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Influence of key operational parameters on biohydrogen production from fruit and vegetable waste via lactate-driven dark fermentation

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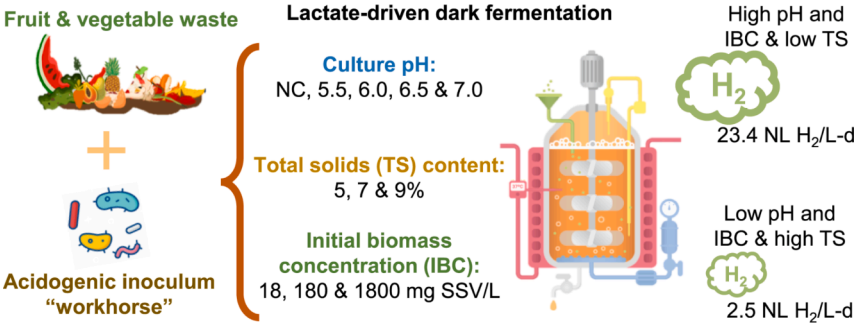
HIGHLIGHTS

- First study exploring the effect of 3 key process parameters on the LD-DF of FVW.
- Boosted H_2 production at pH 7, 5% total solids and 1.8 g VSS/L cell concentration.
- H_2 production agreed with the lactate uptake and acetate and butyrate production.
- Moderate H_2 yield (50 mL/g VS) but an outstanding rate (976.4 mL/L-h) was achieved.
- Lactate-based DF is a promising route to transform FVW into H_2 at high rates.

GRAPHICAL ABSTRACT



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ABSTRACT

This study aims at investigating the influence of operational parameters on biohydrogen production from fruit-vegetable waste (FVW) via lactate-driven dark fermentation. Mesophilic batch fermentations were conducted at different pH (5.5, 6.0, 6.5, 7.0, and non-controlled), total solids (TS) contents (5, 7, and 9%) and initial cell biomass concentrations (18, 180, and 1800 mg VSS/L). Higher hydrogen yields and rates were attained with more neutral pH values and low TS concentrations, whereas higher biomass densities enabled higher production rates and avoided wide variations in hydrogen production. A marked lactate accumulation (still at neutral pH) in the fermentation broth was closely associated with hydrogen inhibition. In contrast, enhanced hydrogen productions matched with much lower lactate accumulations (even it was negligible in some fermentations) along with the acetate and butyrate co-production but not with carbohydrates removal. At pH 7, 5% TS, and 1800 mg VSS/L, 49.5 NmL-H_2/g VS_{fed} and 976.4 NmL-H_2/L-h were attained.

1. Introduction

Nowadays, the inadequate management of fruit and vegetable waste (FVW) is a global concern due to its negative social impacts, economic losses and environmental damage (Ganesh et al., 2022). In the European Union, it is estimated that ≈ 21 kg of unavoidable FVW are generated

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per person annually (De Laurentiis et al., 2018). Besides postconsumer generation, FVW is also generated during its entire production chain. In line with the Sustainable Development Goals 2030, the European Commission actions aimed at reducing food waste generation and promoting its circularity through the Circular Bioeconomy Strategy (European commission, 2020). In this context, FVW is a promising feedstock to produce value-added products and renewable bioenergy through a biorefinery approach (Patel et al., 2019).

The dark fermentation (DF) process is likely the most promising route for producing renewable hydrogen from organic waste, owing to its multiple advantages such as no light requirement, simple operation, relatively high energy yield, and high compatibility with other biotechnologies (e.g., bioplastics-accumulating fermentation, anaerobic digestion, among others) within a biorefinery scheme (Mohan et al., 2016; Chen et al., 2022). Although fermentative hydrogen production from food waste (including FVW) has been previously investigated, some technical limitations, such as high process instability and low hydrogen production yields and rates, must be overcome for further technology scale-up. These bottlenecks have been mostly associated with the excessive growth of lactic acid bacteria (LAB) and consequently with an accumulation of lactate (HLac) (Pu et al., 2019; Santiago et al., 2019; Im et al., 2020). However, recent studies on the DF of food waste have acknowledged the possibility to effectively produce hydrogen from HLac (Gomez-Romero et al., 2014; Noblecourt et al., 2018; Villanueva-Galindo and Moreno-Andrade, 2021). Lactate-driven DF, herein referred to as LD-DF, relies on the cooperative associations between LAB, whose main end-product is HLac, and some specialized hydrogen-producing bacteria (HPB) that can metabolize HLac (Detman et al., 2021; García-Depraect et al., 2021). Harnessing the interactions between LAB and lactate-utilizing HPB (LU-HPB) could enable a more practical and efficient hydrogen production process (Sharmila et al., 2022). However, to the best of the authors' knowledge, the LD-DF of FVW has not been systematically investigated. Additionally, although it is well-known that operational parameters such as pH, total solids (TS) content and microbial density impact the fermentative hydrogen production performance (Ramos et al., 2012; Lee et al., 2014; Moon et al., 2015; Florio et al., 2017; Cappai et al., 2018; Ghimire et al., 2016; Ghimire et al., 2018; Chen et al., 2022), there is a knowledge gap about their impact on the LD-DF using FVW as substrate. Previous studies showed that operational pH values ranging from 5.5 to 7.0 are more conducive to hydrogen production via LD-DF, but the optimal pH is rather dependent on the substrate type, microbial structure involved, and so on (García-Depraect et al., 2021). It has been also reported that high TS contents (>15%) might redirect the carbon flux towards HLac accumulation (Ghimire et al., 2018). Similarly, the initial cell biomass concentration can also influence the hydrogen production outcome (Das et al., 2011). It is therefore crucial to deeply understand how those operational parameters would impact the LD-DF of FVW.

The present study aims at investigating the influence of culture pH, TS content, and initial biomass concentration on the hydrogen production performance from the LD-DF of FVW. To the best of authors' knowledge, this is the first effort made to elucidate the role of such key operational parameters on the FVW-to-hydrogen bioconversion via LD-DF. The findings and discussion herein presented could help in the further development of continuous high-rate DF reactors based on lactate-utilizing, hydrogen-producing pathways, thus tackling process limitations related to the excessive proliferation of LAB.

2. Materials and methods

2.1. Inoculum and substrate

The source of the inoculum herein used was digestate derived from a pilot (100 L) anaerobic digester treating restaurant food waste under mesophilic conditions. The recovered digestate was pretreated by heat shock at 90 °C for 20 min to irreversibly inactivate methanogens (García-Depraect et al., 2022a). Likewise, the enrichment of hydrolytic/acidogenic bacteria was carried out by successive culture passages according to García-Depraect et al. (2019a) but using a growth medium composed of (g/L) lactose 10.0, NH_4Cl 2.4, K_2HPO_4 2.4, $MgCl_2{\cdot}6H_2O$ 2.5, KH_2PO_4 0.6, $CaCl_2{\cdot}2H_2O$ 0.15, and $FeCl_2{\cdot}4H_2O$ 0.035. The resulting inoculum harbored bacteria (such as LAB and LU-HPB) able to perform the LD-DF (García-Depraect et al., 2022a). On the other hand, the fresh fruits and vegetables utilized for mimicking FVW were purchased from a local marketplace. The composition of FVW was adapted from Abubackara et al. (2019) and consisted of banana 14.5, eggplant 12.8, carrot 9.7, tomato 8.4, cucumber 7.5, onion 7.1, radish 6.5, potato 6.2, capsicum 5.7, apple 5.3, cabbage 4.7, grape 3.1, orange 3.1, lemon 2.7, and pumpkin 2.4% (*w/w*). Prior to use, the substrate was blended with an electrical blender (Sammic, XM-32, Azkoitia, Spain) to reduce its particle size and to get a homogenous FVW slurry. No additional tap water was required during blending. The FVW was then collected in 1L plastic bags and frozen at −20 °C to avoid any deterioration. The physicochemical properties of the substrate are presented and discussed in section 3.1.

2.2. Experimental set-up and operational conditions

A series of batch tests was carried out to investigate the influence of pH, TS content and initial cell biomass concentration on the performance of LD-DF of FVW. All fermentation assays were conducted in two identical 1.25-L custom-made fermenters with a working volume of 0.7 L. Each fermenter was equipped with a pH controller (BSV Electronic S. L., EVopH-P5, Spain), a pH electrode (BSV Electronic S. L., HO35-BSV01, Spain), a liquid and a gas sampling port, and a custom-made biogas-flow meter operating under the liquid displacement principle (see Supplementary material).

The effect of pH on the performance of LD-DF of FVW was investigated by controlling the operational pH at 5.5, 6.0, 6.5 and 7.0, which have been found to be conducive for the LD-DF (García-Depraect et al., 2021). A set of fermentations with no pH control was also carried out. The initial pH of the culture broth was 4.6, while the operational pH was adjusted and controlled at the target set-point by adding 6 N NaOH (or 3 N HCl if needed) with an accuracy of ±0.1. All fermentations devoted to investigating the effect of pH were performed with an initial biomass concentration of 18 mg volatile suspended solids (VSS)/L and 5% TS. After the determination of the optimal pH for hydrogen production, a second series of batch tests aimed at evaluating the impact of the initial TS concentration on process performance was conducted. Such fermentations were carried out at an initial TS content of 5, 7 and 9%, with a constant biomass concentration of 18 mg VSS/L and a fixed pH of 7. It should be noted that an initial TS of 9% implied the use of undiluted FVW. Finally, the influence of the initial cell biomass concentration was ascertained by testing three different concentrations, i.e., 18, 180, and 1800 mg VSS/L. VSS was used as a proxy of biomass concentration and the lower value of 18 mg VSS/L was the average initial biomass concentration at which the experiments done to study the effect of pH and TS content were carried out, thus 180 and 1800 mg VSS/L represented a 10 and 100 fold biomass increment, respectively. In such fermentations, the pH and TS content were set at 7 and 5%, respectively. See Supplementary material for a detailed summary of operational conditions and corresponding initial substrate concentrations and initial food-to-microorganism (F/M) ratios.

The fermenters were placed in a controlled-temperature room set at 37 ± 1 °C, magnetically agitated at ~ 300 rpm, and sealed under air atmosphere. Tap water was used to adjust the desired TS content, while fermenters were inoculated with 10% (*v/v*) of a fresh hydrogenogenic inoculum. The preparation of the inoculum was different depending on the condition tested. Thus, the inoculum preserved by refrigeration (4 °C) was reactivated before each use via two successive subculture passages for the assessment of the influence of pH and TS content. Briefly, an aliquot of 0.1 L of the refrigerated inoculum was cultivated

for 19 h at 37 ± 1 °C, ~150 rpm, with no pH control, in a closed air atmosphere using a 2.1-L fermenter (1 L working volume) and the defined mineral growth medium previously described in section 2.1. Then, the resulting biomass was harvested from the culture broth by centrifugation (10000 rpm for 10 min) and grown for 17 h under similar conditions to obtain a fresh and active hydrogenogenic microbial community.

The inocula employed in the tests aiming at evaluating the influence of biomass concentration on the LD-DF process were taken from a steady state continuous hydrogenogenic culture to ensure an identical metabolic state among the different biomass concentrations tested. The continuous fermentation was conducted in a 3-L Biostat-A fermentor (Sartorius Stedim, Spain) with a working volume of 2 L and equipped with an automated control system (Sartorius stedim biotech Biostat A). Mechanical agitation was set at 300 rpm, while pH was controlled at 5.5 ± 0.05 by adding 3 N NaOH and temperature at 35 ± 0.5 °C. The fermenter was inoculated with refrigerated inoculum at 10% *v/v*. Following a 24 h batch culture, the feeding regime was switched to a continuous mode, keeping a constant hydraulic retention time (HRT) of 12 h and an organic loading rate (OLR) of 180 g chemical oxygen demand (COD)/L-d. A pseudo-steady state was defined when hydrogen productivities recorded during at least 3 consecutive HRTs remained within ±10%. Biomass concentration was estimated by means of a correlation curve between VSS and optical density (OD). The culture broth was diluted or concentrated (by centrifugation at 10000 rpm for 10 min) depending on the initial biomass concentration targeted, using the mineral medium described in section 2.1 but without any carbon source.

All operational conditions were tested in duplicate, and the fermentation time was 48 h (corresponding to the period of time when the cumulative hydrogen production plateaued). Liquid and gaseous samples were taken and analyzed periodically throughout the fermentation. The key performance indicators of the process included the hydrogen yield, maximum volumetric hydrogen production rate (VHPR), hydrogen content in the acidogenic off-gas, as well as the removal efficiency of volatile solids (VS) and total carbohydrates, and the profile of organic acids and the acidification degree. The kinetics of hydrogen production obtained using the modified Gompertz model were also included as a process performance indicator, as shown in section 2.5.

2.3. Analytical techniques

Total carbohydrates content was analyzed using the phenol–sulfuric method, while protein content was determined by total Kjeldahl nitrogen (TKN) analysis using a nitrogen-to-protein factor of 6.25. Likewise, pH and the concentration of COD, VS and TS were measured according to standard methods (APHA, 2005). Lipid content was measured according to the PNTNAG-006 SERIDA fat protocol. OD was measured at 600 nm using a UV–vis spectrophotometer (BMG LABTECH, SPECTROstar Nano, Germany). The concentrations of organic acids, including formate (HFor), acetate (HAc), isobutyrate (i-HBu), butyrate (HBu), propionate (HPr), HLac, isovalerate (i-HVal), valerate (HVal), isocaproate (i-HCa), hexanoate (HHex) and heptanoate (HHep), were analyzed by high-performance liquid chromatography (HPLC) using a Waters alliance model e2695 (Massachusetts, USA) equipped with a Waters Alliance 2998 PDA UV–vis detector (Massachusetts, USA) set at 210 nm, a IR detector for ethanol measurement, and a Thermo scientific Carbohydrate H^+ 8 μm HyperREZ XP column (England, UK). The column temperature was set at 75 °C, while the mobile phase was composed of 25 mM H_2SO_4 at a flow rate of 0.7 mL/min. Sodium L-lactate (Sigma-Aldrich part number 71718, USA) and an organic acids mix (Sigma-Aldrich CRM46975, USA) were employed as standards for the quantification of organic acids. The composition of the gas phase was determined by gas chromatography (GC) using a Varian CP-3800 GC-TCD (PaloAlto, USA), as reported elsewhere (García-Depraect et al., 2022b). The volume of acidogenic-off gas was normalized to 0 °C and 1 atm.

2.4. Data analysis

Hydrogen production kinetics were analyzed using the modified Gompertz model (Eq. (1)) previously described by Ramos et al. (2012), where, $H(t)$ represents the total amount of hydrogen (in NmL) produced at time t (h), *Hmax* represents the maximal amount (in NmL) of hydrogen produced, *Rmax* is the maximum hydrogen production rate (in mL/h), and λ stands for the lag time (in h). Each experimental condition was tested in duplicate, and the plotted data corresponds to the average and standard deviation recorded. The acidification degree was calculated according to Eq. (2), where COD eq. is the net sum of COD equivalent (in g/L) of all the organic acids measured at the end of fermentation and $TCOD_{FVW}$ is the total COD concentration (in g/L) of the FVW fed. Finally, a COD mass balance analysis was performed according to Eq. (3). COD equivalent for biomass was estimated as 5% of the total COD of the influent (García-Depraect et al., 2019b).

$$H(t) = H_{max} * exp\left[- exp\left(\frac{2.71828 * R_{max}(\lambda - t)}{H_{max}} + 1 \right) \right] \quad (1)$$

$$Acidification\ degree(\%) = \frac{\text{COD eq.}}{TCOD_{FVW}} x100 \quad (2)$$

$$Total\ initial COD = COD_{organic\ acids} + COD_{residual\ sugars} + COD_{H_2} + COD_{biomass} + COD_{Not\ determined} \quad (3)$$

3. Results and discussion

3.1. Physicochemical characterization of the FVW

Physicochemical features of the feedstock such as the content of carbohydrates, nitrogen, lipids, etc., are among the most important factors governing the performance of the DF process. The FVW exhibited a low pH of 4.6 ± 0.1, which implies the need of using an alkaline additive such as sodium hydroxide to maintain the operational pH at suitable values (higher than 5.5 in most cases) for hydrogen production (Habashy et al., 2021). The FVW had a high organic matter concentration, with an associated total COD concentration of 111.5 ± 5.1 g/L, of which 75.7% was found to be in a solubilized form. This indicated that most organic matter in the FVW was readily available to microorganisms. Indeed, the VS/TS ratio accounted for 94% (95.2 ± 2.0 g TS/L and 89.5 ± 1.5 g VS/L), while the total carbohydrates content was 78.9 ± 2.9 g/L. Thus, hydrolysis was not likely the rate limiting step in the production of hydrogen during DF. As expected, the lipid content was low (1.2% *w/w* on a dry basis), discarding any need for a delipidation pretreatment or DF inhibition issues associated with the accumulation of lipids in the culture broth (Alibardi and Cossu, 2016; Tarazona et al., 2022). The FVW had a TKN concentration of 2.4 ± 0.1 g/L, corresponding to 15.5 ± 0.7% *w/w* protein. In this context, the C/N ratio accounted for 31.6 ± 0.5, which agrees with the literature reporting acceptable hydrogen productions at C/N ratios between 21 and 45 (Gomez-Romero et al., 2014; Rangel et al., 2021). The content of phosphorus was 3.7 g/L, while the ash content was on average closed to 6%, suggesting the presence of essential minerals in the simulated FVW used. Indeed, this study did not require to supplement the DF process with any external (micro)nutrient to achieve a high hydrogen production performance (as discussed in section 3.2). Finally, it should be noted that although FVW is heterogenous in nature, the physicochemical

characterization of the FVW herein used as feedstock grossly agrees with data reported in the literature: an acidic, carbohydrate-rich, readily fermentable substrate with a low lipids level, which represents an excellent feedstock for fermentation-based biorefinery processes (Gomez-Romero et al., 2014).

3.2. Effect of pH on the LD-DF of FVW

The operational pH exerted a markedly impact on both the extent and rate of hydrogen production (Fig. 1, Table 1). The final cumulative hydrogen production increased with increasing the operational pH from 1897.5 ± 370.4 NmL/L at pH 5.5 up to 3443.1 ± 46.4 NmL/L at pH 7 (Table 1). Such an improved hydrogen production response according to the increasing operational pH values agrees with previous studies reported in literature (Dareioti et al., 2014; García-Depraect et al., 2019a). As expected, no hydrogen production was observed under non-controlled pH conditions due to the low pH prevailing during the fermentation process, which dropped from 4.6 down to 3.4, highlighting the need for a pH control system. At pH of 5.5, the hydrogen yield averaged 41.5 ± 8.3 NmL/g VS added, and increased by 15.4, 43.6 and 76.4% when the pH was kept constant at 6, 6.5 and 7, respectively (Table 1). Higher pH values also resulted in a slightly higher hydrogen content in the acidogenic off-gas, rising from 41.4 ± 0.4 to 49.7 ± 1.2%. In contrast, the removal efficiencies for VS were not significantly affected by the operational pH, reaching values between 49.3 ± 0.5 and 54.6 ± 1.5%, except for fermentations carried out without pH control (16.2 ± 4.2%). The removal of carbohydrates ranged from 74.3 ± 1.3 to 82.4 ± 0.5% for all pH conditions tested. Additionally, no clear relationship between hydrogen production and the carbohydrates removal efficiency was observed, which implies that the hydrogen production efficiency cannot be explained merely by the consumption of carbohydrates.

Table 1 shows that the modified Gompertz model adequately represented the experimental data with correlations coefficients (R^2) higher than 0.99, although the diauxic hydrogen production observed required the use of the model with two consecutive steps. It was observed that the higher the pH, the lower the lag phase, which ranged between 6.3 and 8.0 h, pointing out the easily fermentable nature of FVW. A higher operational pH resulted in enhanced VHPRs, thus the highest VHPR of 535.7 ± 10.1 NmL H_2/L-h was obtained at pH 7. That operational condition exhibited a diauxic behavior in hydrogen production with a second VHPR of 262.4 ± 146.5 NmL H_2/L-h. Indeed, a diauxic hydrogen production was also observed for pH 5.5 and pH 6. The occurrence of different metabolic pathways for hydrogen production could explain the diauxic hydrogen production behavior herein recorded, as previously discussed by An et al. (2018).

The main organic acids identified in the culture broth were HLac, HFor, HAc, HBu, and HPr regardless of the pH condition (Fig. 2). No measurable amounts of ethanol were detected for all pH conditions tested, likely due to the operational conditions and biocatalyst used. Non-controlled pH conditions resulted in a marked accumulation of HLac of up to 18.3 ± 0.04 g/L followed by HAc (9.2 ± 0.2 g/L), which suggests that heterolactic fermentation became dominant (Dareioti et al., 2014). Extreme acidic conditions also enabled HPr-type fermentation with up to 4 g HPr/L. Contrarily, at a fixed pH of 5.5, the concentration of HLac began to increase during the first 10 h, peaking at 7.1 ± 0.3 g/L at 24 h of cultivation and thereafter it was totally consumed after 32 h of fermentation. Interestingly, relative low levels of HLac in the culture broth matched with the formation of HAc and HBu. A gradual accumulation of HLac during an early stage of fermentation and its further metabolization accompanied by the production of HAc and HBu was also observed at pH 6, 6.5 and 7 (Fig. 2). Notably, HFor was rapidly accumulated in the fermentation broth and re-consumed during the first 4–8 h of cultivation regardless of the pH conditions. Another observation is that HPr was accumulated, mainly during the stationary phase of hydrogen production, for pH 6, 6.5, and 7, but not for pH 5.5. In general, the more neutral operational pHs not only enabled a higher hydrogen production but also a higher recovery of carboxylic acids (see Fig. 2f), sustaining acidification degrees of 26.1 ± 1.5, 39.6 ± 2.8, 49.3 ± 0.2, and 72.9 ± 0.9 at pH 5.5, 6, 6.5, and 7, respectively. According to the global COD mass balance, 0.2–4.1% and 12.8–18.6% of the total COD of the substrate were diverted to hydrogen and residual sugars depending on the pH tested, considering that biomass growth accounted for 5% of the total influent COD. The recovery of COD accounted for 52.0–97.9% (see Supplementary material).

Culture pH is one of the most important operational parameters in the DF process due to its role in shaping microbial communities and their metabolic activities (Tian et al., 2019; Habashy et al., 2021). According to Eqns. (4) to (6), HLac can be metabolized into hydrogen and HAc or HBu depending on the prevailing pathway (García-Depraect et al.,

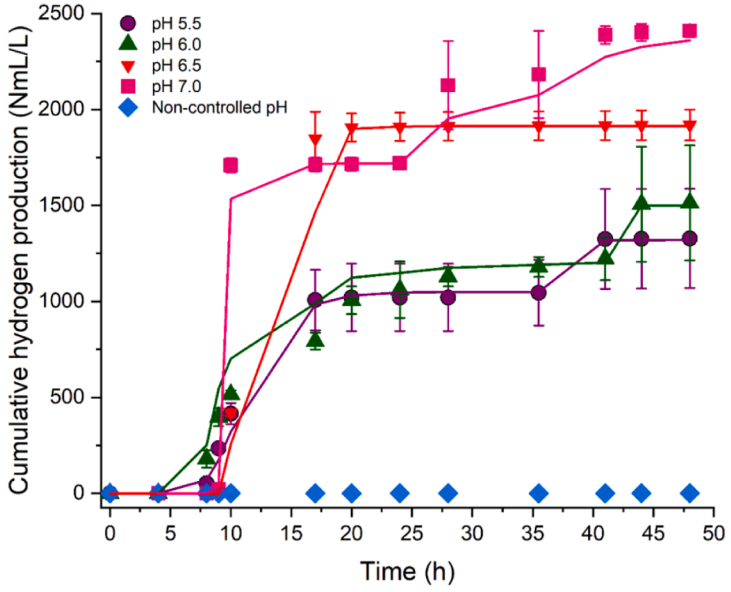

Fig. 1. Time course of cumulative hydrogen production at different operational pH values. Continuous lines represent the predicted data obtained from the modified Gompertz model.

Table 1
Summary of operational performance indicators at different operational pH, initial TS content and initial biomass concentration.

Parameter		Production NmL H_2/L	Yield NmL H_2/g VS fed	VHPR NmL H_2/L-h	[a] H_2 %	VS removal %	[b] CHO removal %	λ h	R_{max} NmL H_2/h	P NmL H_2	R^2
pH, 5% TS and 18 mg VSS/L initial biomass concentration	[c] NC	0	0	0	0	16.2 ± 4.2	77.7 ± 2.0	–	–	–	–
	5.5	1897.5 ± 370.4	41.5 ± 8.3	300.8 ± 47.5/ 197.4 ± 46.8	41.4 ± 0.4	51.2 ± 1.9	74.3 ± 1.3	8.0 ± 0.1,	210.5 ± 33.2/ 138.2 ± 32.8	1027.2 ± 172.6, 1326.7 ± 262.4	0.9997
	6.0	2162.0 ± 428.5	47.9 ± 10.6	322.7 ± 23.3/ 336.1 ± 42.3	43.2 ± 3.6	53.8 ± 3.5	78.1 ± 0.8	7.4 ± 0.4	225.9 ± 16.3/ 235.3 ± 29.6	1510.0 ± 299.9	0.9900
	6.5	2742.4 ± 114.7	59.6 ± 2.7	450.0 ± 40.4	45.3 ± 5.0	49.3 ± 0.5	82.4 ± 0.5	6.8 ± 0.2	315.0 ± 28.3	1914.0 ± 75.7	0.9919
	[d] 7.0	3443.1 ± 46.4	73.2 ± 1.0	535.7 ± 10.1/ 262.4 ± 146.5	49.7 ± 1.2	54.6 ± 1.5	77.7 ± 1.5	6.3 ± 0.0	375.0 ± 7.1/ 183.7 ± 102.5	2457.2 ± 118.7	0.9943
TS content (%), pH 7.0 and 18 mg VSS/L initial biomass concentration	7	4051.3 ± 3.0	61.9 ± 0.2	685.7 ± 161.6	67.7 ± 0.1	42.3 ± 2.5	77.3 ± 2.1	12.8 ± 0.1	480.0 ± 113.1	2846.7 ± 63.7	0.9903
	9	264.7 ± 64.0	3.3 ± 0.8	102.9 ± 36.4	52.5 ± 4.6	31.4 ± 0.7	72.7 ± 2.7	13.2 ± 0.4	72.0 ± 25.5	126.4 ± 37.4	0.9908
Initial cell biomass concentration (mg VSS/L), pH 7.0 and 5% TS	18	2726.2 ± 683.8	60.4 ± 15.8	717.8 ± 57.9	65.5 ± 10.7	46.4 ± 4.1	84.8 ± 0.5	9.5 ± 0.1	502.4 ± 40.5	1871.9 ± 402.0	0.9945
	180	1980.1 ± 251.7	41.2 ± 4.6	1035.7 ± 10.1	52.9 ± 0.3	48.7 ± 1.0	84.0 ± 0.8	8.5 ± 0.1	725.0 ± 7.1	1381.0 ± 169.9	0.9939
	1800	2329.7 ± 191.3	49.5 ± 4.7	976.4 ± 48.7	45.8 ± 0.4	51.1 ± 1.8	84.6 ± 0.2	6.8 ± 0.1	683.5 ± 34.1	1627.6 ± 131.0	0.9971

Note: [a] peak hydrogen concentration; [b] CHO: carbohydrates; [c] NC: non-controlled pH; [d] This condition also represented 5% TS.

2021). Thus, the fact that HLac always remained at relatively low levels, while hydrogen is produced as the concentration of HAc and HBu increased, strongly suggested the occurrence of HLac-utilizing, hydrogen-producing pathways (Noblecourt et al., 2018; García-Depraect et al., 2021; Villanueva-Galindo and Moreno-Andrade, 2021). The hydrogen production performance during LD-DF relies on the balance between LAB and LU-HPB, which is indeed influenced by the operational pH (García-Depraect et al., 2019a; Fuess et al., 2019; Detman et al., 2021). It has been argued that LAB and LU-HPB can coexist via lactate cross-feeding interactions (García-Depraect et al., 2021). In this context, it is also worth mentioning that all DF tests were carried out using a seed inoculum harboring LAB and LU-HPB, which jointly are metabolically able to sustain LD-DF (García-Depraect et al., 2022a). It was therefore suggested that pH-neutral conditions supported an improved balance and syntrophy between LAB and LU-HPB (Jung et al., 2021; Kim et al., 2022). However, although, as mentioned before, there was no clear trend in the efficiency of carbohydrates removal and the amount of hydrogen produced, some hydrogen might have been formed from carbohydrates. Furthermore, it must be stressed that heterolactic fermentation and/or homoacetogenic pathway might also have contributed to the high levels of HAc herein measured (Dareioti et al., 2014). Further molecular analyses can help to better understand the carbon flow.

$$CH_3CH(OH)COOH + 0.5CH_3COOH \rightarrow 0.75CH_3CH_2CH_2COOH + CO_2 + 0.5H_2 + 0.5H_2O \quad (4)$$

$$CH_3CH(OH)COOH + H_2O \rightarrow CH_3COOH + CO_2 + 2H_2 \quad (5)$$

$$2CH_3CH(OH)COOH \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2 \quad (6)$$

3.3. Effect of initial TS content on the LD-DF of FVW

The TS content of FVW was also found to impact both the extent and rate of hydrogen production (Table 1, Fig. 3). Particularly, the hydrogen production efficiency severely decreased with increasing the TS content from 3443.1 ± 46.4 NmL/L at 5% TS to 264.7 ± 64.0 NmL/L at 9% TS (4.1 to 0.2% of the initial COD), with associated hydrogen yields ranging from 3.3 ± 0.8 to 73.2 ± 1.0 NmL H_2/g VS fed (Table 1). The hydrogen content in the biogas peaked at 49.7 ± 4.1, 67.7 ± 0.1 and 52.5 ± 4.6% at a TS content of 5, 7, and 9%, respectively. The lowest VS removal efficiency of 31.4% was achieved at a TS concentration of 9%, which was 34.7 and 73.9% lower than those recorded at 7 and 5% TS, respectively. Finally, the removal efficiency of carbohydrates ranged from 72.7 ± 2.7 to 77.7 ± 1.0%, regardless of the TS content tested, highlighting the fact that hydrogen production via LD-DF and the consumption of carbohydrates were uncoupled. However, carbohydrates were still needed to provide HLac as further hydrogen precursor (García-Depraect et al., 2021). The enhancement in hydrogen production performance herein found when decreasing the TS content in the substrate was also endorsed kinetically. Thus, the lag phase ranged between 6.3 and 13.2 h, while the VHPR varied between 102.9 ± 36.4 and 685.7 ± 161.6 NmL H_2/L-h. A TS content of 5% sustained the shortest adaptation time, the highest hydrogen yield, and a VHPR similar to that computed at 7% TS (Table 1).

The major organic acids detected in the fermentation broth included HLac, HAc, HBu, HPr and HFor, and their accumulation dynamics depended on the TS content (Fig. 4). There was no evidence of ethanol-type fermentation regardless of the condition tested. A slightly accumulation of HPr (2.6–2.9 g/L) at the end of the fermentation was only observed at 5 and 7% TS contents, likely because those conditions enabled hydrogen production. The organic acids profile at 5% TS was discussed in section 3.2. An accumulation of HLac (up to 33.0 ± 4.5 g/L) and HAc (up to 7.9 ± 1.1 g/L), but not of HBu that remained at 0.8 ± 0.3 g/L at the end of the process, was observed at 9% TS (the condition

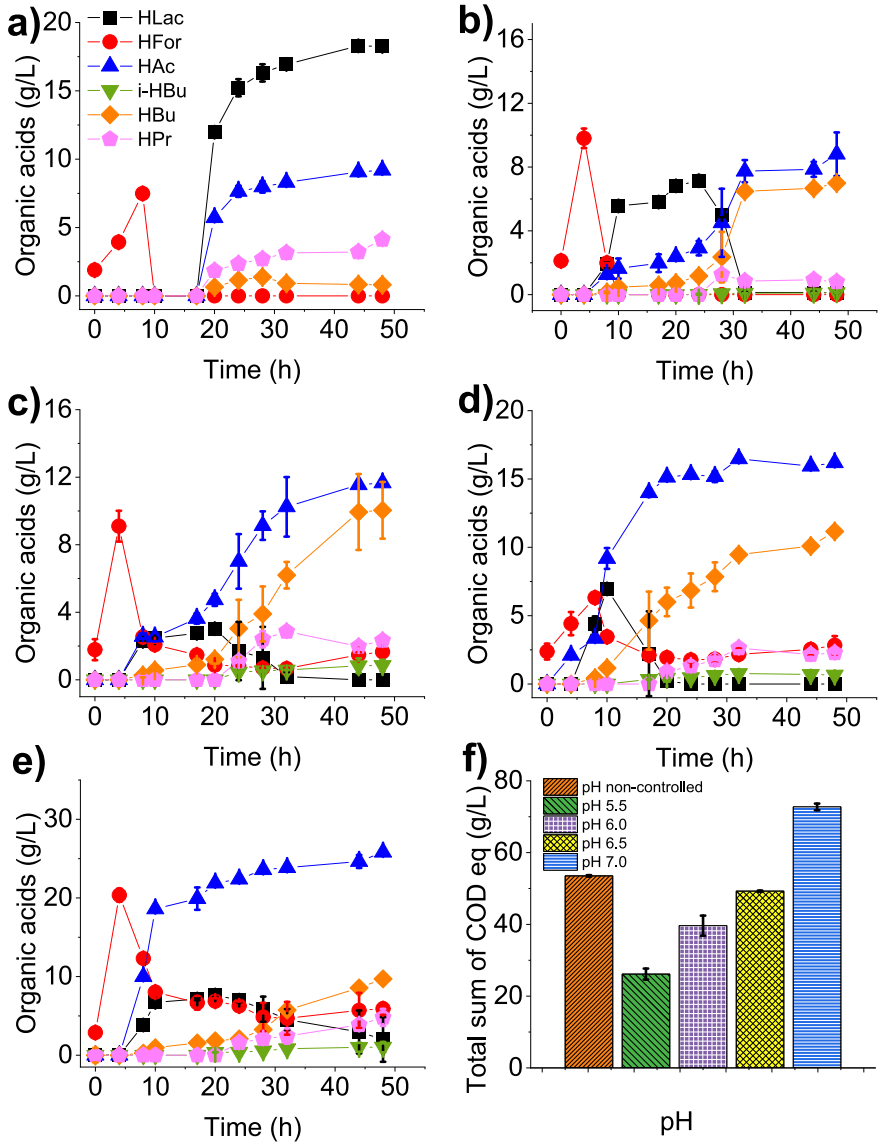

Fig. 2. Time course of organic acids at different operational pH values. a) non-controlled, b) pH 5.5, c) pH 6.0, d) pH 6.5, e) pH 7.0, and f) sum of COD equivalent for each organic acid measured at the end of the fermentation.

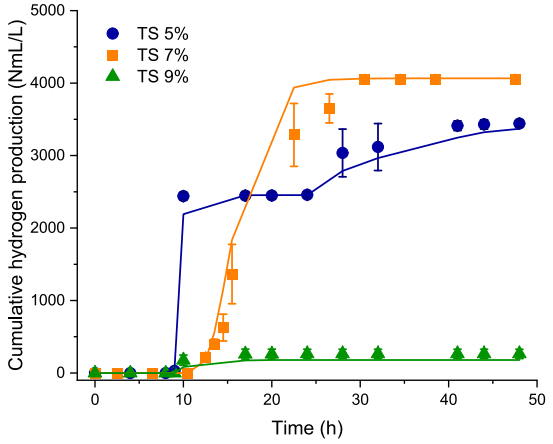

Fig. 3. Time course of cumulative hydrogen production at different TS concentrations. Continuous lines represent the predicted data obtained from the modified Gompertz model.

supporting the lowest hydrogen production). HLac and HAc accumulated up to 11.2 ± 1.0 g/L and 8.7 ± 0.02, respectively, at 7% TS, but HLac was gradually consumed to 9.0 ± 0.1 during the acceleration phase of hydrogen production. At first sight, this HLac accumulation along with the effective hydrogen production recorded at 7% TS seems to be contradictory to the LD-DF pathway. However, the fact that HLac can be produced but also consumed simultaneously may explain the behavior observed for HLac at 7% TS, which also tended to decrease at the end of fermentation and its maximum concentration in the culture broth was comparatively much lower than that measured at 9% TS. The acidification degree at 5% TS was the highest (72.9 ± 0.9%), while a TS content of 7% supported the lowest acidification degree (28.4 ± 0.9%), followed by 9% TS (42.4 ± 3.4%). This highlighted the relevance of the prevailing metabolic fluxes, especially the HLac flux, during LD-DF. In this context, the TS content of FVW may shape microbial populations and cause a shift in metabolic pathways (Chen et al., 2022). It is well recognized that the efficient bioconversion of complex particulate substrates to hydrogen needs a suitable balance between hydrolytic and fermentative bacteria. It has been previously hypothesized that the presence of particulate material may alter the balance between LAB and

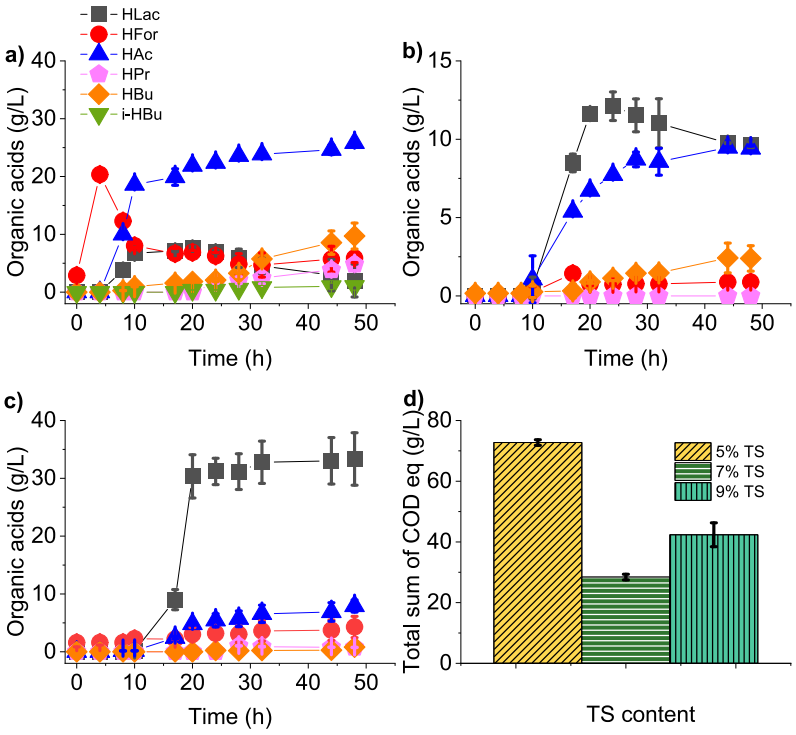

Fig. 4. Time course of organic acids at different TS contents. a) 5%, b) 7%, c) 9%, and d) sum of COD equivalent for each organic acid measured at the end of the fermentation.

LU-HPB during LD-DF, leading to higher LAB activities at higher TS contents, while lower TS contents may boost the activity of LU-HPB (García-Depraect et al., 2019c), thus explaining why low TS contents were ascertained in this study as more conducive to hydrogen production.

3.4. *Effect of initial biomass concentration on the LD-DF of FVW*

The role of initial cell biomass concentration on the performance of LD-DF of FVW was investigated in a third series of batch tests at different biomass concentrations (i.e., 18, 180, and 1800 mg VSS/L). Interestingly, the inoculum concentration did not exert a clear effect on the extent of hydrogen production but on its rate (Fig. 5, Table 1). The average cumulative hydrogen production varied between 1980.1 and

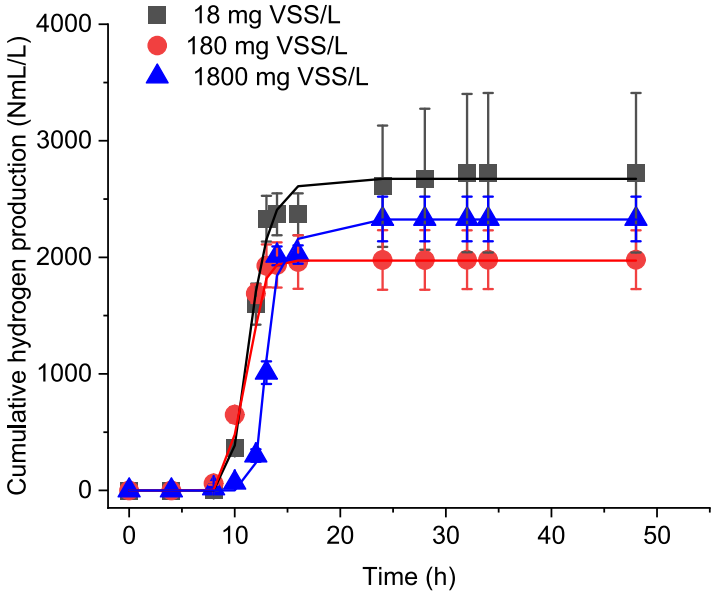

Fig. 5. Time course of cumulative hydrogen production at different inoculum concentrations. Continuous lines represent the predicted data obtained from the modified Gompertz model.

2726.2 NmL/L, with corresponding average hydrogen yields of 49.5–60.4 NmL/g VS fed. The highest hydrogen production was attained at 18 mg VSS/L (Table 1). The hydrogen content in the acidogenic off-gas ranged from 45.8 to 65.5%. It must be highlighted that a biomass concentration of 18 mg VSS/L resulted in a larger variation in the volume of hydrogen produced compared to that in the other tested conditions, likely due to the low initial microbial density in the fermenter. The VS and carbohydrates removal efficiencies were close to 50 and 84%, respectively, regardless of the initial microbial density (Table 1). Finally, the lag phase in hydrogen production decreased from 9.5 to 6.8 h as the biomass concentration increased, while VHPRs of 717.8 ± 57.9, 1035 ± 10.1 and 976.4 ± 48.7 NmL H_2/L-h were attained at 18, 180 and 1800 mg VSS/L, respectively.

Considering that 10- and 100-fold biomass increments showed very similar VHPRs, and the slightly lower hydrogen yield observed in the former assays (Table 1), it is suggested to maintain a high concentration of desirable bacteria in LD-DF systems to produce hydrogen more efficiently. However, more organic matter may be devoted to biomass production rather than hydrogen production at too high cell concentrations (Das et al., 2011; Wu et al., 2012). It should be also noted that the DF process is impacted not only by the initial biomass concentration but also by its physical state (lag, log, or stationary); where a rapidly dividing state may sustain superior performances (Das et al., 2011). The seed herein employed as the workhorse was obtained from a fermenter producing hydrogen continuously and stably from a lactose-based mineral medium. The performance of the seed reactor in terms of hydrogen productivity, biomass concentration and profile of organic acids is shown in the Supplementary material. It must be noted that the larger biomass concentration entailed a low F/M ratio (see Supplementary material), and therefore, the increase in VHPR at larger microbial densities could be explained by the high number of microorganisms that may make faster the bioconversion.

HAc, HLac, HFor, HPr and HBu were the main organic acids identified during the assessment of the influence of the biomass concentration (Fig. 6). HAc was the most dominant intermediate regardless of the biomass concentration, similarly to the previous fermentations displaying high hydrogen production performances (see sections 3.2 and 3.3). For all conditions tested, no ethanol was detected throughout the fermentation. HPr was accumulated, especially at the late stage of fermentation, at concentrations ~1.0 g/L regardless of the biomass concentration tested. Significant concentrations of HLac were also observed, mainly at early stages of fermentation, although this organic acid remained at very low levels during the late stages of fermentation independent of the condition tested. A concurrent HAc and HBu accumulation during HLac uptake was also recorded. Thus, the metabolic patterns observed reinforced the idea that, under the conditions studied, the main route for hydrogen production involved the consumption of HLac. However, it is difficult to determine the exact contribution of different HLac-utilizing, hydrogen-producing pathways since HLac could have been produced and consumed to different extents. Overall, the empirical findings observed in this assay provided a strong indication that FVW was an effective substrate for hydrogen production despite the prevalence of HLac in the fermentation broth, which strongly suggests the presence of HLac producers, showing the advantageous LD-DF process herein proposed.

3.5. *Significance of the study and perspectives*

One of the main bottlenecks of the DF process is the impairment in hydrogen production caused by the overgrowth of LAB. LAB are

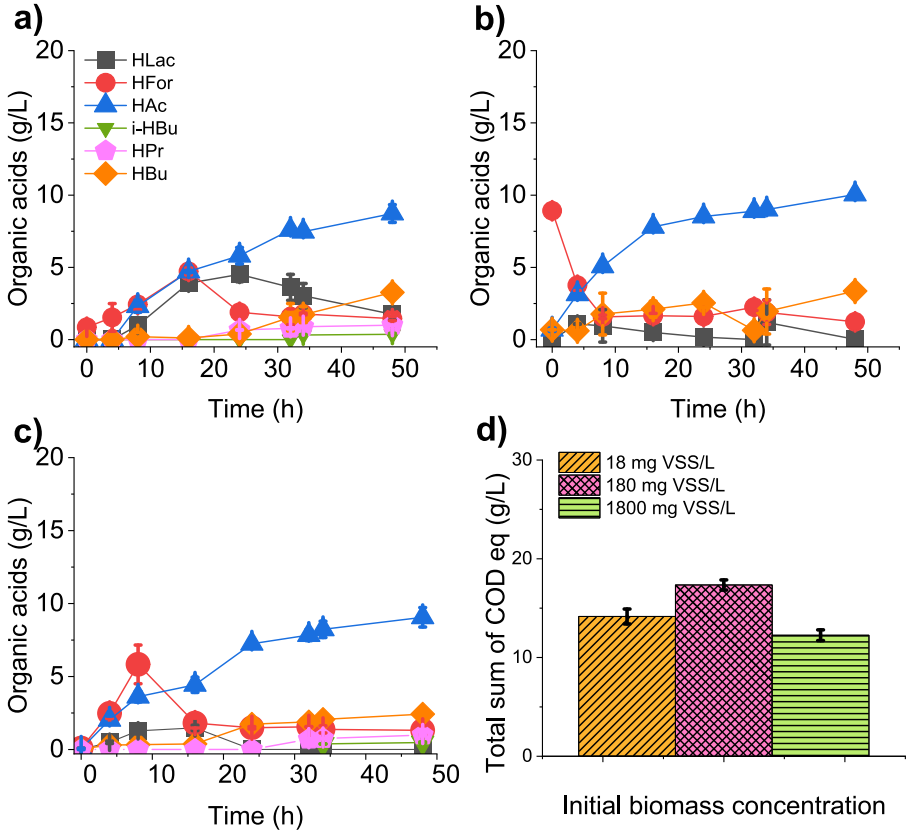

Fig. 6. Time course of organic acids at different inoculum concentrations. a)18, b) 180, c) 1800 mg VSS/L, and d) sum of COD equivalent for each organic acid measured at the end of the fermentation.

commonly seen as unfavourable bacteria to DF due to substrate competition, excretion of bacteriocins, and because HLac is not associated with hydrogen production. Recently, hydrogen production from HLac, herein referred to as LD-DF has been reported as an alternative pathway to cope with LAB associated issues (García-Depraect et al., 2021). Indeed, there is evidence of the occurrence of HLac-utilizing, hydrogen-producing pathways using food waste as substrate (Gomez-Romero et al., 2014; Noblecourt et al., 2018; Villanueva-Galindo and Moreno-Andrade, 2021). The novelty of the present study lies in conducting systematic research to evaluate the effect of pH, TS content, and initial biomass concentration on the performance of LD-DF of FVW. Those are key operational parameters extensively studied (especially pH and TS content) via conventional DF but not for LD-DF. Hydrogen is typically produced directly from carbohydrates via acetic- and butyric-type fermentation. In contrast, HLac (formed from carbohydrates) is hydrogen precursor during LD-DF. From an ecological perspective, LD-DF would benefit from LAB through a cooperative association with LU-HPB. Such a syntrophy is absent when the microbial structure governing the DF process does not have the potential to metabolize HLac into hydrogen, which often results in the accumulation of HLac in the fermentation broth and hydrogen inhibition. LAB commonly thrive and proliferate in DF reactors, on the one hand, because of their ubiquitous nature (for instance they are found in many cases as part of the indigenous microbiota of substrate) and, on the other hand, because they can grow well in the process and environmental conditions at which DF reactors are operated and also may outcompete HPB due to their more rapid substrate uptake and/or higher growth rate using complex substrates (García-Depraect et al., 2021). Substrates and inocula are commonly subjected to pretreatments (e.g., alkali, acid, temperature shock) as a measure to avoid the over proliferation of LAB (Capson-Tojo et al., 2016). From a practical process point of view, LD-DF enables to avoid any pretreatment, thus leading to a more cost-effective process.

The results herein presented indicated that LD-DF is a feasible fermentation route to produce hydrogen from FVW, and the operational parameters tested govern both hydrogen production and its associated metabolic pathways. Furthermore, the potential of the LD-DF process herein proposed could be measured by comparing the hydrogen production yields and rates recorded in this study with those already reported for FVW in literature. Studies on the valorization of FVW into hydrogen via DF are still scarce. Gomez-Romero et al. (2014) reported a VHPR of 234.1 NmL H_2/L-h with an associated hydrogen yield of 142 NmL H_2/g VS fed using FVW. Abubackara et al. (2019) reported a hydrogen yield of 27.2 and 20.8 NmL H_2/g VS fed from the thermophilic DF of autoclaved and non-autoclaved FVW, respectively. The present study achieved a hydrogen yield of ≈ 50 NmL H_2/g VS fed but an outstanding VHPR of 976.4 NmL/L-h. That unprecedent hydrogen production rate shows the potential advantage of the LD-DF to be further optimized. Future studies should investigate the continuous hydrogen production and related microbial communities, with a special emphasis on selecting suitable operational and environmental parameters to boost the HLac-to-hydrogen bioconversion.

4. Conclusions

This study revealed that the FVW-to-hydrogen bioconversion via LD-DF is strongly impacted by culture pH, TS content, and initial biomass concentration. Superior hydrogen productions were associated with HLac consumption along with HAc and HBu production rather than carbohydrates consumption. Findings indicated that a more neutral pH along with a higher initial microbial density and lower solid contents in the substrate mediated superior hydrogen production rates. The highest VHPR of 976 NmL/L-h was achieved at pH 7, 5% TS and 1800 mg VSS/L. Overall, the LD-DF process represents an effective route to produce hydrogen from FVW, while tackling LAB inhibition issues.

CRediT authorship contribution statement

Leonardo J. Martínez-Mendoza: Conceptualization, Methodology, Investigation, Writing – original draft. **Raquel Lebrero:** Supervision, Writing – review & editing. **Raúl Muñoz:** Conceptualization, Methodology, Writing – review & editing, Funding acquisition. **Octavio García-Depraect:** Conceptualization, Methodology, Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2022.128070.

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