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Biohydrogen, Volatile Fatty Acids, and Biomethane from Mezcal Vinasses—A Dark Fermentation Process Evaluation

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Abstract: Mezcal is a drink made in Mexico, the production of which generates vinasses with a high content of organic matter (OM) that is not utilized. However, these residues have the potential to be drawn upon in dark fermentation (DF) processes to obtain biogas rich in biohydrogen, biomethane, and volatile fatty acids (VFAs) with the potential to become biofuels. In the present work, the effect of reaction time (RT) and organic load (OL) was assessed based on the efficiency of removing OM, the production of VFAs, and the generation and composition of biogas in a process of DF fed with mezcal vinasses. The results show that increasing the RT and decreasing the OL increases COD removal but decreases biohydrogen production. The maximum production of H_2 (64 ± 21 NmL $H_2/L_{reactor}$) was obtained with the lowest RT (1 d) and the highest OL (13.5 $gCODm^3d^{-1}$), while the highest accumulation of VFAs (2007 ± 327 mg VFA/L) was obtained with an RT of 3 d. It was determined that RT and OL are key parameters in DF processes for biohydrogen and VFA production.

Keywords: mezcal vinasses; vinasses revalorization; dark fermentation; biogas; biohydrogen

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1. Introduction

Mezcal is a Mexican alcoholic drink that is frequently handcrafted. During its production, vinasses, a type of highly polluting wastewater, are generated [1]. The disposal of this waste to the environment without treatment can have serious side effects, due to its high concentration of organic matter (35,000–122,000 mg COD/L), acidity (pH 3.5–3.94), and high discharge temperature (70 to 90 °C). Some of the aforementioned side effects include eutrophication of water bodies, inhibition of the proliferation of various organisms, and soil erosion due to the emergence of anoxic zones [1–3].

For the treatment of these types of vinasses, as well as similar ones, various biological and physicochemical treatments have been evaluated at the laboratory level: Retes-Pruneda [2] obtained organic matter (OM) removal efficiencies of 92.5% for COD, with a hybrid treatment system consisting of a flocculation stage with alginates and a fungal treatment; Robles-González et al. [4] reached removal efficiencies of 85% for OM, 83% for phenols, and 32% for aromatic compounds with a combined treatment of ozonation and activated sludge. Meanwhile, for residues similar to tequila vinasses, anaerobic processes with average OM removal efficiencies that exceed 70% of the influent COD have been reported [5]. Therefore, these types of processes have the potential to be implemented while managing mezcal vinasses for decontamination. However, a strategy that has not been contemplated for the management of mezcal vinasses, but that has presented encouraging

results with similar effluents, is its revalorization to obtain compounds of high-energy value. A treatment that has the potential to revalorize residues with a high concentration of OM is dark fermentation (DF), which is an anaerobic process for carbohydrate metabolism used by hydrogen-genic microorganisms to produce energy. DF emerges as an alternative for H_2 production [6]. The main purpose of DF is not the removal of OM, but the production of biogas rich in hydrogen (from 31 to 72% of H_2 of the total composition of the gas produced) as an alternative energy source [7–9]. In a DF process, 12 to 17% of the OM available in the influent is bio-transformed into H_2, while the remaining OM remains in the effluent in the form of by-products such as volatile fatty acids (VFAs) and alcohols [7,10]. VFAs can be used as substrates for other bioprocesses to generate different biocombustibles; for example, in a methanogenic process to produce methane, or as substrates in microorganism fuel cells (MFCs) to generate electricity. Some of the most studied bacterial genera in the DF process belong to the genus *Clostridium*, which conducts butyric fermentation, and *Enterobacter,* which conducts acid-mixed fermentation [11,12]. Some factors that can affect the DF process are the operating temperature (35 ± 2 °C), the OM concentration of the substrate (>1000 mgCOD/L), the Organic Load (OL = KgCOD/m^3d^{-1}), the Specific Organic Load (SOL), which refers to the relationship between the OL and the population of the microorganisms in the reactor (SOL = OL/g VSS), the pH (5.5 ± 0.2), and the Hydraulic Retention Time (HRT) which is defined as the flow (Q = m^3d^{-1}) between the volume of the reactor (V = m^3)—in reactors working in continuous flow—and the Reaction Time (RT), which refers to the duration of the treatment cycle (RT = d^{-1})—in reactors working in sequential batch flow. The OL determines the amount of organic matter available in the reactor per volume and time units, and it affects the efficiencies of pollutant removal and by-product generation. The effect of both parameters, RT and OL, on the performance of the vinasse fermentation process has not been sufficiently studied. Therefore, in the present work, the effect of the reaction time (RT) and the organic load (OL) on the volumetric production of biogas, the production of biohydrogen and biomethane, and the production of VFAs in a dark fermentation process fed with mezcal vinasses was assessed.

2. Materials and Methods

2.1. Vinasses

Mezcal vinasses produced in an artisan winery located in the municipality of Nombre de Dios, Durango, Mexico, were sampled and stored in 20 L plastic containers at 4 °C, before being used as a substrate for the DF process. The vinasses were characterized according to their concentration of organic matter—measured as COD and BOD_5—VFAs, pH, phenols, total solids (TS), total suspended solids (TSS), volatile suspended solids (VSS), and conductivity according to the standard methods and the Hach methods [13,14].

2.2. Experimental Set-Up

As an experimental unit, a glass sequential batch reactor (SBR) of 800 mL total capacity was used, with an operational volume of 600 mL (Figure 1). This SBR was inoculated with hydrogenic microorganisms obtained and acclimatized to a DF process by feeding it with a 75% mezcal vinasses solution (21,810 ± 583 mg COD/L), according to the methodology described by Díaz-Barajas et al. [15]. The biomass within the system was maintained in suspension and under homogeneous conditions by applying ascending recirculation (216 mL/min). The system was kept at 35 ± 2 °C using a coiled thermal jacket. The pH was stabilized between 5.3 and 5.7 using Ca $(OH)_2$ (Calidra Company, Torreón, Mexico). The concentration of Ca $(OH)_2$ did not exceed 15% in the vinasse treatment, as concentrations beyond this level have been reported to inhibit biogas production in anaerobic digestion processes [16]. The volumetric production of biogas was determined by connecting the biogas output of the reactor to Microflow equipment from the Bioprocess Control brand (BPC Instruments Co. Ltd., Lund, Sweden). For the collection of biogas samples, the outlet duct of the Microflow equipment was connected to glass containers with a capacity of 10 mL.

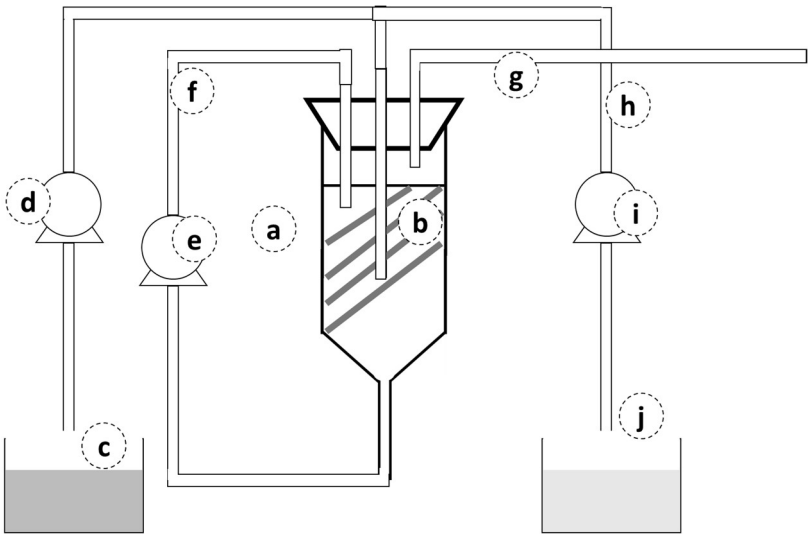

Figure 1. Experimental system: (a) SBR; (b) thermal jacket; (c) substrate; (d) peristaltic pump for feeding; (e) peristaltic pump for recirculation; (f) recirculation of mixed liquor; (g) biogas outlet; (h) extraction of treated liquor; (i) peristaltic pump for the extraction of treated liquor; (j) effluent.

2.3. Effect of Reaction Time and Organic Load on a Dark Fermentation Process with Suspended Biomass

The effect of four reaction times (RTs) of 4, 3, 2, and 1 d (equivalent to HRTs of 8, 6, 4, and 2 d, respectively) and the effect of organic load (OL) between 3.4 and 13.5 g COD/L d on the generation/accumulation of VFAs were evaluated, as well as volumetric production of biogas with high H_2 content in a DF process fed with mezcal vinasses (21,810 ± 583 mg COD/L; 6275 ± 42 mg AGV/L). Each operational period was evaluated for a minimum of five treatment cycles or until constant biogas production was reached.

In each experimental stage, the SBR was sampled at the beginning of the operation and every 24 h until the completion of RT for each cycle. For example, during the fourth day of RT, sampling was conducted at the beginning of the cycle on days 1, 2, and 3, with the final effluent corresponding to day 4. Additionally, when operating the SBR with a 1-day RT, sampling was carried out at 8, 16, and 24 h of operation to determine the effect of RT values of less than 1 d. The sampling consisted of analyzing the vinasses to determine COD, BOD_5, TS, TSS, VSS, and VFAs. Furthermore, the volume of biogas generated was measured, and in certain cycles, a biogas sample was taken to analyze its composition.

2.4. Analytical Tests and Statistical Analysis

Tests for COD and BOD_5, total solids (TS), total suspended solids (TSS), and volatile suspended solids (VSS) were performed according to the Standard methods for water analysis [13]. The conductivity, pH, and temperature of the influent were determined using an Orion 3 Star potentiometer from the Thermo Scientific brand (Long Branch, NJ, USA). The VFA content was determined by the esterification method (8196) for a HACH DR5000 spectrophotometer [14]. The composition of the generated biogas was analyzed using an SRI 8610C gas chromatograph (St. Torrance, CA, USA), equipped with a thermal conductivity detector and a 30 m long (0.53 mm ID) Carboxen-1010 PLOT column (St. Torrance, CA, USA). The operating conditions of the chromatograph were established as follows: the carrier gas was nitrogen at a flow rate of 4.5 mL/min; the injector temperature was 200 °C; the column temperature was 100 °C; and the detector temperature was set at 230 °C. The results obtained for each evaluated RT were compared with ANOVA statistical tests (Tukey's test) performed with the GraphPad 8 software to determine the presence of significant differences between the operational periods evaluated in terms of COD removal, VFAs generation, and biogas production (Supplementary Material).

3. Results and Discussion

3.1. Influent Characterization

The mezcal vinasses used in this study were generated in the State of Durango (Mexico) and share similarities with the mezcal vinasses generated in another region of Mexico (State of Oaxaca), such as an acid pH and a high discharge temperature, as well as OM concentrations higher than 30,000 mg COD/L (Table 1). Due to the similarity between these residues, it is expected that the results obtained in this experimental work can be replicated with the vinasses generated in other wineries that produce artisanal mezcal or use similar residues.

Table 1. Characterization of the mezcal vinasse and inoculum used in this study, produced in the State of Durango, and its comparison with other similar vinasses.

Parameter	Mezcal (Durango) *	Inoculum (Durango) *	Mezcal (Oaxaca) [1]	Mezcal (Oaxaca) [2]	Mezcal (Oaxaca) [3]
pH	3.82 ± 0.16	6.63 ± 0.23	3.6–3.8	-	-
Temperature (°C)	70	30	90	-	-
Volatile Fatty Acids (mg/L)	5815 ± 714		-	-	-
COD (mg/L)	32,966 ± 3088	5542 ± 459	56,230–122,860	42,000	35,000–50,000
BOD_5 (mg/L)	11,700 ± 1272		26,500–33,600	25,576	35,000
Total solids (mg/L)	43,084	25,200 ± 4242	26,830–947,130	45,860	-
Total Suspended Solids (mg/L)	31,788	7073 ± 1141	8400–83,130	-	-
Volatile suspended solids (mg/L)	920	4951 ± 797	1130–6850	-	-
Conductivity (mS/cm)	5.81	6.9	2.6–4.2	-	-

* Present study.

3.2. Effect of Alkali Addition to the Influent

When solid organic substrates that are difficult to biodegrade are used to generate biogas, such as rice straw, sugarcane bagasse, etc., $Ca(OH)_2$ can be used as a pretreatment to unfold the chemical structure of lignin and accelerate the hydrolysis of these organic solids into soluble sugars. These sugars serve as a substrate for anaerobic digestion, thus favoring an increase in biogas production. In such cases, an excess of alkali has been found to hinder methane generation. Gu et al. [16] reported that pretreating rice straw with 5 to 10% $Ca(OH)_2$ improves biogas production by approximately 35%. However, when applying a pretreatment with a concentration of 15%, methane production decreases. This inhibition is attributed to the alkali likely exceeding the desired degradation, causing a dissolution of cellulose and hemicellulose, thereby reducing the amount of sugar available for AD. In the present investigation, a liquid organic waste—mezcal vinasses—was used as a substrate in a DF process. Mezcal vinasses have a high concentration of soluble organic matter and do not require alkaline pretreatment to release fermentable sugars. Nonetheless, $Ca(OH)_2$ in solution (5%) was added to adjust the acidic pH of the vinasses under treatment (3.6–3.8) to a value of 5.5, which is optimal for DF. This addition was about 25 mL per L of reactor, which would be equivalent to a final vinasses concentration of 1.25% of $Ca(OH)_2$, so according to Gu et al. [16], it is not expected that there would be an inhibition due to the addition of an alkali.

3.3. Effect of Reaction Time and Organic Load in a Dark Fermentation Process

3.3.1. Effect on the Organic Matter Removal (COD)

As the RT decreased, between day 4 and day 1, and the OL increased from 3.4 to 13.5 g COD/L d, a decrease in the OM removal efficiency was observed in the system. According to an ANOVA test, the removal efficiencies achieved can be classified into two statically different groups. (Figure 2): group a (RT of 4 and 3 d and OL between 3.4 and 4.5 g COD/L d) with average removal efficiencies of COD between 22 ± 5 and 24 ± 1%, and group b (RT of 2 and 1 d and OL between 6.7 and 13.5 g COD/L d) with average COD removal percentages between 12 ± 3 and 9 ± 2%.

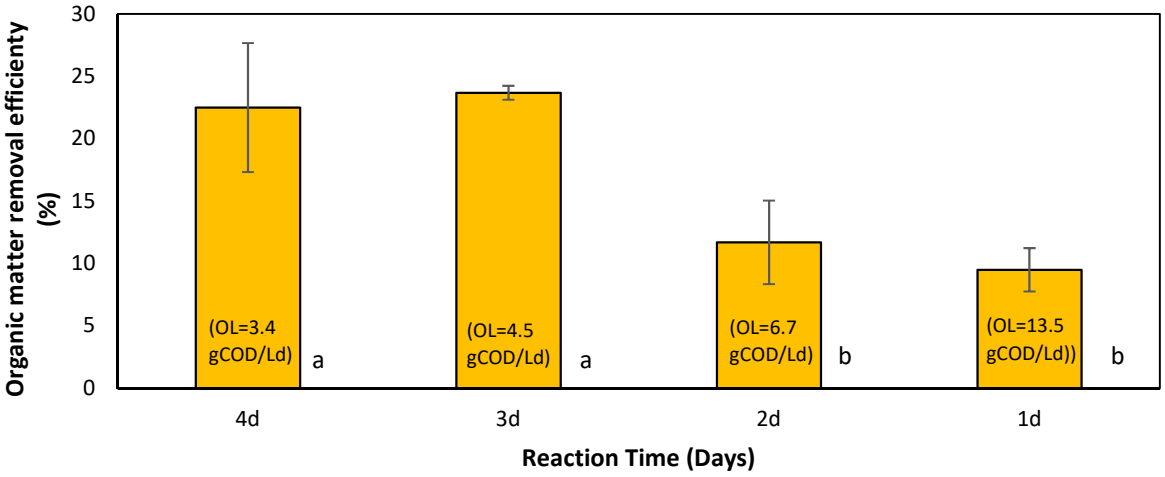

Figure 2. Organic matter removal efficiency is measured as COD by varying the reaction time and organic load. Different subscripts on the side of each column (a and b) indicate that there is a statistically significant difference.

This difference in removal efficiency may indicate that the decrease in RT could inhibit the proliferation of methanogenic microorganisms within the system, in such a way that a lower concentration of OM was transformed into methane, favoring the accumulation of OM in the effluent in the form of by-products such as VFAs. On the other hand, the increase in OL also increased the concentration of toxic pollutants, which could inhibit the removal rate of OM.

3.3.2. Effect of Reaction Time on the Production of Volatile Fatty Acids

When varying the RT of the DF process, different behaviors were observed in the generation of VFAs. With the RT of 4 and 3 d, two increases in VFAs production were observed, occurring on day 1 and day 3 of the operation. However, with the 2- and 1-day RT, a single period of continuous VFAs production was observed, lasting for 1.5 d for the 2-day RT and 18 h for the 1-day RT (Figure 3). This difference in behavior may be attributed to the presence of different carbohydrates in the vinasses, each with different degrees of complexity. With a longer RT, a greater variety of complex carbohydrates can be transformed into VFAs (generated during the second increase in the production of VFAs). On the other hand, with a shorter RT, only the simplest compounds can be transformed into VFAs, resulting in a single generation period.

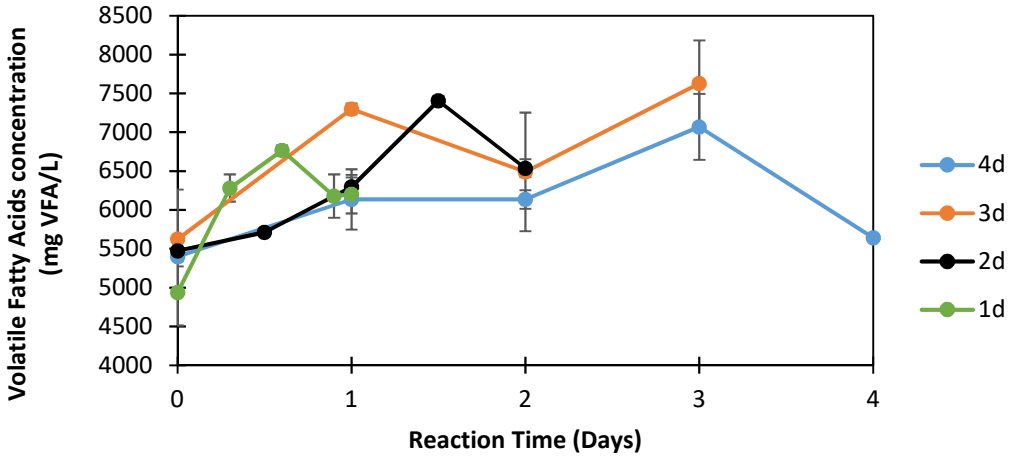

Figure 3. Production kinetics of volatile fatty acids by reducing the period of operation in the dark fermentation reactor with suspended biomass.

The 3-day RT presented the highest production of VFAs at the end of the treatment cycle (2006 ± 327 mg VFA/L = 36 ± 9%), followed by the 2-day RT (1268 ± 328 mg VFA/L = 26 ± 9%), 1-day RT (1064 ± 249 mg VFA/L = 19 ± 5%), and 4-day RT (240 ± 170 mg VFA/L = 4 ± 3%) (Figure 4).

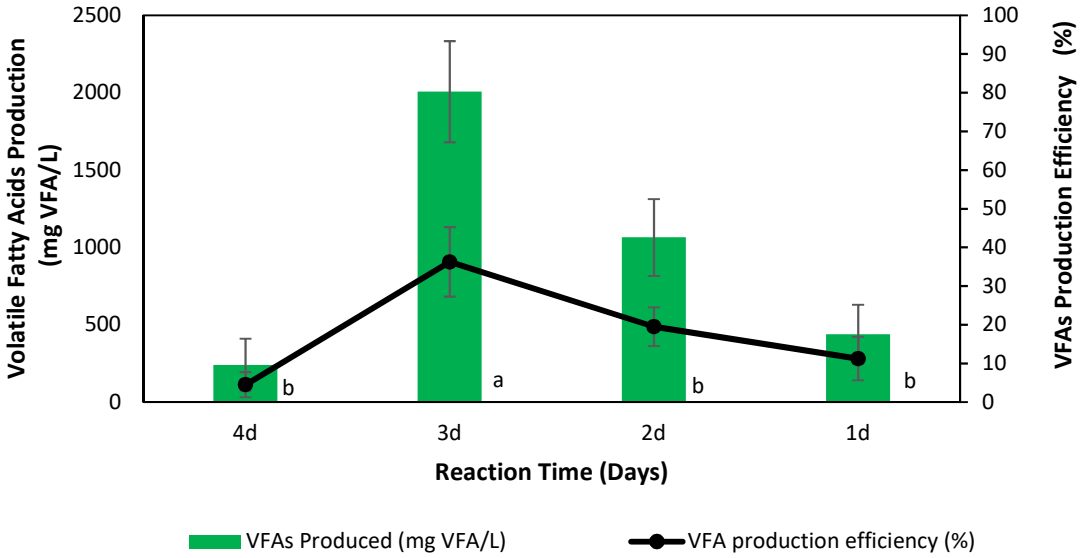

Figure 4. Production and production efficiency of volatile fatty acids at the end of the cycle with different reaction times. Different subscripts on the side of each column (a and b) indicate that there is a statistically significant difference.

An ANOVA analysis was performed on the results to determine if there were statistically significant differences regarding the generation of VFAs when different RTs were applied. Moreover, it could be observed that two groups were formed. In the first group (group a), the RT of 3 d showed a generation of VFAs between two and eight times higher than that observed in the second group (group b), comprising RTs of 1, 2, and 4 d. This difference in the final concentration of VFAs is partly because the RTs included in group b reached their highest production before the end of their respective treatment cycle (Figure 3) and the VFAs could be consumed towards the end of each operational period, while with the RT of 3 d, the VFAs could accumulate to their maximum concentration, ending the treatment cycle before starting the period of consumption of the VFAs.

Even though the maximum production of VFAs was obtained at the end of the treatment cycle with the 3-day RT, when analyzing the maximal VFAs concentration reached at any time during the treatment cycle, it was observed that there were no statistically significant differences between the four RTs evaluated. All of them generated around 2000 mg VFA/L at different times (Table 2). For the RT of 4 d, the maximum production of VFAs was obtained on day 3, on day 1.5 for the RT of 2 d, and for the RT of 1 d, the highest peak was obtained at the time of 0.66 d (16 h). This indicates that, despite not showing significant differences in the maximum production of VFAs between the RT evaluated, the operational period did affect whether they could be accumulated or consumed towards the end of the treatment cycle. In other words, in all RTs, the same concentrations of VFAs were generated, but these were consumed more with an RT of 1, 2, or 4 d with a 3-day RT.

Despite not presenting significant differences in the maximum production of VFAs, if the objective of the treatment is to maximize VFA generation, for example, to feed a subsequent anaerobic digestion (AD) process, it is recommended to consider an RT of 1.5 d. This recommendation stems from the observation that, when operating with a 2 d RT, a level of production similar to that achieved with the 4- and 3-day RTs was obtained in half the time (Figure 3). However, the maximum production of VFAs achieved in this DF process (2006 ± 327 mg VFA/L) was 50% lower than that achieved with tequila vinasses

(Marino-Marmolejo et al.) [17]. As mentioned before, no inhibition was expected due to the $Ca(OH)_2$ adjustment of pH, as the final concentration of $Ca(OH)_2$ in the vinasses was 1.25% which is ten times lower than the inhibition threshold. The authors Mariano-Marmolejo et al. [17] reached an average production of VFAs of 4114 mg VFA/L in a DF process fed with tequila vinasses in an Upflow Anaerobic Sludge Blanket (UASB) reactor with an HRT of 6 h and a predominance of *Clostridium* sp., which are hydrogen-producing acidogenic microorganisms. This better performance in the tequila vinasses study may be attributed to the limitation of the biomass by *Clostridium* sp. bacteria, along with a low HRT, which could inhibit the development of methanogenic bacteria that utilize the VFAs generated by the acidogenic microbiota as a carbon source. However, it is important to highlight that they applied a high OL (30 gCOD/L d), almost twice the maximal OL applied in this research (13.5 gCOD/L d).

Table 2. Maximum production of VFAs by varying the retention time.

Reaction Time (d)	Period of Maximum Production of VFAs (d)	Maximum Production of VFAs (mg VFA/L)	VFAs Production Efficiency (%)
4	3	1670 ± 552	23 ± 6
3	3	2007 ± 327	36 ± 9
2	1.5	1932 ± 198	26 ± 2
1	0.66	1831 ± 364	38 ± 11

3.3.3. Effect of Reaction Time and Organic Load on Volumetric Biogas Production

Reducing the operation time of the DF process from 4 to 3 d and increasing the OL from 3.4 to 4.5 gCOD/L d, favored an important increase (34%) in the accumulated production of biogas at the end of each cycle, which went from 325 ± 7 to 436 ± 42 $NmL/L_{reactor}$ (Figure 5). However, by varying the RT from 3 d to 2 and 1 d and increasing the OL from 6.7 to 13.5 gCOD/L d, no statistically significant differences were observed in the accumulated biogas production. However, it did have a favorable effect on the speed of biogas production (Figure 6), which increased from 81 ± 2 to 411 ± 13 $NmL/L_{reactor}d$, which can be confirmed from the kinetic parameters of biogas production with the different RT (Table 3).

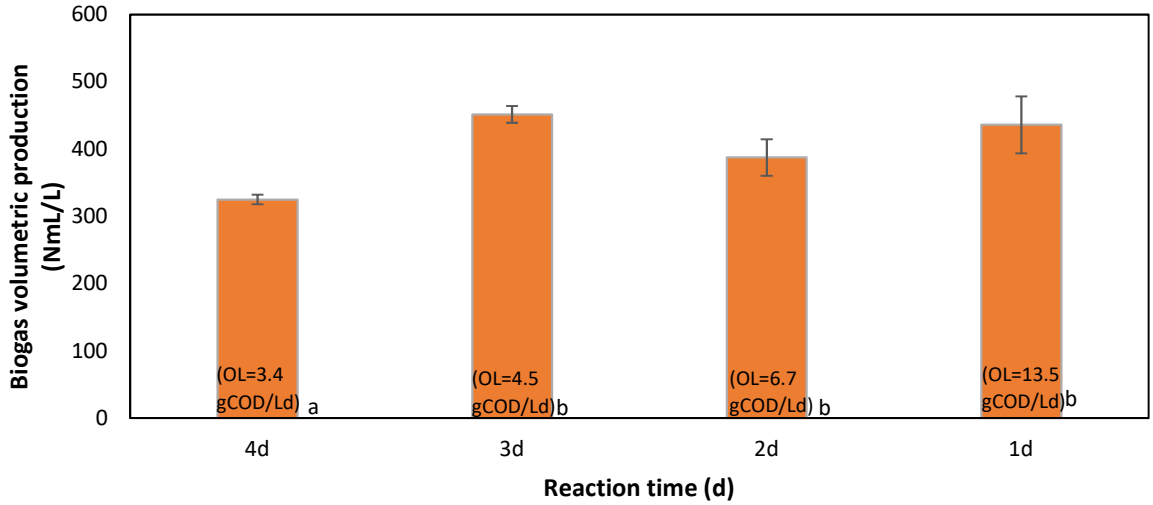

Figure 5. Accumulated volumetric production of biogas at the end of each cycle by varying the reaction time and the organic load (OL). Different subscripts on the side of each column (a and b) indicate that there is a statistically significant difference.

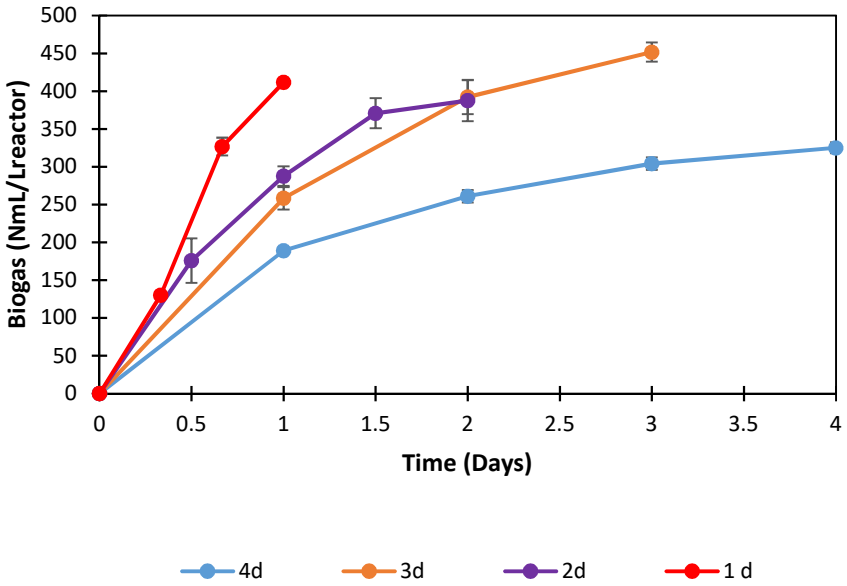

Figure 6. Biogas production by varying the reaction time in the dark fermentation reactor.

Table 3. Kinetic parameters in the volumetric production of biogas per liter of the reactor by varying the reaction time and the organic load.

Reaction Time (d)	OL (g COD/L d)	H Max (NmL/L)	R ($NmL/L_{reactor}h$)	Lag (h)
4	3.4	119	8	3
3	4.5	146	13	15
2	6.7	145	15	11
1	13.5	170	31	8

It seems that, by shortening the RT and increasing the OL, the microorganisms were pushed to degrade the carbohydrates present in the vinasses faster than with a higher RT and lower OL, increasing the speed of biogas generation in the first few hours and limiting the development of methanogenic biomass.

The highest biogas production rate of 411 ± 13 $NmL/L_{reactor}d$ obtained with the 1-day RT was 42% lower than the one reported for tequila vinasses by other authors such as Toledo-Cervantes et al. [18], who generated 709 ± 36 $NmL/L_{reactor}d$ in a DF fed with tequila vinasses in a UASB reactor operated with a 29 h HRT. However, they operated with an OL of 22.34 g COD/L d, which is 65.5% higher than the OL applied in the present study. It seems that there is a relationship between the biogas production rate and the OL applied to the system.

To summarize, the decrease in RT from 4 to 3d resulted in a 34% increase in biogas production. However, reducing the RT from 3 to 1d and increasing the OL from 4.5 to 13.5 gCOD/L d did not show statistically significant variations in the accumulated volumetric production of biogas. Nonetheless, an improvement in the biogas production rate was observed, increasing from 8 to 31 $NmL/L_{reactor}h$.

3.3.4. Effect of the Reaction Time on the Production of Biohydrogen and Biomethane

The effect of different RTs on the composition of the biogas generated in the DF process fed with mezcal vinasses was analyzed (Figure 7). By applying a 4-day RT, H_2 could be only obtained during the first day of operation with a concentration close to 10 ± 0.2 $NmL/L_{reactor}$ (Figure 7a). With the RTs of 3 and 2 d, H_2 was detected throughout each day of its operation cycle (Figure 7b,c). With these RTs, a progressive decrease in the concentration of H_2 was observed as the treatment cycle lasted for longer, reaching

its maximum concentration of 23 ± 1 and 63 ± 3 NmL/$L_{reactor}$ during the first day of treatment with cycles lasting 3 and 2 d, respectively (Figure 7b,c). These results suggest that the greatest accumulation of biohydrogen that can be achieved in a DF fed with mezcal vinasses was obtained in the first 24 h of operation. A possible explanation could be that the RT defines the populations that can be established within the reactor based on their duplication speed. If the RT is prolonged beyond the time that acetogenic bacteria require to duplicate, it can allow for the establishment of slower-growing microorganisms such as methanogenic *Archea* that carry out a methanogenic process consuming the H_2 and VFAs generated during DF. Thus, when the RT lasted for more than 1 d, the methanogenic *Archaea* colonized the reactor and consumed the by-products generated by the acidogenic bacteria. Therefore, it can be stated that the greatest accumulation of biohydrogen of 64 ± 21 was obtained when operating the DF with an RT of 1 d (Figure 7d).

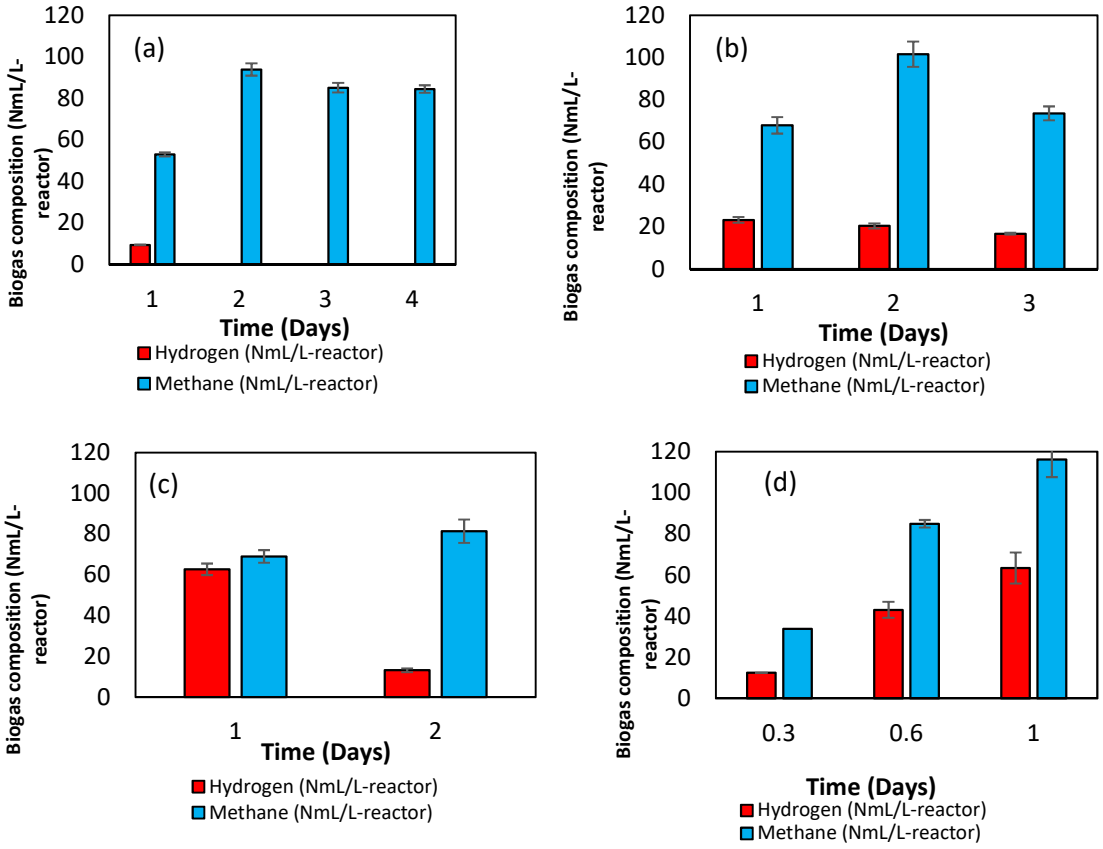

Figure 7. Composition of the biogas generated in a dark fermentation process by varying the retention time. (**a**) RT of 4 d (HRT 8 d); (**b**) RT of 3 d (HRT 6 d); (**c**) RT of 2 d (HRT 4 d); (**d**) RT of 1 d (HRT of 2 d).

To summarize, the decrease in RT from 4 to 1d resulted in an increase in biohydrogen production from 0 to 64 ± 21 NmL $H_2/L_{reactor}$. Apparently, the greater accumulation of H_2 occurs because the reduction in RT limits the growth of methanogenic microorganisms. As a result, a lower concentration of VFAs and hydrogen is transformed into a methanogenic process.

When analyzing the composition of the biogas generated (Table 4), it was observed that, with the RTs of 3, 2, and 1 d, it was possible to detect hydrogen and methane at the end of the cycles, reaching the highest concentration with the RT of 1 d generating 64 ± 21 NmL $H_2/L_{reactor}$d and 116 ± 10 NmL $CH_4/L_{reactor}$d. This gaseous mixture (biohydrogen and biomethane) is known as biohytane and has presented encouraging results as a fuel. Biohytane is mainly generated from two-stage AD processes [19,20]. The generation of biohytane

in a single-stage process of dark fermentation has been only recently reported for tequila vinasses by Serrano-Mesa et al. [21].

Table 4. Composition of the biogas generated with different RT at the end of the treatment cycle.

Reaction Time (d)	Biogas Composition at the End of the Cycle (NmL/$L_{reactor}$)		
	H_2	CH_4	CO_2
4	0	84 ± 2.0	240 ± 5
3	17 ± 0.4	74 ± 2.0	361 ± 10
2	13 ± 1.0	81 ± 6.0	291 ± 20
1	64 ± 21.0 (15%)	116 ± 0 (27%)	250 ± 0 (58%)

The maximum concentration of biohydrogen generated at the end of a treatment cycle, as reported in this study, is significantly lower than that reported for another type of vinasses (tequila) by various authors, such as Buitrón et al. [22], who generated 1378 ± 96 mL $H_2/L_{reactor}$d in a DF by applying 6 h of HRT in a SBR fed with tequila vinasses and also produced 281 ± 17 mL $CH_4/L_{reactor}$d in a second stage (methanogenic process) fed with the DF effluent. When the production of these gases is normalized, it results in the production of 648 ± 45 NmL $H_2/L_{reactor}$d and 132 ± 8 NmL $CH_4/L_{reactor}$d. Thus, by comparing the normalized generation of H_2, it can be seen that the concentration generated in the present study, with an RT of 24 h (HRT of 48 h), was 93% lower than that reported by Buitrón et al. [22] with an HRT of only 6 h. However, methane generation in the present study (HRT of 48 h) represents 87% of the methane obtained by Buitrón et al. [22] in the second stage of AD with a 24 h HRT.

Other authors such as Toledo-Cervantes et al. [18] evaluated DF processes (HRT of 29 h) also for tequila vinasses, obtaining biohydrogen production rates of 448 ± 23 NmL $H_2/L_{reactor}$d (41.8 ± 1.8%) in the absence of methane. Another case is the one evaluated by García-Depraect et al. [7], in which a dark co-fermentation of tequila vinasses (80% v/v) and residual water from a nixtamalization process (20% v/v) was carried out, with a maximum biohydrogen production of 2133 NmL $H_2/L_{reactor}$ (3720 NmL $H_2/L_{reactor}$d; 155 NmL $H_2/L_{reactor}$h; 72.6%), applying an HRT of 28.5 h. In both studies, the application of an HRT between 28.5 and 29 h was chosen, which limited the development of methanogenic communities (in such a way that CH_4 was not generated in the biogas), favoring the accumulation of biohydrogen up to 86%.

Other studies conducted on tequila vinasses, such as those reported by Buitrón et al. [8] and García-Becerra et al. [7], have achieved biogas with biohydrogen concentrations ranging between 37 and 64% in DF processes using attached biomass evaluated at an HRT of 12 and 6 h, respectively. Taking this into consideration, a reduction in the RT to periods equal to or less than 24 h is recommended to maximize the production of biohydrogen from mezcal vinasses in a DF. Furthermore, the integration of complementary treatments that allow for the diversification of energy products and the improvement of organic matter (OM) removal efficiency in agro-industrial residues is suggested.

3.3.5. Effect of Organic Load and Specific Organic Load on Biohydrogen Production

Since the highest biohydrogen production (64 ± 21 NmL $H_2/L_{reactor}$) was obtained with the RT of 1 d, the Organic Load (OL) and the Specific Organic Load (SOL) were determined for this experimental period. During this stage, the SBR was fed with 75% mezcal vinasses (27.04 gCOD/L), and the OL (available OM concentration) was calculated from Equation (1), in which CODa represents the OM concentration in the influent (gCOD/L) and HRT is the hydraulic retention time (d).

$$\mathrm{OL}\left(\frac{\mathrm{g\,COD}}{\mathrm{L\,d}}\right) = \frac{\mathrm{CODa}\left(\mathrm{g}\frac{\mathrm{COD}}{\mathrm{L}}\right)}{\mathrm{HRT\,(d)}} \quad (1)$$

The SOL (available MO concentration based on biomass within the system) was calculated based on the biomass concentration present in the liquor (0.669 ± 0.009 gVSS/L) according to Equation (2).

$$\text{SOL}\left(\frac{\text{g COD}}{\text{gVSS d}}\right) = \frac{\text{CODa}\left(\frac{\text{gCOD}}{\text{L}}\right)}{\text{HRT (d)} \times \text{Biomass}\left(\frac{\text{gVSS}}{\text{L}}\right)} \quad (2)$$

It was determined that this system, when applying an RT of 1 d, operated with an OL of 13.52 ± 0.41 gCOD/L d and an SOL of 20.20 ± 0.61 gCOD/gVSSLd. With these OL and SOL values, 436 ± 42 NmL/$L_{reactor}$ of biogas was obtained with a content of 64 ± 21 NmL $H_2/L_{reactor}$ of biohydrogen and 116 ± 16 NmL $CH_4/L_{reactor}$ of methane. Under these operational conditions, the hydrogen yield using mezcal vinasses as a substrate in dark fermentation was 38.4 ± 5.7 NmL H_2/g $COD_{removed}$ or 1.71 ± 0.25 mmol H_2/g $COD_{removed}$. This performance value is similar to that reported for tequila vinasses: 1.1 ± 0.25 0.1 mmol H_2/g $COD_{removed}$, obtained by operating the reactor with a COD of 44,200 mg/L and an RT of 24H [23], but it is lower than the yield reported for ethanol vinasses: 3.1 ± 1.3 mmol H_2/g $COD_{removed}$, obtained by operating the reactor with a COD 19,512 mg/L and an RT of 24 H [24]. The higher yield being obtained with ethanol is because this substrate is more easily fermentable than tequila or mezcal vinasse.

As has been mentioned, there are no previous studies of dark fermentation for mezcal vinasses, so, when compared with the reports for tequila vinasses, it was observed that biogas production is higher in the latter case. When analyzing these works, it was also observed that the OL and SOL were higher than those reported in the present study. For example, Buitron et al. [22] generated 648 ± 45 NmL $H_2/L_{reactor}$d in a DF process using tequila vinasses (16 gCOD/L). They operated their systems with an OL and SOL of 64 gCOD/Ld and 42.6 gCOD/g SSVd. Comparatively, the OL used in their study was almost five times higher than that used in the present study, while their SOL was two times higher. In other words, their reactors worked with more concentrated wastewater and with a higher population (density) of microorganisms. Therefore, it seems that the increased availability of substrate (OL) and a higher biomass capable of biotransforming this carbon source (SOL) are crucial parameters for achieving a greater biotransformation of organic matter into biohydrogen.

Other authors such as Toledo-Cervantes et al. [18] generated 448 ± 23 NmL $H_2/L_{reactor}$d from tequila vinasses (27 gCOD/L) in a DF process, operating with an average OL of 22.34 gCOD/L d. The presence of a concentration of bioavailable OM that doubles the one used in the present study favored these authors obtaining a biohydrogen production seven times greater than ours. In the same way, García-Depraect et al. [7] generated 34 NmL $H_2/L_{reactor}$h, a biohydrogen production rate 12.7 times higher than that determined in the present study (2.7 NmL $H_2/L_{reactor}$h), from a DF process fed with a mixture of tequila vinasses and wastewater from a nixtamalization process (61.9 gCOD/L), which operated with an OL of 21.22 gCOD/L d. This higher production can be attributed to both the OL and the decreased complexity of the substrate when mixed with a more biodegradable substrate (nixtamal).

Taking this into consideration, it is suggested that DF processes using mezcal vinasses should be operated with organic loads and specific organic loads significantly higher than those used in the present study, ranging between 20 and 60 gCOD/L d of OL and an SOL ≥ 40 gCOD/gSSVd. This implies increasing both the biomass and the concentration of bioavailable OM to enhance the biohydrogen content in the generated biogas.

4. Conclusions

The reaction time (RT) and the organic load (OL) were revealed to be crucial parameters in the generation of VFAs and in the biogas composition that is generated in a DF process fed with mezcal vinasses. The RTs and the OLs studied did not affect the production of VFAs but did affect their consumption or accumulation within the reactor, so their final concentration varied depending on the RT. RT emerged as the most significant parameter

for biogas composition determining the presence or absence of biohydrogen-consuming and biomethane-forming microorganisms. OL was revealed as the most important parameter for biohydrogen yield. The best results of maximum biohydrogen production were obtained with an RT of 1 d and an OL of 13.52 ± 0.41 gCOD/L d being equal to 64 ± 21 NmL $H_2/L_{reactor}$—with a yield of 38.4 ± 5.7 NmL $H_2/g\ COD_{removed}$—and together with a biomethane production of 116 ± 16 NmL $CH_4/L_{reactor}$. Which corresponds to 42% biohytane (15% biohydrogen and 27% biomethane) and 58% CO_2. This is noteworthy because biohytane is typically generated in two-stage anaerobic digestion systems.

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