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Dark Fermentation Process Response to the Use of Undiluted Tequila Vinasse without Nutrient Supplementation

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Abstract: The technical feasibility of valorizing tequila vinasse (TV), a wastewater with high pollution potential, through the production of biogenic hydrogen via dark fermentation, has long been proven in diverse lab-scale reactors that were operated either in batch or continuous mode. However, such systems have mainly been tested with diluted streams and nutrient supplementation, hindering the techno-economic attractiveness of the TV-to-hydrogen concept at large scale. In this study, the feasibility of producing hydrogen from high-strength undiluted TV with no added extra nutrients was evaluated under batch mesophilic conditions. Additionally, the use of two different acidogenic inocula obtained either by heat or heat-aeration pretreatment was investigated to get a greater understanding of the effect of inoculum type on the process. The results obtained showed that the TV utilized herein contained macro- and micro-nutrients high enough to support the hydrogenogenic activity of both cultures, entailing average hydrogen yields of 2.4–2.6 NL H_2/L vinasse and maximum hydrogen production rates of 1.4–1.9 NL H_2/L-d. Interestingly, the consumption of lactate and acetate with the concomitant production of butyrate was observed as the main hydrogen-producing route regardless of the inoculum, pointing out the relevance of the lactate-driven dark fermentative process. *Clostridium beijerinckii* was ascertained as key bacteria, but only in association with microorganisms belonging to the genera *Enterobacter* and *Klebsiella*, as revealed by phylogenetic analyses.

Keywords: bioenergy; bio-hydrogen; *Clostridium*; dark fermentation; inoculum type; lactic acid; nutrient supply; tequila vinasse

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1. Introduction

Hydrogen is a promising energy carrier that could reduce, in the near-to-medium term, the current high dependency on fossil-based energy recovery systems [1]. Nowadays, the hydrogen produced worldwide is mainly used in chemical and petrochemical processes [2]. However, in recent years, hydrogen-powered technologies such as cars and home heating systems have been developed with a few prototypes being already commercially available [1]. Indeed, the Hydrogen Council (https://hydrogencouncil.com) predicts that hydrogen as a fuel will account for 15% of the global energy consumption by 2030. Although hydrogen is currently mainly produced from fossil fuels, there has been an increasing research interest in the production of green hydrogen from renewable sources such as water electrolysis coupled to solar or wind power [2], or by pyrolysis, photofermentation (PF), or dark fermentation (DF) using biomass as the feedstock [3].

DF and PF are ecofriendly processes that are considered advantageous over thermochemical pyrolysis or gasification because they are less energy intensive and flexible enough to use waste and wastewater as feedstocks [4,5]. At this point, it should be noted that

DF is the most promising way to produce biogenic hydrogen since it commonly supports higher hydrogen production rates and yields at lower capital and operational costs when compared to PF [3,6]. Additionally, DF not only can be coupled to the mature anaerobic digestion technology to further increase bioenergy recovery via biogas production [7], but also to emerging biotech processes such as bioelectrochemical systems, bioplastics production, photofermentation, among others [8,9].

DF is a complex process, and its efficiency depends on the substrate characteristics, culture conditions and biological factors [10]. Substrate is typically characterized in terms of pH, solids, carbohydrates, proteins, lipids, as well as micronutrients and putative toxic compounds. Although substrates might contain nutrients supporting microbial growth and nutritional requirements, they also might be deficient in key nutrients that are necessary to produce hydrogen efficiently. While it is true that nutrient supplementation may lead to an enhanced DF process, this strategy may be prohibited for economic reasons at industrial levels. Regarding culture conditions, the most important operational parameters include temperature (including mesophilic, thermophilic, and even hyperthermophilic temperatures), pH (typically set between 5.5 and 6.0), oxidation–reduction potential (ORP), substrate-to-biomass ratio (S_0/X_0), organic loading rate (OLR), and hydraulic retention time (HRT) [10]. Moreover, the type of reactor configuration strongly impacts the hydrogen production efficiency. Different reactors have been reported in the literature, including anaerobic packed-bed reactor (AnPBR), anaerobic structured-bed reactor (AnSTBR), anaerobic fluidized-bed reactor (AnFBR), expanded granular sludge bed reactor (EGSB), up-flow anaerobic sludge blanket reactor (UASB), anaerobic sequencing batch reactor (AnSBR), continuously stirred tank reactor (CSTR) [10], and, more recently, dynamic membrane reactors (DMBR) [11,12]. Among them, the CSTR is perhaps the most widely used reactor configuration to produce biohydrogen via DF due to easy operation and control, as well as owing to its flexibility to operate under suspended and attached biomass configurations; however, DMBRs have sustained very high hydrogen productivities using model substrates [11,13,14], and even more complex ones such as algal biomass [12]. Last but not least, the type of inoculum used, as well as its pretreatment method, are also major factors influencing the DF process.

To achieve high hydrogen production efficiency and stability, microorganisms with a positive function in the overall process such as hydrogen production (e.g., *Clostridium*), oxygen consumption (e.g., *Enterobacter*, *Klebsiella*), or formation of intermediate hydrogen precursors (e.g., *Lactobacillus*) should be enriched. On the other hand, microorganisms that consume hydrogen, such as propionic fermenters, homoacetogens, and methanogens, should be reduced or, even better, washed out [15]. Inocula are commonly subjected to a pretreatment step to shape the microbial community. Cell washout, acidification, alkalization, aeration, and thermal shock are the most widely used pretreatments [15]. Indeed, as aforementioned, several studies have revealed that the type of inoculum pretreatment is an important factor in the process. For instance, Cai and Wang (2016) compared the performance of an inoculum previously pretreated by heat shock, acidification, or alkalization and observed that the heat pretreatment led to the highest hydrogen yield using glucose as the substrate [16]. Similarly, Toledo-Alarcón et al. (2020) reported that the heat-shock pretreatment was advantageous over the aeration one using glycerol as the substrate [17]. However, the heat-shock pretreatment decreases the bacterial diversity and is not selective enough to distinguish between desirable and undesirable microorganisms [10].

Tequila vinasse (TV), an industrial wastewater generated by the tequila industry, has long been proven as a feasible substrate for DF. In the literature, batch [18], semi-continuous [19], and continuous [7,20–23] reactor configurations have been reported for hydrogen production from TV. This substrate is commonly characterized by having an acidic pH (3.4–4.5), organic matter content between 20 and 100 g chemical oxygen demand (COD)/L, solids concentration ranging from 25 to 50 g/L, and macronutrients and trace elements [24]. Note that all previous studies dealing with the production of hydrogen from TV have implemented extra nutrient supplementation, except those reported by

Toledo-Cervantes et al. (2020) [25] and Arellano-García (2021) [23]. However, in the first report, the fed TV had a low organic matter concentration of 29 g COD/L, while the second study failed to efficiently produce hydrogen from undiluted TV, implying that achieving optimal DF performance using concentrated TV with no added nutrients is a challenging task requiring further research.

With the aim of achieving a cost-effective process within a more realistic scenario that does not require the use of fresh water and extra chemicals, this study aimed to investigate the feasibility of producing hydrogen from high-strength TV (>50 g COD/L) without nutrient enhancement. A systematic comparison of two different hydrogenogenic inocula was also conducted to better understand the role of inoculum type in the hydrogen production kinetics, biochemical pathways, and prevailing microbial communities. The novelty of this study relies on the demonstration that TV with high COD concentrations and with no added extra nutrients can be metabolized into biohydrogen via the lactate-driven DF.

2. Materials and Methods

2.1. Tequila Vinasse

TV was kindly supplied by a factory producing tequila 100% agave located in Tequila, Mexico. The suspended solids contained in the TV were removed by centrifugation (Gea Westfalia™ D2–06-107, Germany). After centrifugation, the TV was stored at 4 °C until usage. Table 1 shows the physicochemical parameters measured from the centrifuged TV, which are further discussed in detail in Section 3.1.

Table 1. Physicochemical characterization of the tequila vinasse used in the current study.

Parameter	Value
pH	3.43
Acidity (g $CaCO_3$/L)	6.8 ± 0.1
Total COD (g/L)	52.1 ± 2.8
Soluble COD (g/L)	50.3 ± 4.3
Total organic carbon (g/L)	23.5 ± 0.02
Total reducing sugars (g/L)	8.1 ± 0.1
Total carbohydrates (g/L)	11.9 ± 0.3
Total nitrogen (mg/L)	182.5 ± 17.6
Total phosphorous (mg/L)	297.5 ± 42.4
Sulfate (mg/L)	225.0 ± 0.0
Total solids (g/L)	37.1 ± 0.6
Total volatile solids (g/L)	33.8 ± 0.6
Total suspended solids (g/L)	5.5 ± 0.8
Copper (mg/L)	1.0
Iron (mg/L)	29.8
Manganese (mg/L)	11.2
Zinc (mg/L)	1.2
Sodium (mg/L)	47.1
Nickel (mg/L)	1.0
Magnesium (mg/L)	374.0
Molybdenum (mg/L)	1.0
Sulfur (mg/L)	61.9
Potassium (mg/L)	655.0
Calcium (mg/L)	493.0

2.2. Inocula

Two different inocula were tested for hydrogen production from TV. The first one was anaerobic granular sludge obtained from an UASB reactor treating organic waste, which was subjected to repeated heat shock-aeration pretreatments in order to kill methanogens while preserving facultative bacteria as well as the sporulating, hydrogen-producing *Clostridium* spp., as reported by García-Depraect et al. (2017) [26]. The resulting inoculum,

hereafter referred to as HATI, is a proven working horse to produce hydrogen via DF [7]. During the experimental period, the HATI inoculum was preserved by refrigeration (at 4 °C), as recommended elsewhere [27]. To inoculate metabolically active bacteria, the microbial consortium was reactivated before each fermentation run. Precultures were incubated for 24 h at 37 °C using a growth medium containing the following constituents (in g/L): lactose 10.0; NH_4Cl, 2.4; K_2HPO_4, 2.4; $MgSO_4 \cdot 7H_2O$, 1.5; KH_2PO_4, 0.6; $CaCl_2 \cdot 2H_2O$, 0.15; $FeSO_4 \cdot 7H_2O$, 0.05 [28]. On the other hand, the second inoculum tested was anaerobic granular sludge derived from a full-scale internal circulation reactor digesting TV under mesophilic conditions, which was pretreated by heating at 104 °C for 24 h and then ground in a blender and sieved through a 850 µm mesh screen [29].

2.3. Experimental Setup and Operating Conditions

A 3 L jacketed glass reactor (Applikon™, The Netherlands) was used with the following components: a mechanical stirring device (Applikon™, The Netherlands) that was kept at 150 rpm, a pH controller (BC electronics™ pH 7865, Italy) to maintain pH at 6.0 ± 0.05 by dosing a sodium hydroxide (10 N) solution as needed, a thermostat (Lauda™ RA 8, Germany) to maintain the temperature at 35 °C, and a gas flow meter (Bioprocess control™ µFlow, Sweden) to record gas production (Figure 1).

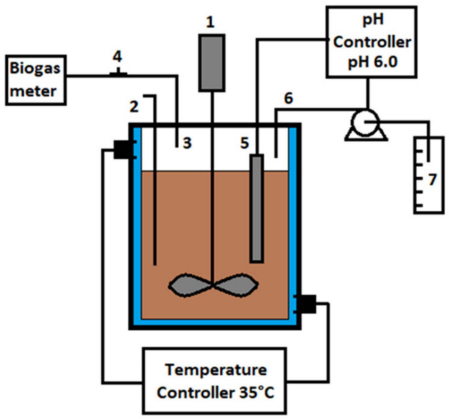

Figure 1. Schematic diagram of the reactor set-up. Mechanical stirring device (1), liquid sampling port (2), gas outlet (3) and sampling port (4), pH meter (6), and graduated cylinder containing 10 M NaOH (7).

Fermentation runs were carried out in triplicate for each inoculum tested. For runs using the HATI inoculum (runs 1–3), 0.3 L (equivalent to 10% of the working volume) of the precultured microbial consortium and 2.7 L of TV were loaded into the reactor, as previously outlined by García-Depraect and León-Becerril (2018) [18]. In the other case (runs 4–6), 20 g/L of the HTI inoculum and 3 L of TV were fed to the reactor, as previously described by Buitrón and Carvajal (2010) [29]. Here, it is worth mentioning that both specific methodologies employed with each type of inoculum are widely used in the DF of TV. The differences in the way that the runs were carried out, namely, inoculum to substrate ratio and the amount of substrate, were further considered in the analysis and discussion for the sake of comparison. Liquid samples were taken periodically to measure total reducing sugars (TRS), volatile fatty acids (VFAs) and lactate. COD and solids were measured at the start and end of the process, whereas biogas production was monitored throughout the run. Finally, to measure the hydrogen content in the evolved biogas, 25 µL of gas were periodically sampled during the gas production phase and analyzed by gas chromatography.

2.4. Analysis

The analyses for COD, pH, total nitrogen, total phosphorous, and solids were performed according to standard methods [30]. TRS was measured using the 3,5-dinitrosylsalicilic

acid (DNS) method, employing glucose for the calibration curve [31]. Hydrogen content in the evolved acidogenic off-gas was measured with a gas chromatograph (PerkinElmer™ Clarus 580, USA) using the operating conditions described by [26]. VFA and lactate were measured by high-performance liquid chromatography (HPLC) according to the procedure described by [18]. Microbial communities present at the rapid phase of hydrogen production were characterized by high-throughput sequencing using the Illumina Miseq platform (Illumina, USA). Genomic DNA was extracted from 0.3 g sample using the MoBio PowerSoil DNA extraction kit (Mo Bio Laboratories Inc., USA) according to the manufacturer's instructions. The quality and quantity of the extracted DNA were assessed with a NanoDrop-2000 spectrophotometer (Thermo Fisher Scientific, USA), then the DNA samples were analyzed by RTL Genomics (RTL, USA) for 16 rRNA gene sequencing using the universal primers 28F and 388R for bacteria. Bioinformatic data analysis was performed according to RTL's protocol [22]. Bacteria with less than 1.0% of relative abundance were grouped together as others.

2.5. Data Analysis

Hydrogen production kinetics were fitted to the modified Gompertz model (Equation (1)), where e is the Neperian number, CHP is the maximum cumulative hydrogen production (NmL), P_{max} is the maximum hydrogen production rate (NmL/h), t is the incubation time (h), and λ is the lag phase duration (h). CHP was further divided by the volume of TV fed to obtain normalized results. P_{max} was divided by the working volume to obtain the maximum hydrogen production rate (HPR, NL/L-d). The gas volume herein reported is normalized to 0 °C and 1 atm. The observed correlation coefficient (R^2) value was used to assess the quality of fitting, while sensitivity analysis was used to validate the estimated kinetics parameters [32]. The cultivation time needed to achieve 90% of total hydrogen production (t_{90}) was also estimated as described in Equation (2) [33]. The t_{90} value is an indicator of the time needed for the fermentation to end, making it useful to evaluate the performance between runs. Finally, significant differences between runs were determined using a homoscedastic Student's *t*-test with a 95% confidence level (*p*-value < 0.05).

$$H_2(t) = CHP * \exp\left\{-exp\left[\frac{P_{max} * e}{CHP}(\lambda - t) + 1\right]\right\} \quad (1)$$

$$t_{90(H2)} = \frac{CHP}{P_{max} * e}[1 - ln(-ln\ 0.90)] + \lambda \quad (2)$$

3. Results and Discussion

3.1. Evaluation of Nutrients Present in TV

As shown in Table 1, the centrifuged TV presented a relatively high COD content, mainly in the soluble form. As expected, the higher fraction of solids (more than 85% of the total solids) were mostly measured as dissolved solids. Besides fermentable carbohydrates, the TV also contained macro and micronutrients including N, P, Fe, Ni, Mg, Ca, among others, which together were able to support the DF process regardless of the type of inoculum tested, as will be discussed in the following sections. It is well known that microorganisms need several nutrients to grow, and the bacteria involved in DF are no exception. Nutrient deficiencies in the substrate can be the bottleneck for the growth of hydrogen-producing bacteria. Firstly, N and P are needed for cellular growth, including the synthesis of enzymes; Argun et al. (2008) determined that a C/N ratio of 200 and a C/P ratio of 1000 are optimal for hydrogen production from wheat starch [34]. Hydrogen production by *Clostridium* sp. occurs through the action of the hydrogenase and pyruvate-ferredoxin oxidoreductase enzymes [35]. Both proteins contain Fe atoms [36,37], meaning that enough Fe is needed for hydrogen production. It has been previously determined that $FeCl_3$, at a concentration of 800 mg/L, optimized the hydrogen production [38]. Mg, Na, Zn, and Ca are also important trace elements for the growth of anaerobic bacteria since

they are required as enzyme cofactors and in cellular transport processes. Lin and Lay (2005) proposed an optimal formulation containing Mg, Na, Zn, Fe, and N [39].

Table 2 shows a comparison between the nutrients (relative to the carbon content) present in the TV herein used and the optimal nutrient formulation obtained from other studies aiming to maximize the hydrogen production by varying nutrient formulation. The TV showed calcium and sodium levels below those previously ascertained as optimal for hydrogen production, whereas the relative amounts of P, Mg, and Zn were found to be higher than the ones reported in the literature as the optimal. Regarding N content, the concentration present in the TV is lower than the optimal one reported by Oztekin et al. (2008) [40] and Pérez-Rangel et al. (2020) [41], but higher than that reported by Lin and Lay (2005) [39] and Argun et al. (2008) [34]. Likewise, the relative Fe content in the used TV fell well within the values observed elsewhere [39,40]. At this point, it should be noted that it is difficult to make a fair comparison among different hydrogenogenic systems, given that the DF is a complex process itself, and the high variability in set-ups and operating conditions including the type of inoculum and substrate. Furthermore, the physicochemical composition of TV changes considerably from factory to factory; even it may vary from batch to batch within the same factory depending mainly on the characteristics of the agave used to produce tequila. It has been previously reported that special focus must be paid in the content of N and Fe of TV since both might be limiting for hydrogen production [28]. This means that, in the case of nutrient-limiting conditions, tailored nutrient supply should be pursued rather than an indiscriminate use of extra chemicals, which is costly and, consequently, prohibitive to industrial applications.

Table 2. Nutrients present in the tequila vinasse used in this study and optimal nutrient formulation for hydrogen production previously reported in other studies. Values are expressed as parts of nutrient per 100 parts of organic carbon.

Substrate	N	P	Mg	Fe	Ca	Zn	Na	Reference
Wheat powder	0.5	0.1	-	-	-	-	-	[34]
Anaerobic sewage sludge	0.2	-	0.2	0.04	7	0.003	5	[39]
Wheat starch	2	0.8	-	1.5	-	-	-	[40]
Lignocellulose	5.7	0.3	-	-	3.8	-	-	[41]
Tequila vinasse	0.8	1.3	1.6	0.10	2.1	0.005	0.2	This study

3.2. Hydrogen Production Performance

The cumulative hydrogen production from TV using either HATI or HTI is shown in Figure 2. Similar hydrogen production profiles were sustained by the inocula tested; in all the cases, they adjusted well to the modified Gompertz model ($R^2 > 0.99$). Methane was not detected throughout all the runs, suggesting that both inoculum pretreatment methods were effective in removing methanogens. The feasibility of producing hydrogen from high-strength, undiluted TV without nutrient supplementation was herein demonstrated for the first time. As seen in Table 3, the use of HATI led to slightly better performance indicators such as higher CHP (2.6 NL H_2/L vinasse), HPR (1.9 NL H_2/L-d), higher hydrogen content in the biogas (71%), and shorten lag phase (63.3 h). However, no significant differences (p-value > 0.05) were found when compared to the performance exhibited by the HTI inoculum. Some runs were finished before a clear plateau was reached; in those cases, slightly higher hydrogen productions could be expected. Hydrogen yields were similar between the inocula used, ranging from 73.6 to 78.2 NmL H_2/g VS added, which are equivalent to 47.8 to 50.7 NmL H_2/g COD added. In terms of substrate consumption, the soluble COD removal efficiency ranged from 15% to 17%, while the removal of reducing sugars was between 47 and 51%. The fact that it was feasible to produce hydrogen from TV without the need of adding fresh water to dilute the feeding and/or extra nutrients encourages its use as a DF feedstock.

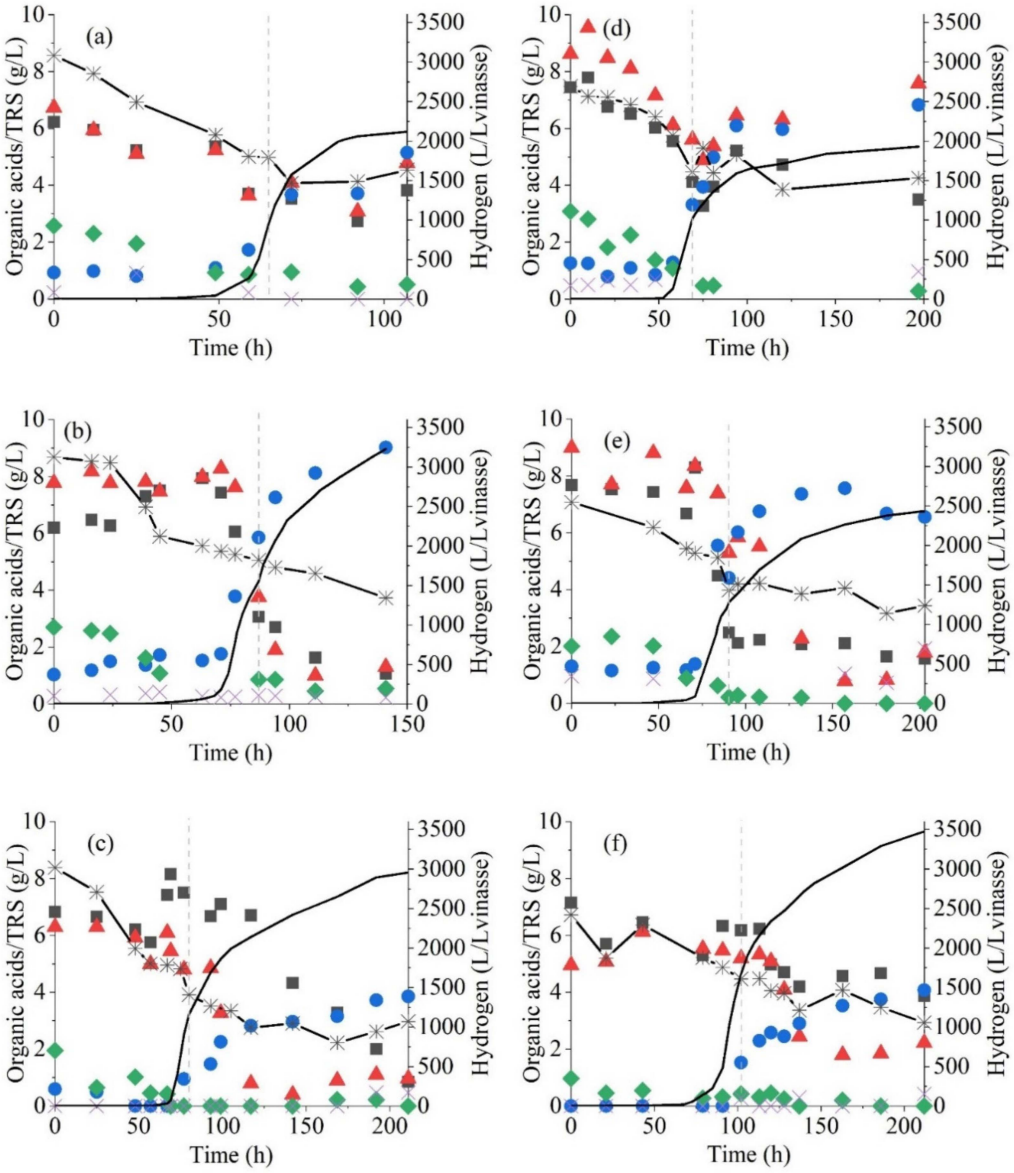

Figure 2. Time course of cumulative hydrogen production (-), total reducing sugars (*), and organic acids such as formate (♦), acetate (■), lactate (▲), propionate (×), and butyrate (●) for the runs conducted with the microbial consortia HATI (run 1 (**a**), run 2 (**b**), and run 3 (**c**)), and HTI (run 4 (**d**), run 5 (**e**), and run 6 (**f**)). Vertical dotted lines show the sampling time for the microbial community analysis.

Regarding other studies dealing with the DF of TV, a comparison of operating conditions and performances is shown in Table 4. García-Depraect and León-Becerril (2018) [18] reported a HPR of 3.8 NL H_2/L-d and a hydrogen production yield of 124 NmL H_2/g VS added, which are roughly twofold higher than those obtained in the present study, likely due to the different TV used by the authors, which indeed was amended with nutrients. Buitrón et al. reported HPR values of 1.2 and 1.7 NL H_2/L-d in semi-continuously [19] and continuously fed [20] reactors, respectively, which are quite similar to those observed in this study; however, the authors diluted the TV and supplemented it with several nutrients to enhance hydrogen production. In another study, Toledo-Cervantes et al. (2020) [25] reported the hydrogen production from undiluted TV (28.1 g COD/L) without extra nutrients, achieving a HPR of 0.5 NL H_2/L-d and a hydrogen yield of 0.75 L H_2/L of TV. Using undiluted TV (26.2–36.1 g COD/L) without nutrient supply, unstable and very low HPR values (0.06 ± 0.07 NL H_2/L-d) were obtained in a CSTR with a stirring rate of 200 rpm

and a hydraulic retention time of 6 h [23]. Higher HPR values of up to 12.5 NL H_2/L-d have been reported in CSTR systems fed with TV, but at the expense of supplementing extra nitrogen and iron [7,21].

Table 3. Comparison of the modified Gompertz model kinetic parameters and other hydrogen production performance indicators obtained with the different microbial consortia tested.

Performance Indicator	Inoculum		*p*-Value
	HATI	HTI	
CHP (NmL H_2/L vinasse)	2644 ± 538	2490 ± 804	0.797
HPRmax (NL H_2/L-d)	1.89 ± 0.72	1.43 ± 0.16	0.339
Lag phase (h)	63.3 ± 5.3	66.9 ± 11.8	0.660
R^2	0.996 ± 0.005	0.995 ± 0.002	0.805
t_{90}	104 ± 23	117 ± 29	0.570
H_2 content (% *v*/*v*)	71 ± 7	64 ± 10	0.350
Hydrogen yield (NmL H_2/g VS added)	78.2 ± 15.9	73.6 ± 23.8	0.797
Hydrogen yield (NmL H_2/g COD added)	50.7 ± 10.3	47.8 ± 15.4	0.797
Soluble COD removal (%)	15 ± 1	17 ± 4	0.657
TRS removal (%)	47 ± 9	51 ± 7	0.437

Table 4. Comparison of different reported dark fermentation systems fed with tequila vinasse.

Operation Mode	pH	Temperature (°C)	Inoculum Pretreatment	Hydrogen Production Rate (NL H_2/L-d)	Nutrient Supplementation	Feeding Concentration (g COD/L)	Reference
Batch	5.5	35	Heat-aeration	3.8	Yes	58	[18]
Semi-continuous	4.7	35	Heat	1.2	Yes	16	[19]
Continuous	5.5	35	Heat	1.7	Yes	8.5	[20]
Continuous	5.5	35	Heat-aeration	12.5	Yes	42	[21]
Continuous	5.5	N.R.	Heat	0.06	No	26	[23]
Semi-continuous	5.5	55	Heat	0.5	No	29	[25]
Batch	6.0	35	Heat-aeration	1.9	No	52	This study

N.R.: not reported.

As shown in Figure 2 and Table 3, some variability between runs was observed in hydrogen production. The use of HATI and HTI exhibited variation coefficients in CHP of 20 and 34%, respectively, which are higher than the 7% reported in a batch DF reactor fed with concentrated TV amended with nutrients [18]. Future batchwise studies should thus analyze in detail the variability of the hydrogen performance and the associated metabolic pathways between different batch runs, where low variability or, in other words, high process consistency, should be sought. Likewise, the development of novel integrated approaches devoted to the long-term stability in continuous hydrogen-producing reactors warrants more research. The importance of nutrient addition in the DF of TV was studied by García-Depraect et al. (2019) and showed that TV with a reduced nutrient formulation (containing only N and Fe sources) led to a 39% lower hydrogen yield than when a complex formulation was used [28]. The impact of nutrient addition in the stability and performance of hydrogen-producing reactors continuously fed with TV should be determined in further studies. Similarly, a cost-benefit analysis between the potential higher stability and hydrogen production efficiency and the cost of adding nutrients should be performed.

3.3. Effect of the Inoculum Type on the Microbial Community Composition

The heat-shock pretreatment used in this study (105 °C for 24 h) was aimed at eliminating all non-spore-forming microorganisms while allowing spore-forming, hydrogen-producing bacteria, *Clostridium* spp., to survive [29]. As seen in Table 5, the only spore-forming microorganisms detected (during the accelerated phase of hydrogen production) in the runs inoculated with the inoculum HTI were bacteria affiliated to the *Clostridium* genus. Other detected non-spore-forming bacteria such as the genera *Prevotella*, *Enterococcus*,

Klebsiella, and *Enterobacter* could be autochthonous microorganisms present in the TV. Indeed, besides *Clostridium* genus, *Klebsiella*, *Eubacterium*, *Streptococcus*, *Lactobacillus* have been found to be part of the native microflora of TV [28]. The microbial community analysis of the HTI did not show the presence of a spore-forming facultative anaerobe that could survive the heat-shock treatment (*Bacilliales* sp.). *Clostridium* sp. as a strict anaerobe needs an oxygen-free environment to grow, which can be achieved either by inert gas sparging or with the aid of microorganisms that deplete the oxygen. In this regard, it is reasonable to infer that the production of hydrogen using the HTI inoculum was depended on the presence of facultative anaerobes in the substrate, which allowed *Clostridium* to thrive by creating anaerobic conditions.

Table 5. Microbial community composition at the accelerated phase of hydrogen production.

Microbial Species	Facultative	Sporulating	H_2 Producer	HATI			HTI		
				Run 1	Run 2	Run 3	Run 4	Run 5	Run 6
Prevotella sp.	No	No	No	0.0	0.0	0.0	4.0	0.0	0.0
Sporolactobacillus terrae	No	Yes	No	0.3	0.1	1.4	0.5	0.0	0.1
Enterococcus casseliflavus	No *	No	No	0.0	0.0	0.0	13.7	2.4	0.0
Lactobacillus casei	No *	No	No	18.6	7.0	14.7	0.1	0.0	0.0
Lactobacillus harbinensis	No *	No	No	11.9	2.4	8.1	0.0	0.0	0.0
Lactobacillus rhamnosus	No *	No	No	0.0	0.8	1.1	0.0	0.0	0.0
Clostridium beijerinckii	No	Yes	Yes	25.8	8.7	40.4	65.7	37.9	34.8
Clostridium sp.	No	Yes	Yes	4.2	0.9	4.7	4.4	5.1	2.9
Enterobacter sp.	Yes	No	Yes	10.9	25.0	5.6	0.0	16.3	56.1
Klebsiella sp.	Yes	No	Yes	18.8	54.6	13.4	0.0	32.4	1.2
<1.0%	-	-	-	1.3	0.1	1.0	3.2	0.0	0.0
Unclassified	-	-	-	8.2	0.5	9.7	8.2	5.9	4.9

* Aerotolerant microorganisms that can grow in the presence of oxygen but not metabolize it [35].

On the other hand, the HATI culture underwent a different pretreatment that consisted of repeated light heat-shock and micro-aeration cycles. This pretreatment successfully eliminates methanogens while preserving other non-hydrogen-consumers, which, in turn, may enhance the overall hydrogen production performance [18,26]. *Lactobacillus* sp. are non-spore-forming, aerotolerant bacteria, which means that they can grow in the presence of oxygen, but unlike facultative anaerobes, they do not metabolize it [35]. This could explain the presence of *Lactobacillus* in the runs performed with the HATI inoculum but not with the HTI one, which was one remarked difference in the microbial community composition. It has been previously determined the microbial community composition of the HATI inoculum, embracing diverse genera such as *Enterobacter*, *Klebsiella*, *Prevotella*, *Lactobacillus*, *Clostridium*, *Acetobacter*, *Citrobacter*, *Propionibacterium*, *Actinomyces*, *Sporolactobacillus*, among others [18,26]. Therefore, the presence of facultative anaerobes in these runs could be mainly attributed to the inoculum origin; however, the indigenous microflora of the TV would also have an impact on the overall process performance, a fact that deserves further investigation.

3.4. Effect of the Inoculum Type on the Metabolic Profiles

As seen in Figure 2, the consumption of TRS during the DF process reached values around 50%, 11–12 g equivalent glucose, regardless of the type of inoculum. It is worth mentioning that a higher TRS consumption (82% on average) occurred during the lag phase of hydrogen production. This is indicative that the DF of TV was, to a significant extent, uncoupled from carbohydrates consumption. This uncoupling behavior can be well supported with the lactate-based hydrogen-producing pathways, which have been previously reviewed comprehensively in [10]. Indeed, the observed hydrogen production corroborated well with the consumption of lactate and acetate and the formation of butyrate. The lactate-driven hydrogen fermentation has been reported to take place using

TV [7,18,25,28], but also with others such as cheese whey [33], sugarcane vinasse [42], olive mill waste [43], among others.

Using TV and HATI as the major substrate and the inoculum, respectively, it has been shown that lactic acid bacteria (LAB) mainly affiliated to the genus *Lactobacillus* are responsible for the conversion of carbohydrates to lactate (or lactate plus acetate when the heterolactic pathway became dominant) in a primary fermentation, while hydrogen-producing bacteria (HPB) of the genus *Clostridium* play a major role in hydrogen production from the lactate in a secondary fermentation [44]. Other authors have reported similar microbial dynamics during the lactate-driven DF process [18,45,46]. At this point, it should be noted that, besides *Lactobacillus* and *Clostridium*, other bacteria such as *Klebsiella*, *Prevotella, Enterococcus*, and *Sporolactobacillus*, were also coexisting microorganisms during the process.

As previously mentioned, the consumption of lactate and acetate associated to the concomitant production of butyrate and hydrogen was observed in all the tested runs regardless of the type of inoculum used, except in runs 1 and 4, likely due to the prevalence of other co-existing metabolic pathways such as lactic fermentation. Indeed, an increase in lactate concentration was observed in runs 1 and 4, which coincided with the lowest hydrogen production yield recorded by the inocula used. Interestingly, the presence of LAB in runs 4–6 (those inoculated with the inoculum HTI) was not clear; although the presence of LAB at the rapid phase of hydrogen production was negligible, the microbial composition has been widely reported to be highly dynamic in batch reactors [18,45,46]. The hydrogen production observed was thus supported by the lactate and acetate initially contained in the TV. Those high concentrations of lactate and acetate are suggestive of heterolactic fermentation, which would happen during the collection, handling, or even storage of the TV. Indeed, earlier reports have shown diverse feedstocks with high concentrations of lactate and acetate [47–49]. In the literature, the presence of LAB in DF is a cause of controversy due to its different effects on hydrogen production [10,50,51]. In some cases, it has been reported that LAB hinders the production of hydrogen by excreting bacteriocins and/or competing for the substrate (carbohydrates) [52–54]; however, in other cases, it has been reported that LAB enhance hydrogen production by providing hydrogen precursors, i.e., lactate [28,33,42].

As seen in Table 5, *C. beijerinckii*, a known hydrogen producer, was present in all the runs tested regardless of the inoculum used; thus, it was proposed to be the main HPB thanks to its capacity to metabolize lactate into hydrogen [55]. *Clostridium* sp. cells are known to be the more common hydrogen producers in DF reactors, yielding 1.5–3 mol H_2/mol hexose [50]. Thus, it would be expected that a higher abundance of *Clostridium* spp. correlates well with a higher hydrogen production performance. However, in practice, that correlation is not always observed, or at least not a high correlation, due to the presence of other coexisting HPB or non-HPB, meaning that HPB abundance should be rather interpreted in a comprehensive way. Herein, the relative abundance of *C. beijerinckii* was not necessarily correlated with a high hydrogen production. The first run of HTI (run 4) showed the highest *C. beijerinckii* relative abundance (65.7%) but the lowest hydrogen production (1.7 NL H_2/L of vinasse), which could be explained by the absence of *Enterobacter* sp. and *Klebsiella* sp. A similar trend was observed by Cheng et al. (2011), where the abundance of *Clostridium* spp. (mainly *C. pasteurianum*) was directly related to the hydrogen production yield when using sucrose as the substrate. However, when using sugarcane molasses as the substrate, no significant correlation of *Clostridium* spp. and hydrogen production performance was found due to the dominance of LAB and *Klebsiella* sp. [56].

In this study, the highest hydrogen yield of 3.4 NL H_2/L vinasse was recorded in run 6 using HTI as the inoculum, which showed the highest abundance (56%) of *Enterobacter*. Likewise, high abundance (55%) of *Klebsiella* in run 2 using HATI resulted in 3.1 NL H_2/L vinasse, pointing out the importance of thriving beneficial bacterial associations like *Clostridium-Enterobacter-Klebsiella* that can boost the DF process. An increase in the hydrogen production due to the proliferation of *Enterobacteriaceae* in codominance with

Clostridium was also observed by Pattra et al. [57]. Although *Clostridium* sp. are believed to be the most efficient hydrogen producer, some studies outline that *Enterobacter* sp. can also yield a high hydrogen production [58–60], on some occasions even higher than *Clostridium* sp. [61]. Considering that, it is interesting to point out that future studies should study the role of *Enterobacteriaceae*, whose fermentation end metabolites include ethanol, succinate and 2,3-butanediol [35], to better explain the hydrogen production performance. The symbiotic relation between *Enterobacteriaceae* and *Clostridium* is explained by the fact that *Enterobacteriaceae* are facultative anaerobes that generate an anaerobic environment by consuming oxygen [62]. The main hydrogen producers, *Clostridium* sp., as strict anaerobes, need the mentioned anaerobiosis to grow [35,50]. An oxygen-free environment can be achieved by nitrogen sparging or by microorganisms that deplete oxygen (facultative anaerobes). However, previous studies have stressed that nitrogen sparging not only increases the hydrogen production costs, but also dilutes the hydrogen content in the acidogenic off-gas [59,63], hence, the importance of facultative anaerobes in DF is highlighted.

4. Conclusions

The feasibility of producing hydrogen from undiluted TV without nutrient supplementation using two different fermentative inoculum sources was demonstrated for the first time in this study. Interestingly, the main hydrogen-producing pathway was found to be uncoupled from the metabolization of carbohydrates regardless of the inoculum used. Consequently, hydrogen gas was thus produced from the consumption of lactate, and acetate acting as an electron acceptor, with the concomitant formation of butyrate, reinforcing the emerging concept of favoring the lactate-driven DF process for the effective production of hydrogen from TV. It should be realized that, although there was evidence that it is possible to produce up to 1.9 NL H_2/L-d or 2.6 NL H_2/L vinasse using high-strength, undiluted TV without nutrient supplementation, those values are lower than previously reported maximum values using TV supplemented with nutrients. The nitrogen and iron content of TV is of special importance, and should be verified to avoid DF inhibition. Besides hydrogen producers, the presence of facultative bacteria was important in the process. Particularly, a high DF process performance was not correlated with *Clostridium* but with the microbial association of *Clostridium-Enterobacter-Klebsiella*, which was favorable for oxygen depletion and hydrogen production.

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