affected these results as $bioH_2$ producing microorganisms were probably washed out [8, 47] and microorganisms indigenous to the substrate probably proliferated.

Given that during the 20 cycles a high amount of $bioH_2$ was accumulated, CRTT showed a higher yield (223 mL H_2 g^{-1} TVS) and productivity (8 mL H_2 L^{-1} h^{-1}) than BRTT (Table 3). Garcia-Peña et al. used fruit and vegetable residues and reported a yield of 60 mL H_2 g^{-1} TVS and a productivity of 1.58 mL H_2 L^{-1} h^{-1} [40]. In contrast, Reyna-Gómez obtained a yield of 25 mL H_2 g^{-1} TVS and a productivity of 2.6 mL H_2 L^{-1} h^{-1} when they performed the co-digestion of fruit peels and sewage sludge [30]. The values obtained in the present study were slightly higher than those previously reported. However, when NEJ and tequila vinasses were used as substrates, a yield of 91 mL H_2 g^{-1} COD, and productivity of 102 mL H_2 L^{-1} h^{-1} were achieved [34].

Volatile Fatty Acids

Figure 6 shows the VFAs produced during cycles 7, 10, and 14. The highest concentration in the three cycles corresponded to acetic acid, with values between 13 and 17.5 mmol. These data agree with the results obtained for $bioH_2$ (9–20% in the biogas). In addition, in the three cycles, CH removal was approximately 70%. Our results showed that other metabolic pathways were present in cycle 10, after which the concentration of propionic acid was increased to 8 mmol. The highest VFA concentration was observed during this cycle. It is likely that this process negatively affected $bioH_2$ production. Propionic acid inhibits $bioH_2$ generation during DF. To produce propionic acid, molecular H_2 must be consumed [43, 48]. This process has been previously reported by Cruz-López et al. They observed high acetic

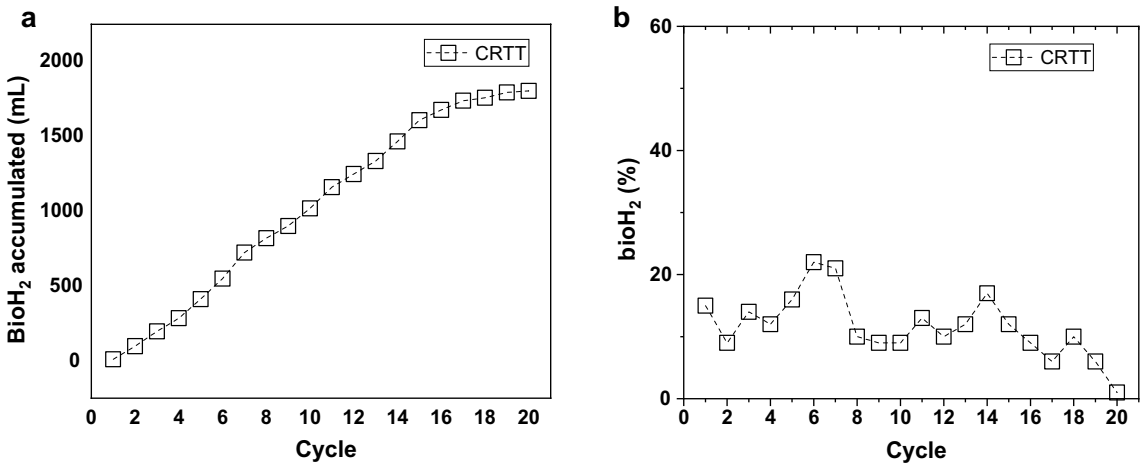


Fig. 5 **a** $BioH_2$ accumulated during DF performed in CRTT. **b** $BioH_2$ in biogas during DF carried out in CRTT. (40 RPM, 35 ± 1 °C, pH 5.50 ± 0.05, HRT 6 h)

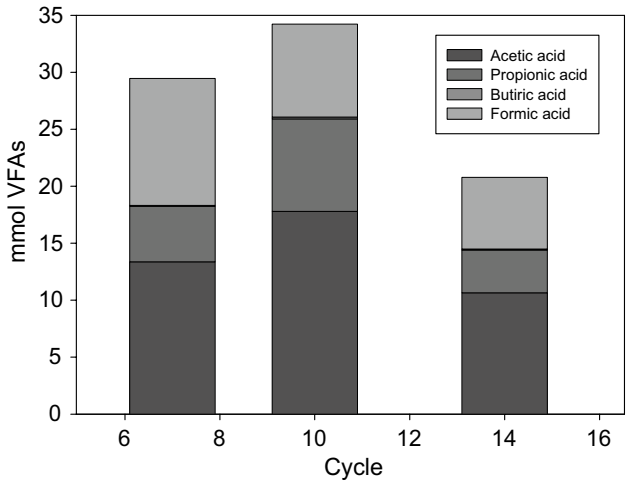


Fig. 6 VFAs production (mmol) when DF took place in CRTT (40 RPM, 35 ± 1 °C, pH 5.50 ± 0.05, HRT 6 h)

acid concentrations when a continuous bioreactor was operated at an HRT of 9 h during the co-digestion of cheese whey and brewery wastewater [8]. Similarly, in cycle 10, the concentration of propionic acid was increased to 550 mg L^{-1} [8] and acetic acid was the predominant acid. For this reason, $bioH_2$ production remained constant.

Microbial Composition in Bioreactors

To characterize the microbiome, samples were collected from the reactor at different reaction times. DNA extraction was performed, and nucleic acids were identified by amplicon sequencing using the MiSeq Illumina platform. Figure 7 shows the microbial diversity at the species level for BRTT and BRUV. In the BRTT, *Clostridium* was the dominant species with 87 and 68% after 24 and 72 h, respectively. As shown in this Figure, after 24 h, the predominant species was *Clostridium butyricum* (87%), followed by *Clostridium beijerinckii* with only 1% of total *Clostridium*. Moreover, after 72 h, higher *Clostridium* diversity was observed. In this case, *Clostridium lundense* was present in 25%, *Clostridium* sp. in 13%, *C. beijerinckii* in 12%, *C. butyricum* in 9%, *Clostridium sporogenes* in 5%, and *Clostridium oryzae* in only 1%.

The presence of *C. butyricum* was confirmed 24 h after the BRTT. This species is known as a $bioH_2$ producer when mesophilic conditions prevail [40]. It is also known for its high $bioH_2$ conversion rate regardless of substrate complexity. A maximum $bioH_2$ yield of 3.6 mol H_2 mol^{-1} hexose was reported when *C. butyricum* was used to digest organic residues. This value is similar to the maximum theoretical $bioH_2$ yield of 4 mol H_2 mol^{-1} hexose [49]. Other authors have used *C. butyricum* as an inoculum in addition to pure glucose and hydrolyzed starch as substrates where yields of 1.98 and 1.5 mol H_2 mol^{-1} glucose were produced, respectively [50, 51]. The *C. butyricum* genome contains the *hydA* gene, which encodes hydrogenases. These enzymes are involved in monosaccharide metabolism and $bioH_2$ production [52].

In the BRTT, *C. beijerinckii* was the predominant species. Both *C. butyricum* and *C. Beijerinckii* are strict anaerobic bacteria that have been identified as highly efficient $bioH_2$ producers. This is due to their theoretical yields of approximately 4 mol $bioH_2$ mol^{-1} glucose. However, the maximum experimental value appeared to be 2 mol $bioH_2$ mol^{-1} glucose [47, 53]. *C. beijerinckii* is predominantly found in microbial consortia used in $bioH_2$ production under mesophilic conditions. García-Depraect and León-Becerril used a continuous stirred-tank reactor (CSTR) loaded with tequila vinasses at a temperature of 35 °C. They reported a yield of 124 mL H_2 g^{-1} TVS and identified *C. beijerinckii* as the predominant species [54]. Rambabu et al., performed the fermentation of rice industry wastewater in a BR with the same bacteria under mesophilic conditions (37 °C)

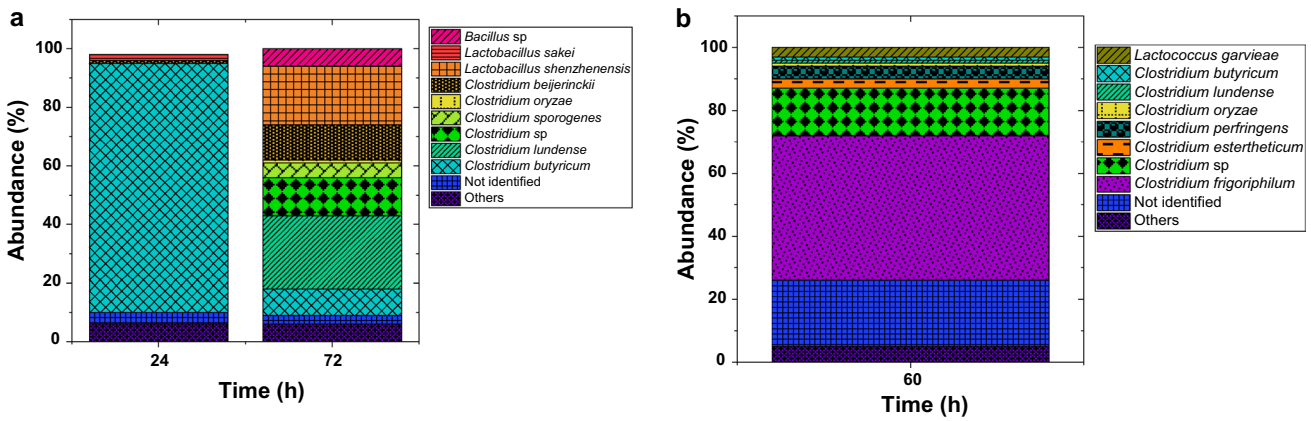


Fig. 7 Microbial diversity at different times during DF process: **a** BRTT **b** BRUV (40 RPM, 35 ± 1 °C, pH 5.50 ± 0.05)

and reported a yield of 215 mL H_2 L^{-1} [55]. Thus, if the proper conditions are met, the presence of these two species guarantees efficient bioH_2 production.

In our experiments, *C. lundense* was present in higher proportions in the BRTT after 72 h. Even when this species does not produce bioH_2, it is usually studied because it can hydrolyze lipids. Thus, it may participate in the hydrolysis of DF [56]. These microorganisms are spore-forming bacteria that can grow under mesophilic conditions and at pHs between 5.5 and 7 [56, 57]. In the present study, it was also determined that *Lactobacillus shenzhenensi* represented 20% of the BRTT microbiota after 72 h. Even when *Lactobacillus* is not able to form spores [58], their presence in the final stages of DF may be due to the protection provided by GS [42]. It was also observed that the percentage of *Lactobacillus shenzhenensi* increased from 1 to 20% during the last 48 h of bioreactor operation. The positive effects of lactic acid bacteria on DF have been extensively discussed [37]. In the present study, our data indicated that *Lactobacillus* inhibited bioH_2 production because of competition with bacteria of the genus *Clostridium* [40].

Similar to BRTT, 73% of BRUV microbiota corresponded to *Clostridium*. After 60 h of BRUV operation, among the predominant species, *Clostridium frigoriphilum* (46%), *Clostridium* sp. (15%), *Clostridium perfringens* (4%), *Clostridium estertheticum* (3%), and *C. oryzae* (1%) have all shown potential as bioH_2 producers; however, as shown in Fig. 7, only 1% was featured by *C. butyricum*, which is a highly efficient bioH_2 producer [59]. According to these results, the low bioH_2 production observed in the BRUV was caused by the low rate of BioH_2-producing *Clostridium*. The predominant species in this reactor, *C. frigoriphilum*, and *C. estertheticum*, have been reported to be tolerant to low temperatures and are responsible for the decomposition of vacuum-packaged frozen meat [60]. The presence of these species was probably due to the nature of the ABW used during substrate co-digestion. In these experiments, meat was used to prepare a synthetic solution to simulate the abattoir wastewater [61].

In addition, it is likely that the presence of CH_4 in BRUV was responsible for the low bioH_2 production. No methanogens were identified during this stage of the study. However, after 60 h of DF, 21% of the microbiota were not characterized. Some methanogenic species may be present in this portion of the microbiota. These microorganisms also participated in organic matter degradation.

The CRTT microbiota was analyzed in cycles 7 and 14. The highest CH consumption (75%) was observed in cycle 7. In addition, the maximum bioH_2 production (17% bioH_2 in the biogas, 131 mL total bioH_2) was observed in cycle 14.

Figure 8 presents CRTT microbial diversity at the species level during cycles 7 and 14, where *Clostridium* represented the most predominant genera at concentrations of 59 and 62%, respectively. During cycle seven, the distribution was *Clostridium* sp., (47%), *C. frigoriphilum* (5%), *C. oryzae* (4%), *C. butyricum* (1%), *C. beijerinckii* (1%), and *Clostridium carboxidivorans* (1%). To the best of our knowledge, this is the first time *Clostridium carboxidivorans* have been identified in these reactor types. These bacteria are classified as acetogenic, and produce acetate, ethanol, butyrate, and butanol [62]. Therefore, it is considered to be a bioH_2-producing bacteria. However, *C. butyricum*, *C. acetobutyricum*, and *C. beijerinckii* [63] displayed higher efficiencies than the former. During cycle 14, lower *Clostridium* diversity was observed. Specifically, *Clostridium* spp. and *C. oryzae* were found in proportions of 97 and 3%, respectively. However, a high proportion of these bacteria might be responsible for the high bioH_2 production. Also, in cycle 7, a total of 4% *Ethanoligenens* sp., were quantified. These bacteria produced up to

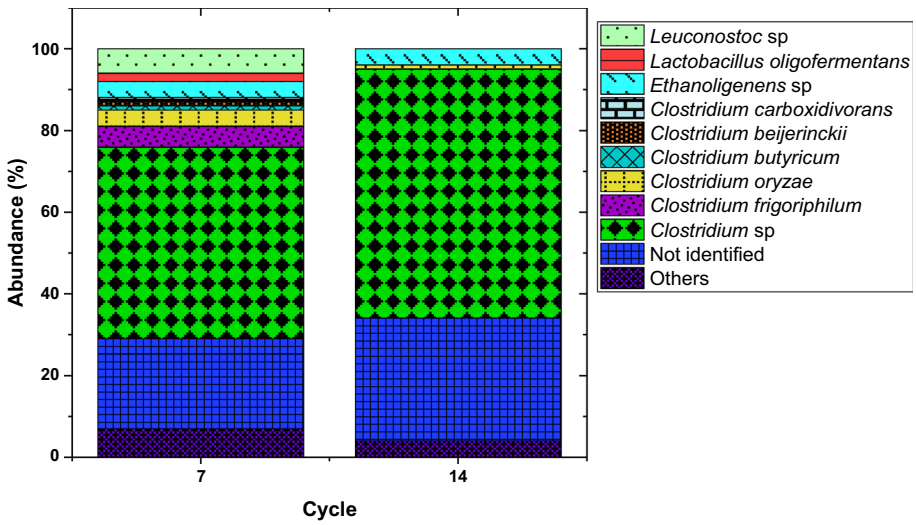


Fig. 8 Microbial diversity at cycles 7 and 14 when DF was performed in CRTT (40 RPM, 35 ± 1 °C, pH 5.50 ± 0.05, HRT 6 h)

2.6 mol $bioH_2$/mol substrate [40, 55]. They also showed high hydrolytic capacity. Therefore, they show high performance during CH removal, and consequently, elevated CH consumption was observed in this cycle [40]. Other bacteria that were identified included *Leuconostoc* sp. (6%) and *Lactobacillus oligofermenans* (2%), which hinder $bioH_2$ production as they compete for the substrate with $bioH_2$-producing species [45]. Notably, *Lactobacillales* produce different CH-degrading enzymes including fructokinase, lactate dehydrogenase, and glucose 6 phosphate dehydrogenase [64]. In contrast to cycle 7, reduced microbial diversity was observed in cycle 14 because only *Clostridiaceae* and a small percentage of *Ethanoligenens* spp. were identified. The absence of *Lactobacillus* resulted in low $bioH_2$. In addition, a high proportion of *Clostridium* species was observed during this cycle.

Conclusions

In this study, the effect of inoculum pretreatment on $bioH_2$ production was determined. Our data indicated that the best pretreatment corresponded to TT because it resulted in the most effective microbial diversity for $bioH_2$ production compared to UV treatment. In this case, the predominant bacterial species was *Clostridium*, which produces $bioH_2$. In addition, the highest $bioH_2$ productivity (8 mL H_2 L^{-1} h^{-1}) and maximum yield (223 mL H_2 g^{-1} TVS) were obtained when the reactor was operated in continuous mode at an HRT of 6 h. The results presented herein prove that continuous $bioH_2$ production can be achieved through co-digestion of DF with NEJ-ABW. Therefore, we present an alternative method for treating wastewater generated by these industries.

Acknowledgements The authors acknowledge SENER-CONACyT-Sustentabilidad Energética for providing funding (CONACYT Research Project Code No. 249908), UANL for providing financial support through the PAICYT Research Program (485-IT2022), and the Environmental Engineering Research Laboratory of the UANL Institute of Civil Engineering. RCCF thanks CONACYT for the graduate scholarship No. 1080269.

Author Contributions All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by RCCF and LMRG. The first draft of the manuscript was written by RCCF and ACL. All authors commented on previous versions of the manuscript and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Funding This work was supported by SENER-CONACyT-Sustentabilidad Energética and Universidad Autónoma de Nuevo León, Mexico. RCCF has received research support from CONACyT, Mexico.

Data Availability Data are available from the corresponding author on request.

Declarations

Competing Interests The authors declare no competing interests.

Ethical Approval Not applicable.

References

1. Abas, N., Kalair, A., Khan, N.: Review of fossil fuels and future energy technologies. Futures **69**, 31–49 (2015). https://doi.org/10.1016/j.futures.2015.03.003
2. Zhang, L., Zhang, B., Zhu, X., Chang, H., Wang, S.O.H.: Role of bioreactors in microbial biomass and energy conversion. In: Liao, Q., Chang, J., Herrmann, C., Xia, A. (eds.) Bioreactors for microbial biomass and energy conversion, pp. 39–80. Springer, Singapore (2018)
3. Johnston, B., Mayo, M.C., Khare, A.: Hydrogen: the energy source for the 21st century. Technovation **25**(6), 569–585 (2005). https://doi.org/10.1016/j.technovation.2003.11.005
4. Hallenbeck, P.C., Ghosh, D.: Advances in fermentative biohydrogen production: the way forward? Trends Biotechnol. **27**(5), 287–297 (2009). https://doi.org/10.1016/j.tibtech.2009.02.004
5. Akinbomi, J., Taherzadeh, M.J.: Evaluation of fermentative hydrogen production from single and mixed fruit wastes. Energies **8**(5), 4253–4272 (2015). https://doi.org/10.3390/en8054253
6. Alexandropoulou, M., Antonopoulou, G., Lyberatos, G.: Food industry waste's exploitation via anaerobic digestion and fermentative hydrogen production in an up-flow column reactor. Waste Biomass Valor. **7**(4), 711–723 (2016). https://doi.org/10.1007/s12649-016-9544-y
7. Valta, K., Damala, P., Angeli, E., Antonopoulou, G., Malamis, D., Haralambous, K.J.: Current treatment technologies of cheese whey and wastewater by Greek cheese manufacturing units and potential valorisation opportunities. Waste Biomass Valor. **8**, 1649–1663 (2017). https://doi.org/10.1007/s12649-017-9862-8
8. Cruz-López, A., Cruz-Méndez, A., Suárez-Vázquez, S.I., Reyna-Gómez, L.M., Pecina-Chacón, D.E., de León Gómez, H.: Effect of hydraulic retention time on continuous biohydrogen production by the codigestion of brewery wastewater and cheese whey. Bioenergy Res. (2022). https://doi.org/10.1007/s12155-022-10399-0
9. Reyna-Gómez, L.M., Molina-Guerrero, C.E., Alfaro, J.M., Vázquez, S.I.S., Robledo-Olivo, A., Cruz-López, A.: Effect of carbon/nitrogen ratio, temperature, and inoculum source on hydrogen production from dark codigestion of fruit peels and sewage sludge. Sustainable **11**(7), 1–13 (2019). https://doi.org/10.3390/SU11072139
10. Preethi, T.M.M., Usman, J.R., Banu, M.G., Kumar, G.: Biohydrogen production from industrial wastewater: an overview. Bioresour. Technol. Rep. **7**, 100287 (2019). https://doi.org/10.1016/j.biteb.2019.100287
11. Odejobi, O.J., Ajala, O.O., Osuolale, F.N.: Anaerobic co-digestion of kitchen waste and animal manure: a review of operating parameters, inhibiting factors, and pretreatment with their impact on process performance. Biomass Convers. Biorefin. **13**(7), 5515–5531 (2023). https://doi.org/10.1007/s13399-021-01626-3
12. Ghimire, A., et al.: Effects of operational parameters on dark fermentative hydrogen production from biodegradable complex waste biomass. Waste Manag. **50**, 55–64 (2016). https://doi.org/10.1016/j.wasman.2016.01.044

13. Argun, H., Dao, S.: Bio-hydrogen production from waste peach pulp by dark fermentation: effect of inoculum addition Hidayet. Int. J. Hydrogen Energy **42**(4), 2569–2574 (2017). https://doi.org/10.1016/j.ijhydene.2016.06.225
14. García-Depraect, O., Gómez-Romero, J., León-Becerril, E., López-López, A.: A novel biohydrogen production process: co-digestion of vinasse and Nejayote as complex raw substrates using a robust inoculum. Int. J. Hydrogen Energy **42**(9), 5820–5831 (2017). https://doi.org/10.1016/j.ijhydene.2016.11.204
15. Hernández, S.C., Jiménez, L.D., García, J.A.B.: Potential of energy production from slaughterhouse wastewater. Interciencia **43**(8), 558–565 (2018)
16. Del Angel-Acosta, Y.A., Alvarez, L.H., Garcia-Reyes, R.B., Carrillo-Reyes, J., Garcia-Gonzalez, A., Meza-Escalante, E.R.: Co-digestion of corn (nejayote) and brewery wastewater at different ratios and pH conditions for biohydrogen production. Int. J. Hydrogen Energy **46**(54), 27422–27430 (2021). https://doi.org/10.1016/j.ijhydene.2021.05.208
17. España-Gamboa, E., Domínguez-Maldonado, J.A., Tapia-Tussell, R., Chale-Canul, J.S., Alzate-Gaviria, L.: Corn industrial wastewater (nejayote): a promising substrate in Mexico for methane production in a coupled system (APCR-UASB). Environ. Sci. Pollut. Res. **25**(1), 712–722 (2018). https://doi.org/10.1007/s11356-017-0479-z
18. Díaz-Montes, E., Castro-Muñoz, R.: Analyzing the phenolic enriched fractions from Nixtamalization wastewater (Nejayote) fractionated in a three-step membrane process. Curr. Res. Food Sci. **5**, 1–10 (2022). https://doi.org/10.1016/j.crfs.2021.11.012
19. Salmerón-Alcocer, A., et al.: Aerobic treatment of maize-processing wastewater (nejayote) in a single-stream multi-stage bioreactor. J. Environ. Eng. Sci. **2**(5), 401–406 (2003). https://doi.org/10.1139/s03-046
20. Castro-Muñoz, R., Fíla, V., Durán-Páramo, E.: A review of the primary by-product (nejayote) of the nixtamalization during maize processing: potential reuses. Waste Biomass Valor. **10**, 13–22 (2019). https://doi.org/10.1007/s12649-017-0029-4
21. Campechano Carrera, E.M., de Dios Figueroa, J., Cárdenas, G.A., Villa, H.E., Martínez Flores, S.J., Sandoval, J., Luna Bárcenas, J.G.: New ecological nixtamalisation process for tortilla production and its impact on the chemical properties of whole corn flour and wastewater effluents. Int. J. Food Sci. Technol. **47**(3), 564–571 (2012). https://doi.org/10.1111/j.1365-2621.2011.02878.x
22. García-Depraect, O., Rene, E.R., Gómez-Romero, J., López-López, A., León-Becerril, E.: Enhanced biohydrogen production from the dark co-fermentation of tequila vinasse and nixtamalization wastewater: novel insights into ecological regulation by pH. Fuel **253**, 159–166 (2019). https://doi.org/10.1016/j.fuel.2019.04.147
23. Maravilla, A.R., Marion, S.T., Samaniego, J.G., Romero, F.T.: Producción de hidrógeno por digestion anaerobia del nejayote. Fuel **253**, 159 (2016)
24. Zúñiga-Sánchez J.O.: Evaluación del uso del nejayote para la producción de goma xantano por *Xanthomonas campestris* y su escalamiento a nivel semi-piloto. p. 61, (2020)
25. Ong, V.Z., Wu, T.Y.: An application of ultrasonication in lignocellulosic biomass valorisation into bio-energy and bio-based products. Renew. Sustain. Energy Rev. **132**, 109924 (2020). https://doi.org/10.1016/j.rser.2020.109924
26. Stefanovic, B., Rosenau, T., Potthast, A.: Effect of sonochemical treatments on the integrity and oxidation state of cellulose. Carbohydr. Polym. **92**(1), 921–927 (2013). https://doi.org/10.1016/j.carbpol.2012.09.039
27. Khamtib, S., Reungsang, A.: Co-digestion of oil palm trunk hydrolysate with slaughterhouse wastewater for thermophilic bio-hydrogen production by *Thermoanaerobacterium thermosaccharolyticm* KKU19. Int. J. Hydrogen Energy **39**(13), 6872–6880 (2014). https://doi.org/10.1016/j.ijhydene.2014.02.073
28. Yun, J., Kim, T.G., Cho, K.S.: Suppression of methanogenesis in hydrogen fermentation by intermittent feeding. J. Environ. Sci. Heal. Part A **50**(14), 1458–1467 (2015). https://doi.org/10.1080/10934529.2015.1074480
29. Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F.: Colorimetric method for determination of sugars and related substances. Anal. Chem. (1956). https://doi.org/10.1021/ac60111a017
30. Reyna-Gómez, L.M., Cruz-López, A., Alfaro, J.M., Suárez-Vázquez, S.I.: Evaluation of the production of biohydrogen during the co-digestion of organic wastes in an upflow hybrid anaerobic reactor. Chem. Eng. J. **425**, 129235 (2021). https://doi.org/10.1016/j.cej.2021.129235
31. APHA: Standard methods for the examination of water and wastewater. Stand. Methods, (2005). ISBN 9780875532356
32. Madigan M.T., Martinko J.M., Bender K.S., Buckley D.H., Stahl D.A.: Brock—biologia de los microorganismos. In: Metabolismo microbiano, (2015)
33. Casanova-Mina, A.A., Suárez-Vázquez, S.I., Acuña-Askar, K., Alfaro-Barbosa, J.M., Cruz-López, A.: Continuous dark fermentation by industrial food wastewater: the effect of hydraulic retention time on hydrogen production and microbial variation. Biomass Convers. Biorefin. (2023). https://doi.org/10.1007/s13399-023-04596-w
34. Wong, Y.M., Wu, T.Y., Juan, J.C.: A review of sustainable hydrogen production using seed sludge via dark fermentation. Renew. Sustain. Energy Rev. **34**, 471–482 (2014). https://doi.org/10.1016/j.rser.2014.03.008
35. Basak, B., Fatima, A., Jeon, B.-H., Ganguly, A., Chatterjee, P.K., Dey, A.: Process kinetic studies of biohydrogen production by co-fermentation of fruit-vegetable wastes and cottage cheese whey. Energy Sustain. Dev. **47**, 39–52 (2018). https://doi.org/10.1016/j.esd.2018.08.004
36. Vijayaraghavan, K., Ahmad, D., Soning, C.: Bio-hydrogen generation from mixed fruit peel waste using anaerobic contact filter. Int. J. Hydrogen Energy (2007). https://doi.org/10.1016/j.ijhydene.2007.07.001
37. Bernardo Ruggeri, S.S., Tommasi, T.: BioH_2 & BioCH_4 through anaerobic digestion: from research to full-scale applications. Springer, London (2015)
38. Cisneros-Pérez, C., Carrillo-Reyes, J., Celis, L.B., Alatriste-Mondragón, F., Etchebehere, C., Razo-Flores, E.: Inoculum pretreatment promotes differences in hydrogen production performance in EGSB reactors. Int. J. Hydrogen Energy **40**(19), 6329–6339 (2015). https://doi.org/10.1016/j.ijhydene.2015.03.048
39. Garcia-Peña, E.I., Canul-Chan, M., Chairez, I., Salgado-Manjarez, E., Aranda-Barradas, J.: Biohydrogen production based on the evaluation of kinetic parameters of a mixed microbial culture using glucose and fruit-vegetable waste as feedstocks. Appl. Biochem. Biotechnol. **171**, 279–293 (2013). https://doi.org/10.1007/s12010-013-0341-9
40. Dzulkarnain, E.L.N., Audu, J.O., Wan Dagang, W.R.Z., Abdul-Wahab, M.F.: Microbiomes of biohydrogen production from dark fermentation of industrial wastes: current trends, advanced tools and future outlook. Bioresour. Bioprocess. **9**(16), 1–25 (2022). https://doi.org/10.1186/s40643-022-00504-8
41. Abubackar, H.N., et al.: Effects of size and autoclavation of fruit and vegetable wastes on biohydrogen production by dark dry anaerobic fermentation under mesophilic condition. Int. J. Hydrogen Energy **44**(33), 17767–17780 (2019). https://doi.org/10.1016/j.ijhydene.2019.05.106
42. Reyna-Gómez, L.M.: Producción de H_2 a partir de la codigestión de cáscaras de frutas y lodos de una planta tratadora de agua

residual a escala laboratorio. Universidad Autónoma de Nuevo León, San Nicolás de los Garza (2021)
43. Wang, J., Yin, Y.: Progress in microbiology for fermentative hydrogen production from organic wastes. Crit. Rev. Environ. Sci. Technol. **41**(1), 825–865 (2019). https://doi.org/10.1080/10643389.2018.1487226
44. Chong, M.-L., Sabaratnam, V., Shirai, Y., Hassan, M.A.: Biohydrogen production from biomass and industrial wastes by dark fermentation. Int. J. Hydrogen Energy **34**(8), 3277–3287 (2009). https://doi.org/10.1016/j.ijhydene.2009.02.010
45. Tapia-Venegas, E., et al.: Adaptation of acidogenic sludge to increasing glycerol concentrations for biohydrogen production. Appl. Microbiol. Biotechnol. **99**, 8295–8308 (2015). https://doi.org/10.1007/s00253-015-6832-6
46. Kovalev, A.A., et al.: Biochemical hydrogen potential assay for predicting the patterns of the kinetics of semi-continuous dark fermentation. Bioresour. Technol. **376**, 128919 (2023). https://doi.org/10.1016/j.biortech.2023.128919
47. Sivagurunathan, P., Anburajan, P., Kumar, G., Kim, S.H.: Effect of hydraulic retention time (HRT) on biohydrogen production from galactose in an up-flow anaerobic sludge blanket reactor. Int. J. Hydrogen Energy (2016). https://doi.org/10.1016/j.ijhydene.2016.06.047
48. Liang, Z., et al.: Removal of volatile organic compounds (VOCs) emitted from a textile dyeing wastewater treatment plant and the attenuation of respiratory health risks using a pilot-scale biofilter. J. Clean. Prod. (2020). https://doi.org/10.1016/j.jclepro.2020.120019
49. Kivistö, A., Santala, V., Karp, M.: Non-sterile process for biohydrogen and 1,3-propanediol production from raw glycerol. Int. J. Hydrogen Energy **38**(27), 11749–11755 (2013). https://doi.org/10.1016/j.ijhydene.2013.06.119
50. Argun, H., Kargi, F., Kapdan, I.K.: Microbial culture selection for bio-hydrogen production from waste ground wheat by dark fermentation. Int. J. Hydrogen Energy **34**(5), 2195–2200 (2009). https://doi.org/10.1016/j.ijhydene.2008.12.066
51. Baeyens, J., et al.: Reviewing the potential of bio-hydrogen production by fermentation. Renew. Sustain. Energy Rev. **131**, 11–23 (2020). https://doi.org/10.1016/j.rser.2020.110023
52. Wang, M.Y., Olson, B.H., Chang, J.S.: Relationship among growth parameters for *Clostridium butyricum*, hydA gene expression, and biohydrogen production in a sucrose-supplemented batch reactor. Appl. Microbiol. Biotechnol. **78**, 525–532 (2008). https://doi.org/10.1007/s00253-007-1317-x
53. Behera, B.K., Varma, A.: Microbial resources for sustainable energy. Springer, Switzerland (2016)
54. García-Depraect, O., León-Becerril, E.: Fermentative biohydrogen production from tequila vinasse via the lactate-acetate pathway: operational performance, kinetic analysis and microbial ecology. Fuel **234**, 151–160 (2018). https://doi.org/10.1016/j.fuel.2018.06.126
55. Rambabu, K., Bharath, G., Thanigaivelan, A., Das, D.B., Show, P.L., Banat, F.: Augmented biohydrogen production from rice mill wastewater through nano-metal oxides assisted dark fermentation. Bioresour. Technol. **319**, 124243 (2021). https://doi.org/10.1016/j.biortech.2020.124243
56. Cirne, D.G., Delgado, O.D., Marichamy, S., Mattiasson, B.: *Clostridium lundense* sp. Nov., a novel anaerobic lipolytic bacterium isolated from bovine rumen. Int. J. Syst. Evol. Microbiol. **56**(3), 625–628 (2006). https://doi.org/10.1099/ijs.0.63730-0
57. Wikoff, W.R., et al.: Metabolomics analysis reveals large effects of gut microflora on mammalian blood metabolites. Proc. Natl. Acad. Sci. USA **106**(10), 3698–3703 (2009). https://doi.org/10.1073/pnas.0812874106
58. Zou, Y., et al.: *Lactobacillus shenzhenensis* sp. Nov., isolated from a fermented dairy beverage. Int. J. Syst. Evol. Microbiol. **63**(5), 1817–1823 (2013). https://doi.org/10.1099/ijs.0.041111-0
59. Bando, Y., Fujimoto, N., Suzuki, M., Ohnishi, A.: A microbiological study of biohydrogen production from beer lees. Int. J. Hydrogen Energy **38**(6), 2709–2718 (2013). https://doi.org/10.1016/j.ijhydene.2012.11.142
60. Mang, S., Schwaiger, K., Lindner, R., Gareis, M., Dorn-In, S.: High incidence of cold-tolerant *Clostridium frigoriphilum* and *C. algidicarnis* in vacuum-packed beef on retail sale in Germany. Int. J. Food Microbiol. **340**, 109053 (2021). https://doi.org/10.1016/j.ijfoodmicro.2021.109053
61. Barba, F.J., Ahrné, L., Xanthakis, E., Landerslev, M.G., Orlien, V.: Innovative technologies for food preservation. In: Barba, F.J., Orlien, V., Sant'Ana, A.S., Kouba, M. (eds.) Innovative technologies for food preservation: inactivation of spoilage and pathogenic microorganisms, pp. 25–51. Elsevier, Amsterdam (2018)
62. Liou, J.S.C., Balkwill, D.L., Drake, G.R., Tanner, R.S.: *Clostridium carboxidivorans* sp. Nov., a solvent-producing clostridium isolated from an agricultural settling lagoon, and reclassification of the acetogen *Clostridium scatologenes* strain SL1 as *Clostridium drakei* sp. Nov. Int. J. Syst. Evol. Microbiol. **55**(5), 2085–2091 (2005). https://doi.org/10.1099/ijs.0.63482-0
63. Murray M.-Y: Comprehensive biotechnology, Pergamon, ISBN: 9780080885049. Second Ed., (2011)
64. Mazareli, R.C.D.S., et al.: Metagenomic analysis of autochthonous microbial biomass from banana waste: screening design of factors that affect hydrogen production. Biomass Bioenergy **138**, 105573 (2020). https://doi.org/10.1016/j.biombioe.2020.105573

Authors and Affiliations

Roberto Carlos Campos-Flores[1] · Lirio María Reyna-Gómez[1] · Santiago Iván Suárez-Vázquez[1] · Armando Robledo-Olivo[2] · Arquímedes Cruz-López[1]

✉ Arquímedes Cruz-López
ARQUIMEDES.CRUZLPZ@uanl.edu.mx

[1] Universidad Autónoma de Nuevo León, Facultad de Ingeniería Civil, Departamento de Ingeniería Ambiental, Av. Universidad S/N, Cd. Universitaria, 66455 San Nicolás de los Garza, NL, Mexico

[2] Food Science and Technology Department, Universidad Autónoma Agraria Antonio Narro, Unidad Saltillo, Blvd Antonio Narro 1923, Buenavista, 25315 Saltillo, Coahuila, Mexico