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Unlocking the high-rate continuous performance of fermentative hydrogen bioproduction from fruit and vegetable residues by modulating hydraulic retention time

Leonardo J. Martínez-Mendoza, Octavio García-Depraect, Raúl Muñoz *

Institute of Sustainable Processes, University of Valladolid, Dr. Mergelina s/n., 47011 Valladolid, Spain

HIGHLIGHTS

- HRT determined H_2 productivity and yield and the profile of soluble end-products.
- H_2 productivity of 11.8 NL/L-d and H_2 yield of 95.6 NmL/g VS_{fed} achieved at 9 h HRT.
- The major organic acids were lactate (key to metabolism), acetate and butyrate.
- Max energy recovery from fruit-vegetable waste (FVW): 1.2 kJ/g VS_{fed} and 150 kJ/L-d.
- Source-separated FVW is a good feedstock to produce H_2 via dark fermentation.

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ABSTRACT

Harnessing fruit-vegetable waste (FVW) as a resource to produce hydrogen via dark fermentation (DF) embraces the circular economy concept. However, there is still a need to upgrade continuous FVW-DF bioprocessing to enhance hydrogen production rates (HPR). This study aims to investigate the influence of the hydraulic retention time (HRT) on the DF of FVW by mixed culture. A stirred tank reactor under continuous mesophilic conditions was operated for 47 days with HRT stepwise reductions from 24 to 6 h, leading to organic loading rates between 47 and 188 g volatile solids (VS)/L-d. The optimum HRT of 9 h resulted in an unprecedented HPR from FVW of 11.8 NL/L-d, with a hydrogen yield of 95.6 NmL/g VS fed. Based on an overarching inspection of hydrogen production in conjunction with organic acids and carbohydrates analyses, it was hypothesized that the high FVW-to-biohydrogen conversion rate achieved was powered by lactate metabolism.

1. Introduction

To date, hydrogen holds a tremendous potential to mitigate the negative impact resulting from the massive use of fossil-derived fuels not only owing to its elevated energy density (142 MJ/kg), which is roughly 3-fold higher than that of methane, natural gas or gasoline, but also because only water vapor is released during hydrogen combustion (Pathy et al., 2022). Unfortunately, a huge amount of the hydrogen produced nowadays (approximately 60 million tonnes per year) is derived directly or indirectly from fossil fuels (Muradov, 2017). A growing interest in the generation of biogenic hydrogen has arisen over the last years. Biohydrogen is defined as the hydrogen produced from biotechnologies such as bioelectrochemical systems, biophotolysis, dark fermentation (DF), and photofermentation (Kim and Kim, 2011). Amongst them, DF is nowadays regarded as one of the most promising biological routes for valorizing biomass into hydrogen, owing to its mild culture conditions, relatively low operation costs, easy control, and relatively high achievable hydrogen production rates (HPR) and yields (HY) (Luo et al., 2022; Tran and Nguyen, 2022). Indeed, DF is a bioconversion process able to transform multiple biodegradable wastes into hydrogen and organic acids, thus supporting the circular economy concepts of “waste-to-energy” and “waste-to-commodity chemicals” (Mohan et al., 2016; Boshagh, 2021).

Fruit & vegetable wastes (FVW) can be a sustainable feedstock for DF according to its physicochemical characteristics such as high moisture (80–90 %) and carbohydrates (70–90 %) contents, and the inherent

* Corresponding author.
E-mail address: mutora@iq.uva.es (R. Muñoz).

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presence of other macro/micronutrients needed for microbial growth (Kaur et al., 2019). The latest statistical report stated that around 1.3 billion tonnes of food are wasted per year, of which FVW represents approximately 42 % (Ganesh et al., 2022). It is estimated that, in the European Union, almost 22 kg of FVW are yearly generated per capita in households (De Laurentiis et al., 2018). Processing industries, marketplaces and households are the main identified sources of FVW. It should be highlighted that the management and disposal of food waste (FW; including FVW) is a severe problem worldwide. In this context, the DF of FVW is an ongoing endeavor to divert it from landfilling, open field dumping and incineration. These traditional end-of-life options for FW not only causes pernicious greenhouse gas emissions, unpleasant odors or leachates, but also promote a tremendous loss in circularity and economic value (Basak et al., 2018; Magama et al., 2022).

Recent works have been focused on FVW valorization through hydrogen production by DF (Gomez-Romero et al., 2014; Keskin et al., 2018; Gómez Camacho et al., 2019; Soltan et al., 2019; Cieciura-Włoch et al., 2020; Dwivedi et al., 2020; Martínez-Mendoza et al., 2022). For instance, Martínez-Mendoza et al. (2022) studied the influence of key operational parameters, i.e., initial biomass concentration, pH, and initial total solids (TS) content, on the batch hydrogen production of FVW via lactate-driven DF. That study achieved an unprecedented maximum volumetric HPR of 23.4 NL/L-d at pH 7.0, 5 % TS and 1.8 g volatile suspended solids (VSS) biomass concentration. However, the number of studies on the continuous hydrogen production from FVW is very limited. The highest HPR so far reported from FVW is only 1.4 ± 0.4 NL H_2/L-d (Gómez Camacho et al., 2019). Thus, engineering an efficient hydrogenogenic fermenter under continuous mode is imperative for the scale-up and widespread implementation of DF (Park et al., 2015). Some operational strategies used to improve the hydrogen production efficiency include reactor design, bioaugmentation, additives supply, biomass and inoculum pre-treatment, and control of environmental and operational process parameters such as hydraulic retention time (HRT), organic loading rate (OLR), pH, and temperature (Sivagurunathan et al., 2016; García-Depraect et al., 2023). Among them, the HRT, which represents the average time that substrate remains in the reactor, is central for maximizing the HPR (Park et al., 2015; Magama et al., 2022). It is accepted that higher waste-to-hydrogen bioconversion rates can be achieved at low HRTs, albeit a too low HRT can lead to biomass washout and further process collapse (Magama et al., 2022). Despite the relevance of the HRT on hydrogen production, there is a lack of studies that modulate it to maximize the rate of FVW-to-hydrogen bioconversion. Hence, this work aimed at evaluating the impact of HRT on the fermentative hydrogen production from FVW. Soluble and non-soluble (by)products were considered in the assessment of the process performance. A comprehensive energy and mass balance analysis of the process is also presented with the purpose of assessing the baseline for a future design, analysis and optimization of the DF of FVW in the next generation of biorefineries.

2. Material and methods

2.1. Inoculum

A hydrogenogenic mixed culture previously used in mesophilic batch fermentations producing hydrogen from FVW via lactate-driven DF was employed as inoculum (Martínez-Mendoza et al., 2022). Inoculum preparation involved the cultivation of an inoculum aliquot of 0.1 L (preserved at 4 °C) for 19 h at 37 ± 1 °C and ~150 rpm, without pH control, in a 2.1-L gas-tight fermenter with 0.9 L of mineral growth medium composed of (g/L): $CaCl_2 \cdot 2H_2O$ 0.15, $FeCl_2 \cdot 4H_2O$ 0.035, KH_2PO_4 0.6, K_2HPO_4 2.4, lactose 10.0, $MgCl_2 \cdot 6H_2O$ 2.5, and NH_4Cl 2.4. The resulting fresh and active hydrogenogenic culture with a concentration of VSS of 180 mg/L was employed as inoculum.

2.2. Substrate

Simulated FVW mimicking fruits and vegetables derived from a local marketplace was used as the substrate. The substrate composition was prepared as reported in our previous study (Martínez-Mendoza et al., 2022). The FVW was blended without tap water addition using a semi-industrial blender (Sammic, XM-32, Spain) and frozen at −20 °C for storage. The physicochemical characteristics of the blended FVW were as follows: pH 4.5 ± 0.1, 111.3 ± 2.9 g total chemical oxygen demand (COD)/L, 96.7 ± 1.0 g soluble COD/L, 3.1 ± 0.1 g total Kjeldahl nitrogen/L, 3.7 g phosphorus/L, 106.2 ± 0.5 g TS/L, 99.9 ± 0.5 g VS/L; total carbohydrates 82.8 ± 3.0 % *w/w*, protein 19.1 ± 0.6 % *w/w*, lipids 1.2 % *w/w*, and ash 5.9 ± 0.6 % *w/w* (on a dry weight basis). The elemental composition was as follows: carbon 44.2 ± 0.4 %, hydrogen 5.7 ± 0.2 %, oxygen 41.7 ± 0.3 %, and nitrogen 1.4 ± 0.0 %. Prior to feeding, FVW was diluted using tap water to a final TS content of 5 % (Martínez-Mendoza et al., 2022).

2.3. Experimental set-up and process operation

The continuous DF of FVW was evaluated using a polyvinyl chloride fermenter with a total volume of 1.25 L (0.55 L head space) and equipped with a pH control device (BSV, Spain), a pH probe (HO35-BSV01, Spain), gas & liquid sampling ports, and a continuous gas-flow meter (Fig. 1). The fermenter was continuously operated for 47 days, divided in five operational stages (I–V), at decreasing HRT values. The HRT was shortened from 24 to 16, 12, 9 and 6 h by rising the feed flow rate at a constant concentration of 47.0 g VS/L (5 % TS). Hence, the OLR progressively increased from 47.0 to 70.6, 94.1, 125.4, and 188.1 g VS/L-d. The operational parameters of the hydrogenogenic fermenter are summarized in Table 1. The hydrogenogenic fermenter was placed at 37 ± 1 °C in a controlled-temperature room, magnetically stirred at ~ 300 rpm, and maintained at pH 7.0 ± 0.1 (using NaOH 6 M). The hydrogenogenic fermenter was seeded with 10 % *v/v* of a recently collected hydrogenogenic microbial seed and operated in batch mode for 8.5 h prior to continuous operation. Samples of 50 mL were periodically taken from the influent and effluent in order to monitor the acidification degree, the organic acids profile, and the removal efficiency of total carbohydrates. In addition, the flow rate and composition of the acidogenic off-gas generated during the DF process was daily measured and reported as normal volumes (NmL or NL) at standard pressure and temperature conditions (0 °C and 1 atm). The main performance indicators of the procedure included the HPR and HY, the concentration of hydrogen in the acidogenic off-gas, the hydrogen production stability index (HPSI), and the energy production rate and yield (EPR and EY, respectively).

2.4. Analytical procedures and data analysis

Total carbohydrates, protein, pH, biogas composition, organic acids, COD, and solids were determined as reported by Martínez-Mendoza et al. (2022). The HPSI was calculated as reported by García-Depraect et al. (2020) using Eq. (1). The HPSI calculation considers variations in HPR during each operational stage (not including HPR values from the first 3 HRTs in each operational stage). A stability index equals to 1 means a constant HPR, while a deviation value in HPR as large as the average HPR represents a stability index equals to 0. Thus, the higher the HPSI index, the lower the dispersion of hydrogen production.

$$HPSI = 1 - \frac{Standard\ deviation\ HPR}{Average\ HPR} \tag{1}$$

The degree of acidification was calculated as the ratio (in percentage) of the sum concentration of organic acids (in COD equivalent) and the total COD of the influent (Martínez-Mendoza et al., 2022). Whereas the mass balance analysis of COD was presented considering as input the total COD of the influent, and for the output the COD equivalent of

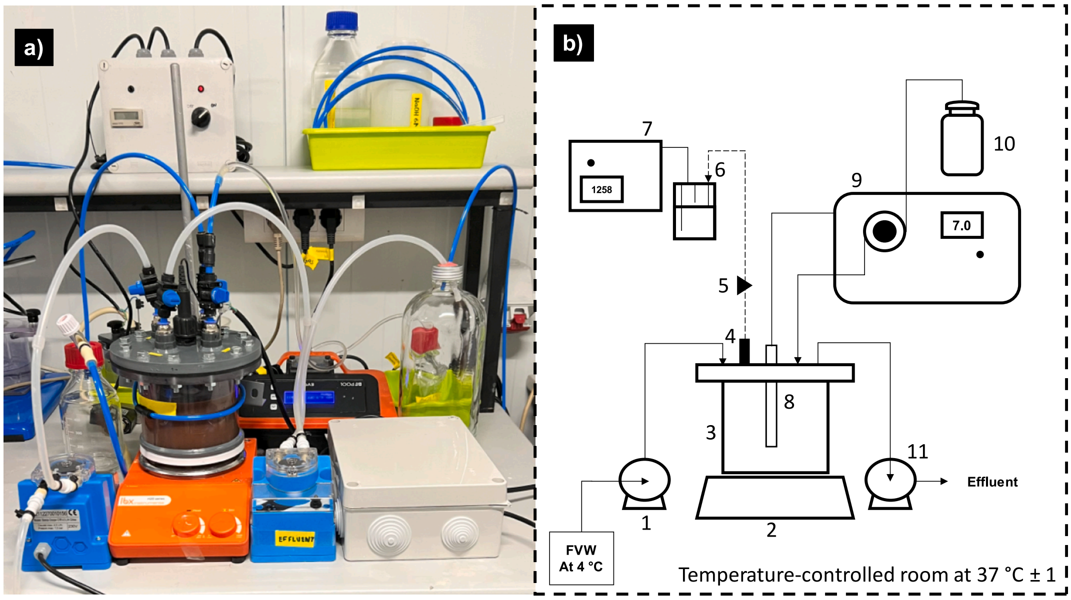

Fig. 1. Photograph (a) and scheme (b) of the continuous dark fermentation set-up used to investigate the effect of hydraulic retention time on the FVW-to-hydrogen biotransformation. Peristaltic pump (1 and 11), magnetic stirrer (2), dark fermenter (3), gas outlet (4), gas sampling port (5), water column (6), gas counter (7), pH probe (8), pH controller (9), 6 N NaOH solution (10).

Table 1
Operational parameters of the continuous hydrogenogenic fermenter.

Parameter	Operational stage				
	I	II	III	IV	V
Time (days)	0–6.0	6.0–18.5	18.5–26.5	26.5–41.5	41.5–46.5
HRT (h)	24	16	12	9	6
OLR (g VS/L-d)	47.0	70.6	94.1	125.4	188.1
HRT cycles	5.9	18.0	16.0	39.7	22.4

Note: OLR: organic loading rate; HRT: hydraulic retention time; VS: volatile solids.

hydrogen (8 g COD/g H_2) and organic acids produced, biomass growth, and residual carbohydrates (assuming glucose as the sugar). The biomass growth COD equivalent was estimated as 10 % of the influent total COD (Chernicharo, 2007). The acetic acid that may be produced from homoacetogenesis ($HAc_{Homoacetogenesis}$) was determined using Eq. (2), as previously described by Montiel Corona and Razo-Flores (2018), where the concentration of organic acids and hydrogen gas are expressed in mmol.

$$HAc_{Homoacetogenesis} = (2 \times [Butyric\ acid] + 2 \times [Acetic\ acid] - [Propionic\ acid] - [H_2])/6 \quad (2)$$

Finally, the energy analysis was estimated in terms of EPR (kJ/L-d) and EY (kJ/g VS), calculated using Eq. (3), (4), respectively, where HPR is expressed in NL H_2/L-d, HV_{H_2} is the hydrogen heating value (286 kJ/mol), and HY stands for the hydrogen yield (NL H_2/g VS fed) (Kumar et al., 2016).

$$EPR = \frac{Average\ HPR}{22.4} \times HV_{H_2} \quad (3)$$

$$EY = \frac{Average\ HY}{22.4} \times HV_{H_2} \quad (4)$$

2.5. Statistical analysis

The experimental data analyzed were obtained under pseudo-steady state in each operational stage, except in stage V where no pseudo-steady state was achieved. When hydrogen productivities remained a minimum of 3 consecutive HRTs within ± 10 % variance, a pseudo-steady state was obtained. An analysis of variance (ANOVA) was performed using a Tukey test with a p-value < 0.05 to evaluate the impact of HRT on the process performance indicators. According to the Shapiro-Wilk test, data showed a normal distribution ($p \leq 0.05$). The software Statgraphics Centurion version 19.2.01 was used to perform the statistical analyses.

3. Results and discussion

3.1. Influence of hydraulic retention time on hydrogen production performance

The hydrogen production performance of a dark fermenter continuously fed with FVW at five different HRTs (i.e., 24, 16, 12, 9, and 6 h) was evaluated for 47 days. Substrate concentration was maintained at 47.0 g VS/L throughout the experiment, and therefore, the stepwise reduction in HRT entailed a stepwise increase in the OLR (from 47.0 to 188.1 g VS/L-d; Table 1). The OLR is the amount of substrate fed per unit volume of reactor per day, thus determining the availability of subtrate for hydrogen production. In the present study, the performance of FVW-DF was assessed in regard to HRT for simplification purposes, but it is important to keep in mind that the combined effect of HRT and OLR may impact the process perfomance of DF (Sivagurunathan et al., 2016). The biogas production rate ranged from 6.0 to 19.7 NL/L-d depending on the HRT assessed, while the hydrogen content in the gaseous phase remained between 52.6 and 65.1 % v/v. The HRT therefore exerted a severe impact on hydrogen production efficiency (Fig. 2, Table 2). Particularly, process operation at an HRT of 24 and 16 h supported similar HPR, HY, and hydrogen content values in the gas phase under pseudo-steady state (p-value < 0.05), which accounted for 3.1 ± 0.2 NL H_2/L-d, 66.6 ± 4.0 NmL H_2/g VS fed and 52.6 ± 2.8 % H_2 v/v, respectively. HPSI indices of 0.94 and 0.86 were obtained in stages I and II, respectively, indicating a high hydrogen production stability. An HRT of 12 h in stage III sustained an increase of 92 and 46 % in HPR and HY, respectively, compared to process operation at 16 and 24 h HRT. The HPSI recorded at 12 h HRT was 0.87. Likewise, the HPR and HY ramped up to 11.8 ± 0.9 NL H_2/L-d and 95.6 ± 5.1 NmL H_2/g VS fed, respectively, during stage IV (9 h HRT), which were 57 and 20 % higher than

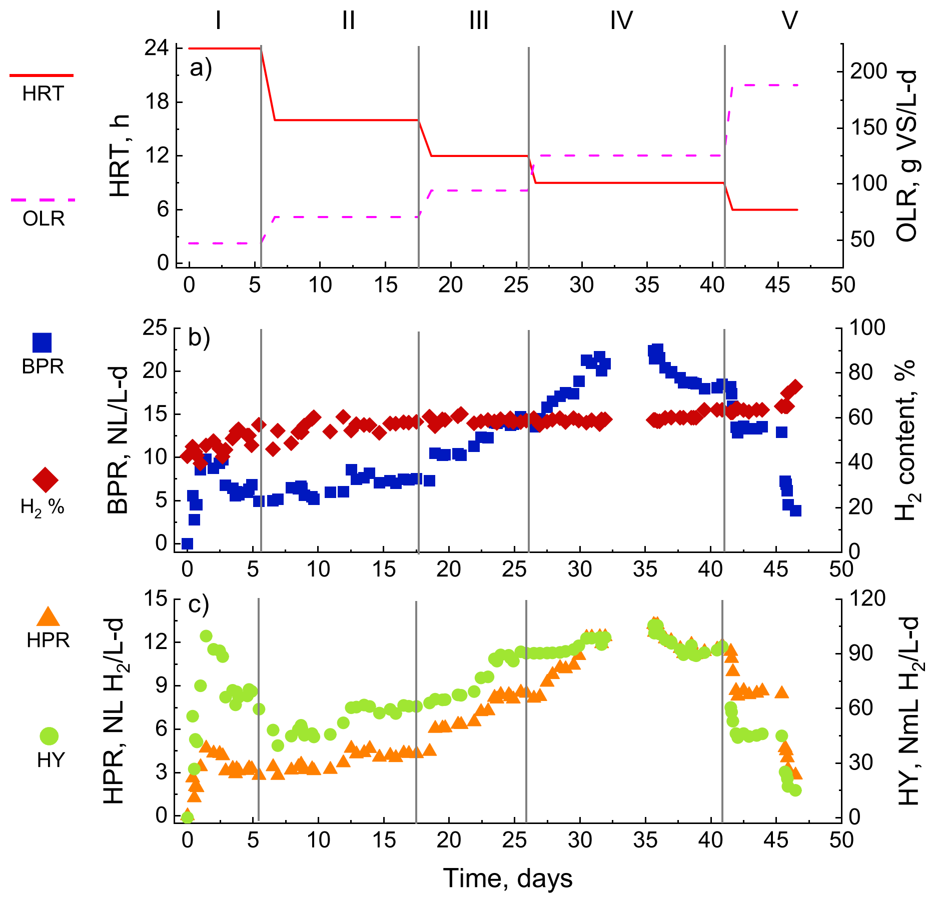

Fig. 2. Time course of a) hydraulic retention time (HRT) and organic loading rate (OLR); b) biogas production rate (BPR) and hydrogen content in the acidogenic off-gas, and c) hydrogen production rate (HPR) and yield (HY), recorded during the continuous dark fermentation of fruit-vegetable waste.

Table 2
Pseudo-steady state operational performance indicators for hydrogen production under different operational conditions using fruit-vegetable waste as substrate.

Parameter	Operational stage				
	I	II	III	IV	V
BPR (NL/L-d)	6.0 ± 0.6	6.9 ± 1.0	12.7 ± 1.7	19.7 ± 1.8	11.1 ± 4.4
HPR (NL H_2/L-d)	3.1 ± 0.2	3.9 ± 0.5	7.5 ± 1.0	11.8 ± 0.9	7.1 ± 2.7
HY (NmL H_2/g VS fed)	66.6 ± 4.0	54.6 ± 6.7	79.5 ± 10.3	95.6 ± 5.1	37.9 ± 14.1
Hydrogen content (% *v*/*v*)	52.6 ± 2.8	56.6 ± 2.4	59.0 ± 1.1	59.8 ± 1.6	65.1 ± 3.3
HPSI	0.94	0.86	0.87	0.91	0.63
Total carbohydrates removal (%)	79.2 ± 0.8	75.1 ± 0.7	74.1 ± 0.3	73.5 ± 0.3	65.6 ± 0.9

Notes: Mean value ± standard deviation. **BPR:** biogas production rate; **HPR:** hydrogen production rate; **HY:** hydrogen yield; **HPSI:** hydrogen production stability index. No pseudo-steady state was achieved in operational stage V. The number of samples analyzed was 8 (days 3.5–5.5), 17 (days 11.9–18.0), 14 (days 18.5–25.9), 25 (days 30.9–40.9) and 13 (days 42.5–46.5) for the operational stage I, II, II, IV, and V, respectively.

those achieved in the previous operational stage. At 9 h HRT and 125.4 g VS/L-d of OLR, the hydrogen content was 59.8 % and the HPSI reached a value of 0.91, which were statistically higher than those achieved in all the previous stages. However, a further decrease in HRT from 9 to 6 h mediated a decrease of the HPR by 40 % and of HY by 60 %, compared to those attained at an HRT of 9 h. This sharp decrease in hydrogen production caused the collapse of the process and might be attributed to organic overloading with an OLR of 188.1 g VS/L-d or biomass washout, as previously observed elsewhere (Chen et al., 2012; Goud et al., 2014; Jung et al., 2022). Biomass washout has been successfully prevented by the use of immobilized systems like the dynamic membrane reactor, in which a self-forming biofilm layer is formed, during the filtration of the fermentation broth, on a low-cost porous support such a stainless screen mesh via the deposition of bacteria and their metabolites (Jung et al., 2022). Note that the hydrogen content in this last stage V was slightly higher than those observed in the previous stages (on average 65.1 %; Fig. 2 and Table 2). It has been indeed reported that the content of hydrogen in the gas produced is not a reliable indicator of the process performance (Martínez-Mendoza et al., 2022; García-Depraect et al., 2023). The temporary high hydrogen content observed before process collapse might be attributed to the active addition of NaOH to control the pH when increasing the OLR, which can modify the equilibrium of the $CO_2/HCO_3^-/CO_3^{2-}$ buffer system and impact on the composition of the acidogenic off-gas.

Although it is difficult to make a fair comparison of data reported on the performance of DF systems as it depends on the particular conditions of a given process, it is necessary to benchmark the fermentative hydrogen production from complex feedstocks, FVW in this case. The baseline for benchmarking herein used were the HY and HPR already reported in the literature for similar FVW feedstocks. In batch experiments, the maximum HPR is estimated as the highest hydrogen production rate (usually acquired from the modified Gompertz model) divided by the working volume. Gomez-Romero et al. (2014) reported a maximum HPR of 5.6 NL H_2/L-d in the batch DF of FVW at pH of 5.5 and 37 °C. Later, Keskin et al. (2018) conducted batch DF tests at 55 °C, initial pH of 7.0 and using a mix of FVW as substrate. The authors reported maximum HPR and HY values ranging from 0.05 to 0.6 NL H_2/L-d and from 31 to 76 NmL H_2/g VS fed, respectively. Other study performing the batch DF (35 °C and pH 6.0) of a mixture composed of 25 % pea, 25 % tomato, 25 % banana and 25 % orange reported a HY of 2.55 ± 0.07 NL H_2/L and a maximum HPR of 5.1 NL H_2/L-d (Soltan et al., 2019). Dwivedi et al. (2020) investigated the effect of pH (5.5–8) and food-to-microorganism (F/M) ratio (0.5–2) on the mesophilic (35–40 °C) hydrogen production from FVW. The authors found pH 7.5 and F/M 1:1 the best condition leading to the highest maximum volumetric HPR of 0.03 NL H_2/L-d. In another study, Cieciura-Włoch et al. (2020) investigated the semi-continuous, mesophilic DF of FVW and reported a HY of 52.1 NmL H_2/g VS at pH 5.5, an OLR of ≈ 15 g VS/L-d, and a solid retention time of 72 h. Gómez Camacho et al. (2019) assessed the continuous DF of a mixture of FVW under mesophilic conditions (35 °C) and reported the highest HPR and HY to be 1.4 ± 0.4 NL H_2/L-d and 74 ± 29 NmL H_2/g VS respectively, at an HRT of 36 h and an OLR of 19.4 ± 5.6 g VS/L-d. More recently, Martínez-Mendoza et al. (2022) achieved a HY of 50 NmL H_2/g VS fed with an outstanding maximum volumetric HPR of 23.4 NL H_2/L-d from the lactate-driven DF of FVW conducted under batch mode at constant pH (7.0) and temperature (35 °C). Here it is worth highlighting that there is a lack of studies evaluating the continuous DF of FVW. In this regard, the HPR achieved in the present study is the highest so far reported, even using FVW without nutrient supplementation and without applying pretreatment or hydrolysis procedures. In comparison to other complex organic waste like FW, the HPR achieved herein with FVW is comparatively higher than the highest hydrogen productivity (7.1 ± 0.4 NL H_2/L-d) so far reported for the continuos DF of FW (Jung et al., 2022), likely due to the existing differences in the physicochemical composition between substrates.

Carbohydrate removal effciency ranged from 65.6 ± 0.9 % to 79.2 ± 0.8 % along the experiment (Table 2). Interesingly, the efficiency of carbohydrate consumption was not correlated either positively or negatively with that of hydrogen production. One possible explanation for that is associated with substrate competition issues between non-hydrogen producing bacteria and hydrogen producers. In this context, it is well known that HRT/OLR can shape the microbial communities involved in the DF process. In general, it has been reported that high HRT (low OLR) values may trigger the dominance of lactic acid bacteria (LAB) over hydrogen-producing bacteria (HPB), thereby leading to poor hydrogen production efficiencies (García-Depraect et al., 2021b; Palomo-Briones et al., 2021). In contrast, low HRTs (high OLR) are often more conducive for the growth of HPB, thus maximizing hydrogen production (García-Depraect et al., 2021a). Here the continuous hydrogen production from FVW was biocatalysed by a mixed bacterial culture, which has the metabolic capacity of transforming carbohydrates into lactate and of producing hydrogen from lactate (García-Depraect et al., 2022; Martínez-Mendoza et al., 2022; Regueira-Marcos et al., 2023). Effective lactate cross-feeding between LAB and some HPB (e.g., *Clostridium butyricum*) is keystone in metabolizing lactate as hydrogen precursor (García-Depraect et al., 2021b). Indeed, lactate was one of the major metabolites detected in the fermentation broth (effluent) throughout the operation (as will be discussed in section 3.2). Thus, it is highly probable that the high FVW-to-hydrogen bioconversion rates herein achieved were powered by beneficial associations between LAB and HPB, which were apparently boosted at 9 h HRT and impaired at either higher or lower HRTs. Further microbiology approaches can shed light on this inference (Kumar et al., 2018).

3.2. Effect of hydraulic retention time on the profile of organic acids

The main soluble metabolites identified for all the operational stages in the effluent were lactate, formate, acetate, propionate, and isovalerate, whereas ethanol was not detected (Fig. 3). For 24 h of HRT, an accumulation of lactate of 10.5 ± 1.2 g/L was obtained as the main metabolite in this operational stage, followed by acetate, butyrate, isovalerate, formate and propionate (with concentrations of 6.7 ± 2.3, 4.7 ± 2.1, 4.6 ± 0.3, 1.3 ± 0.4, and 0.4 ± 0.3 g/L, respectively). The highest and lowest lactate concentration in the effluent was 18.4 ± 1.2 g/L and 13.3 ± 0.9 g/L, observed at 16 and 6 h of HRT, respectively. The higher OLR at the shortest HRT of 6 h did not entail neither an enhanced lactate accumulation, nor higher HY and HPR. A marked lactate accumulation in the fermentation broth has been typically associated with hydrogen inhibition (García-Depraect et al., 2021b; Palomo-Briones et al., 2021). However, in this study, efficient hydrogen production was achieved despite lactate accumulation (stage V). As mentioned before, one explanation for that is that the inoculum used, which has been previously proven to support the lactacte-driven DF of FVW, was able to metabolize lactate into hydrogen. Indeed, this is the first study that use a hydrogenogenic inoculum able to perform lactate-driven DF to produce continuously hydrogen from FVW. Also, during all the operational stages, the concentration of acetate, isovalerate, and propionate decreased with lower HRT by 58, 85, and 50 %, respectively. Formate concentration increased simultaneously from 1.3 ± 0.4 g/L until 3.3 ± 0.9 g/L when hydrogen production sharply dropped at 6 h of HRT. Interestingly, butyrate concentration was virtually constant during the operational stages involving hydrogen production (I–IV), showing a decrease of 38 % during the last operational stage when organic overload or biomass washout could have strongly impaired the hydrogen production. In general, the decrease in HRT led to a marked reduction in the recovery of carboxylic acids, resulting in acidification degrees of 62.4 ± 1.0 %, 56.4 ± 3.6 %, 53.2 ± 1.5 %, 46.9 ± 3.7 %, and 42.9 ± 3.0 % at 24, 16, 12, 9 and 6 h of HRT, respectively (Table 3).

Organic acids, hydrogen gas, residual sugars, and biomass were included in the COD mass balance with a recovery range of 94.2–83.7 % (Table 3), which means that most of metabolites produced during the fermentation were quantified. The small loss of COD can be explained by the presence of other compounds in the FVW used that were not measured or remained unoxidized. According to the COD balance, hydrogen recovery accounted for 3.9–6.1 % of the total COD present in the substrate. The highest hydrogen recovery obtained at high OLR of 125.4 g VS/L-d could be explained by the fact that high substrate availability can boost electron flux to divert to hydrogen production (Wu et al., 2012). High substrate availability can also favor the growth of HPB over homoacetogens such as *Acetobacterium woodii* and *Blautia coccoides*. In this study, the share of acetate that may be produced via homoacetogenesis decreased (from 31.1 ± 3.0 to 4.8 ± 5.0 %) with HRT

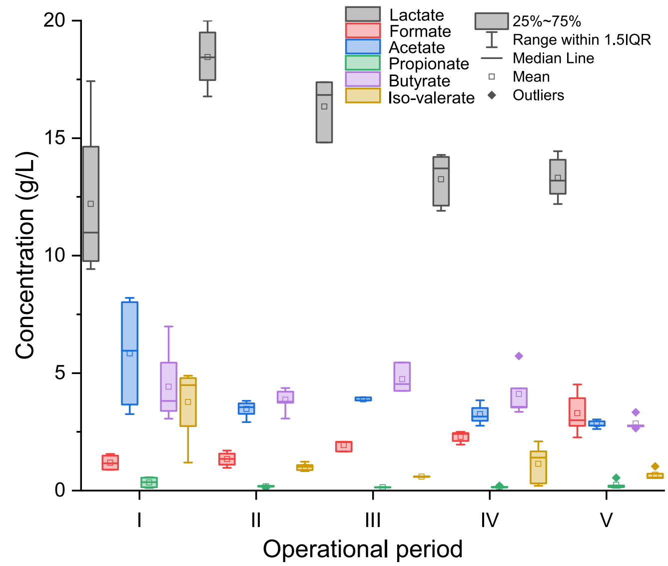

Fig. 3. Box and whisker plot of the concentration of organic acids recorded at pseudo-steady state at different operational stages. IQR: Interquartile range. No pseudo-steady state was achieved in operational stage V.

Table 3
Acidification degree, homoacetogenesis degree, and COD mass balance analysis for the different conditions tested.

Parameter	Operational stage				
	I	II	III	IV	V
Acidification degree (%)	60.3 ± 4.3	56.4 ± 3.6	53.2 ± 1.5	46.9 ± 3.7	42.9 ± 3.0
[a] $HAc_{Homoacetogenesis}$ (%)	31.1 ± 3.0	13.5 ± 1.4	8.2 ± 2.5	4.8 ± 5.0	14.1 ± 5.8
COD equiv. organic acids (g/L)	31.6 ± 2.2	29.5 ± 1.9	27.9 ± 0.8	24.6 ± 2.0	22.1 ± 1.4
COD equiv. hydrogen (g/L)	2.4 ± 0.5	2.0 ± 0.1	3.0 ± 0.0	3.2 ± 0.1	1.1 ± 0.5
COD equiv. residual sugars (g/L)	9.3 ± 0.3	11.2 ± 0.3	11.7 ± 0.1	11.9 ± 0.1	15.5 ± 0.4
[b] COD Recovery (%)	92.8 ± 2.9	91.6 ± 1.9	91.1 ± 1.5	85.7 ± 3.7	83.7 ± 1.9

***Notes*:** Mean value ± standard deviation. [a] $HAc_{Homoacetogenesis}$: putative acetic acid derived from homoacetogenesis; [b] It was assumed that 10 % of the total COD concentration of substrate was diverted to biomass growth (Chernicharo, 2007). COD: chemical oxygen demand. No pseudo-steady state was achieved in operational stage V. The number of samples analyzed was 4, 7, 3, 5 and 4 for the operational stage I, II, II, IV, and V, respectively.

shortening (high OLR), which agrees well with previous observations (Fuentes et al., 2021). However, it went up to 14.1 ± 5.8 % when hydrogen production was imparied at 6 h HRT (Table 3). It should be noted that this way of estimating homoacetogenesis assumes the production of hydrogen via butyric- and acetic-pathways, as well as propioneogenesis and homoacetogenesis as hydrogen sink pathways. Thus, hydrogen production from lactate, which can use acetate as electron acceptor (García-Depraect et al., 2021a), is overlooked in Eq. (2). Overall, in the present study, the observed profile of organic acids suggests that lactate-driven DF was key in achieving high hydrogen production performance using FVW as substate.

3.3. Energy and mass balance analysis

Based on the HPR and HY achieved, the average EPR and EY ranged from 40.0 to 150.3 kJ/L-d and from 0.5 to 1.2 ± 0.1 kJ/g VS fed, respectively (Table 4). A similar EY of 1.5 kJ/g VS was reported from the DF of FW (Ghimire et al., 2015). An energy and mass balance analysis of the process was performed based on a daily feed of 1000 kg of FVW (99.9 kg VS) and considering the operational conditions of operational stage IV (HRT of 9 h and OLR of 125.4 g VS/L-d), which led to the highest HPR and HY. As shown in Fig. 4, 1000 kg of FVW per day would be managed by a working volume of reactor of ≈ 0.8 m^3 and produce up to 9.4 m^3 H_2 per day with an associated HY of 94.1 L H_2/kg VS fed or 9.4 L H_2/kg FVW. In other words, 1255 kg of FVW would be managed by 1 m^3 working volume. Such a hydrogen production would produce 120 MJ per day, impliying an EPR of 150.3 MJ/m^3-d and an EY of 1.2 MJ/kg VS fed or 0.1 MJ/kg FVW. Regarding the soluble by-products, the resulting fermentation broth would contain 13.2, 4.1, 3.2, 2.3, 1.1, and

Table 4
Energy production analysis at pseudo-steady state under different operational conditions for the dark fermentation of fruit-vegetable waste.

Parameter	Operational stage				
	I	II	III	IV	V
EPR (kJ/L-d)	40.0 ± 2.4	49.7 ± 6.8	95.5 ± 12.4	150.3 ± 12.0	90.9 ± 34.0
EY (kJ/g VS fed)	0.9 ± 0.1	0.7 ± 0.1	1.0 ± 0.1	1.2 ± 0.1	0.5 ± 0.2

***Note*:** EPR: energy production rate; EY: energy yield. No pseudo-steady state was achieved in operational stage V. The number of samples analyzed was 8, 17,14, 25 and 13 for the operational stage I, II, II, IV, and V, respectively.

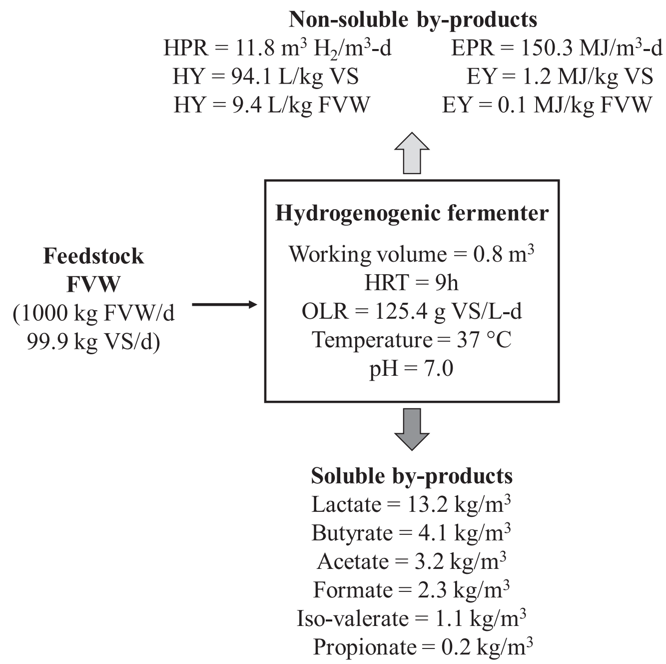

Fig. 4. Energy recovery and mass balance of the continuous dark fermentation of fruit-vegetable waste.

0.2 kg/m^3 of lactate, butyrate, acetate, formate, isovalerate and propionate, respectively. Such building blocks can be further used togheter with the remaining sugars as carbon and energy source for other biotechnologies, for instance, anaerobic digestion that does not requiere a downstream step for the recovery and purification of organic acids (Montiel Corona and Razo-Flores, 2018; García-Depraect et al., 2020).

Although an oustanding HPR was achieved herein, further endeavours are still needed to improve the DF of FVW. Some technical issues, such as process instability and insuficient HPR and HY, must be overcome before process scale-up. Future work should deeply investigate the microbiology of the process and explore promising enhacement strategies such as the use of conductive materials such as magnetite, which has been reported to promote the bioconversion of lactate into hydrogen (Kim et al., 2022).

4. Conclusions

The effect of HRT on continuous FVW-DF was investigated. The HRT exerted a markedly impact on HPR/HY. Low process performance was observed at HRTs above 12 h, while 6 h HRT resulted in process collapse due to organic overloading or biomass washout. The highest HPR (11.8 NL/L-d) and HY (95.6 NmL/g VS fed) were recorded at 9 h HRT. The major by-products were lactate, acetate and butyrate throughout the operation while hydrogen production did not correlate with carbohydrates consumption, which together suggested the occurrence of lactate-driven hydrogen-producing pathway(s). Overall, FVW is a good feedstock for hydrogen production, producing 150.3 kJ/L-d.

CRediT authorship contribution statement

Leonardo J. Martínez-Mendoza: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft. **Octavio García-Depraect:** Conceptualization, Methodology, Validation, Supervision, Funding acquisition, Project administration, Writing – review & editing. **Raúl Muñoz:** Conceptualization, Methodology, Validation, Supervision, Funding acquisition, Project administration, Writing – review & editing.

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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