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Biohydrogen production by lactate-driven dark fermentation of real organic wastes derived from solid waste treatment plants

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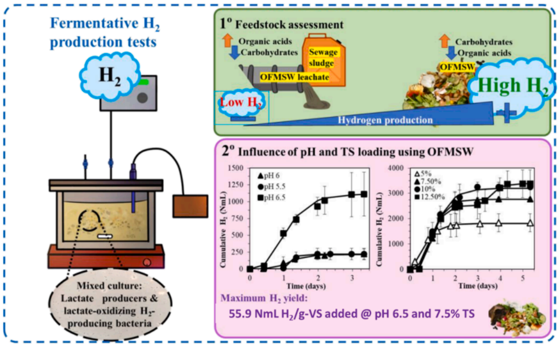
HIGHLIGHTS

- Lactate-driven dark fermentation (LD-DF) of real organic waste was studied.
- Heterogeneous OFMSW was suitable for bioH_2 production via LD-DF.
- A shortage of carbs and/or a high pre-acidification degree inhibits H_2 production.
- pH and initial solids loading have a strong impact on the OFMSW LD-DF.

GRAPHICAL ABSTRACT



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ABSTRACT

This study evaluated the hydrogen production potential through lactate-driven dark fermentation (LD-DF) of organic wastes from solid waste treatment plants, including the organic fraction of municipal solid waste (OFMSW), mixed sewage sludge, and two OFMSW leachates. In initial batch fermentations, only OFMSW supported a significant hydrogen yield (70.1 ± 7.7 NmL-H_2/g-VS added) among the tested feedstocks. Lactate acted as an important hydrogen precursor, requiring the presence of carbohydrates for sequential two-step lactate-type fermentation. The impact of operational pH (5.5–6.5) and initial total solids (TS) concentration (5–12.5 % *w/w*) was also evaluated using OFMSW as substrate, obtaining hydrogen yields ranging from 6.6 to 55.9 NmL-H_2/g-VSadded. The highest yield occurred at 6.5 pH and 7.5 % TS. The LD-DF pathway was indicated to be present under diverse pH and TS conditions, supported by employing a specialized microbial consortium capable of performing LD-DF, along with the observed changes in lactate levels during fermentation.

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1. Introduction

According to the European Commission, renewable hydrogen can be defined as the hydrogen produced by electrolysis of water, reforming of biogas, or biochemical conversion of biomass (European Commission, 2020). Renewable hydrogen holds an enormous potential for industrial and transportation applications. It is nowadays considered the keystone of the Europe's clean energy transition and decarbonization strategy of the European Green Deal (EBA, 2023). Indeed, the European Commission has committed to significantly increase the European production capacity of renewable hydrogen to up to 10 million tons by 2030 (European Commission, 2020). While most current roadmaps for hydrogen production prioritize electrolysis of water using renewable energy, it is essential to promote and develop other technologies, such as the biochemical conversion of biomass.

In this context, dark fermentation (DF) represents an alternative biological route for biogenic hydrogen production. In fact, DF is a light-independent process characterized by significantly higher hydrogen production rates compared to other biological processes such as direct/indirect biophotolysis, photofermentation, and microbial electrolysis cells (MECs). DF has shown a wide range of hydrogen productivities, from 1 NL H_2/L-d to up to 78 NL H_2/L-d (Pugazhendhi et al., 2017). This variability is influenced by a number of factors including microbiology, substrate composition, reactor design, and other environmental and process parameters (e.g. pH, temperature, etc.) (Park et al., 2021). It should be noted that to make the DF process economically viable, it will be necessary to integrate DF with other biotechnologies. These may include anaerobic digestion for biogas and fertilizer production, aerobic fermentation for bioplastic production, photosynthetic processes for the production of high-value compounds such as pigments and industrial chain building blocks, and anaerobic fermentation for organic acid production, among others (Martínez-Mendoza et al., 2022; Sivagurunathan et al., 2018). In brief, DF is a complex biological process based on the fermentation of organic waste and wastewater by multiple species of bacteria that produce hydrogen along with carbon dioxide and other soluble by-products such as acetate and butyrate under anaerobic conditions (Krishnan et al., 2023).

In recent years, several studies reported in the literature have shown that the lactate-driven dark fermentation (LD-DF) process is an alternative fermentation pathway to produce green hydrogen from biomass. Some feedstocks that support the LD-DF process are fruit-vegetable waste (Gomez-Romero et al., 2014; Martínez-Mendoza et al., 2022), food waste (Regueira-Marcos et al., 2023); sugarcane and tequila vinasses (Bernal et al., 2021; Diaz-Cruces et al., 2020; Fuess et al., 2018), cheese whey (Asunis et al., 2019; Blanco et al., 2019), and molasses (Detman et al., 2021). The growing scientific interest in LD-DF relies on the fact that it can exploit the bacterial association between lactic acid bacteria (LAB) and lactate oxidizing hydrogen-producing bacteria (LO-HPB) that exists through the cross-feeding of lactate (García-Depraect et al., 2021). Thus, the LD-DF pathway can cope with the multiple undesirable inhibitory effects caused by the invasion and colonization of LAB, which often outcompete HPB. In this context, different research approaches in the field of LD-DF have been carried out in recent years, such as the study of i) metabolic fluxes (Jung et al., 2021; Munier et al., 2023), ii) pure cultures (Detman et al., 2019; Ohnishi et al., 2022; Sivagurunathan et al., 2023), iii) the addition of nanoparticles and conductive materials (Cui et al., 2022; Gökçek et al., 2023; Kim et al., 2021, 2022; Park et al., 2019); iv) microbial communities (Fuess et al., 2018; García-Depraect et al., 2019; Park et al., 2019; Pérez-Rangel et al., 2021; Rasmey et al., 2023), v) kinetic modeling (Blanco et al., 2019; García-Depraect et al., 2019), and vi) operational parameters (García-Depraect et al., 2019; Martínez-Mendoza et al., 2022; Regueira-Marcos et al., 2023).

Despite the potential of LD-DF, there are only a limited number of studies devoted to the systematic evaluation of real feedstocks for hydrogen production via LD-DF by mixed culture. Conducting laboratory-scale tests using real substrates, rather than synthetic substrates, can provide more reliable data on expected performance of LD-DF in large-scale fermentation systems. This research need is particularly relevant when evaluating the potential for hydrogen production from municipal organic wastes due to their high abundance and availability, low cost, and appropriate content of micro and macronutrients that support fermentation (Sailer et al., 2021; Yang and Wang, 2017). In this context, the knowledge of the performance of LD-DF on real feedstocks collected directly from centralized waste management facilities is still very limited. Indeed, the inherent high heterogeneity of municipal organic wastes, coupled with factors such as pre-acidification or contamination with undesirable microorganisms (and other unknown components), are critical aspects that are difficult to mimic or to assess when simulating them or when the target feedstock is not directly collected at the municipal wastewater treatment plants or centralized solid waste treatment facilities. In this context, it has been recently reported the achievement of high hydrogen production performances from simulated organic wastes, such as fruit-vegetable waste and household-type food waste by using a specialized biocatalyst able to perform LD-DF (Martínez-Mendoza et al., 2022; Regueira-Marcos et al., 2023). However, whether the use of a LD-DF inoculum can drive successful hydrogen production from real complex feedstocks is still an open research question. Therefore, as a down-scale approach, this study aims to evaluate the hydrogen production potential by LD-DF of different real municipal wastes, namely, the organic fraction of municipal solid waste (OFMSW), OFMSW leachate, and mixed sewage sludge under batch conditions. Following a feedstock screening study, the independent effect of pH and initial solids loading on the performance of the LD-DF of OFMSW under batch conditions was also investigated to further improve the resource-to-hydrogen bioconversion.

2. Materials and methods

2.1. Hydrogen-producing consortium

The acidogenic inoculum used in this study was derived from pre-treated digestate obtained from an anaerobic digester operating on food waste under mesophilic conditions. The pre-treatment consisted of subjecting the digestate to 90 °C for 20 min in order to inactivate methanogens. The pretreated culture was then grown for 1 week in a lab-scale chemostat, using cheese whey powder as the substrate. Cheese whey was selected as it serves as an excellent substrate for promoting LD-DF due to its high lactose content and the efficient conversion of lactose to lactate during fermentation. The operating conditions were temperature 37 °C, pH 6.4 ± 0.1, hydraulic retention time (HRT) 6 h, organic loading rate (OLR) 180 g chemical oxygen demand (COD)/L-d, and agitation speed 200 rpm. The resulting acidogenic bacterial consortium, which had the metabolic capacity to produce hydrogen from lactate, was then stored at 4 °C and used as needed (Regueira-Marcos et al., 2023). Prior to use, a 0.1 L aliquot of the inoculum was used for a 24 h microbial reactivation step performed in batch mode in a 2.1 L fermenter with a 1 L working volume, at 37 °C, ~200 rpm, and without pH control (initial pH 6.4). The growth mineral medium used in the bacterial reactivation step consisted of (in g/L): lactose, 10; $CaCl_2 \cdot 2H_2O$, 0.15; $FeCl_2 \cdot 4H_2O$, 0.035; KH_2PO_4, 0.6; K_2HPO_4, 2.4; $MgCl_2 \cdot 6H_2O$, 2.5; and NH_4Cl, 2.4 (Regueira-Marcos et al., 2023). In practical applications, inoculum reactivation with a lactose-based medium is only necessary during fermenter start-up, as substrate provides the necessary nutrients. In addition, the use of a lactose-based medium during the reactivation phase of the microbial consortium ensures a consistent biocatalyst, which is essential for conducting reproducible fermentative hydrogen production experiments.

2.2. Feedstocks

Four different feedstocks were tested, namely OFMSW, two different

OFMSW leachates, and a mixed sewage sludge. Both the OFMSW and the leachates were collected from the urban Waste Treatment and Disposal Centre of Valladolid, Spain, which is managed by FCC Medio Ambiente. The mixed sewage sludge was collected from the Valladolid wastewater treatment plant. Three different batches of OFMSW were collected (i.e., OFMSW-L1, OFMSW-L2, OFMSW-L3), each on a different sampling date. Likewise, the leachates were collected before (leachate-1) and after (leachate-2) the industrial composting of OFMSW.

All OFMSW batches were shredded with tap water using a semi-industrial blender (Sammic, XM-32, Spain) to achieve a smooth paste consistency. Prior to grinding, all visible inert materials (i.e. plastic, metal, wood, etc.) were removed by hand. The resulting pulp was stored at −20 °C to prevent deterioration. Both the leachates and the mixed sewage sludge were tested in their raw form. The raw mixed sewage sludge was also tested for hydrogen production after autoclaving (121 °C for 30 min) using a conventional autoclave (Raypa series AES, Spain), to produce some easily fermentable organics while avoiding the presence of undesirable microbiota such as hydrogen-consuming microorganisms due to its high indigenous microbial load. The leachates and the mixed sewage sludge were stored at 4 °C until use. Table 1 summarizes the physicochemical characterization of all the different feedstocks tested.

2.3. Experimental setup and operating conditions

This study was divided into three different batch trials. First, a series of LD-DF tests were performed to evaluate the hydrogen production potential of each feedstock tested. As a result of this screening test, the subsequent experiments were performed using OFMSW only. In a second set of experiments, the effect of pH on the performance of the LD-DF process was investigated, while in a third set of experiments the corresponding effect of the initial total solids (TS) content was studied. All the experiments were carried out using an identical experimental setup consisting of three identical 1.4-L jacketed fermenters operated in parallel. Each fermenter was equipped with the following instrumentation: a pH controller (BSV, Spain) with a pH probe (HO35-BSV01, Spain), a magnetic stirring plate (LBX S20, Labbox Labware, Spain), and an in-house gas flow meter (see supplementary material). A thermostatic bath (Tectron-Bio 20, Selecta, Spain) was used to heat the fermenters. The fermenters were also equipped with sampling ports for periodic collection of liquid and gaseous samples.

All the fermentation tests conducted in this study were performed in triplicate under the following culture conditions: 10 % (*v/v*) inoculum size with a volatile suspended solids (VSS) concentration of 0.66 ± 0.17 g/L, 0.8 L working volume, 37 ± 1 °C, and ≈ 300 rpm. The feed-to-inoculum ratio (F/M) varied depending on the substrate and the initial TS content evaluated for each batch test. In particular, the first trial assessing the hydrogen production potential of each feedstock was conducted at constant pH of 6 and without dilution of the feedstock, except in the case of OFMSW, where the initial TS content was adjusted to 5 % by dilution with tap water. The F/M ratio for the first set of experiments was as follows (in g VS/g VSS): 286.9 for mixed sludge, 619.8 for leachate-1, 625.1 for leachate-2, and 626.4 for OFMSW-L1. In the next series of batch tests, the influence of pH was investigated by controlling the pH at 5.5, 6.0 and 6.5, while keeping the initial TS content at 5 % (the F/M ratio remained constant at 564.55 g VS/g VSS during the pH tests, using OFMSW-L2). Finally, the influence of the initial TS concentration was studied at 5, 7.5, 10, and 12.5 %, while the pH was controlled at 6.5 (optimum pH found in the pH tests). The F/M ratios (in g VS/g VSS) were 642.3 at 5 % TS, 963.4 at 7.5 % TS, 1284.6 at 10 % TS, and 1605.7 at 12.5 % TS.

Over the course of the fermentation, the cumulative volume of the acidogenic biogas (reported at standard temperature and pressure conditions of 0 °C and 1 atm) and its composition were measured twice daily, while the fermentation broth was sampled once daily to analyze the profile of lactate and volatile fatty acids (VFAs). All the fermentation tests were ended when the cumulative hydrogen production reached a plateau. The process was evaluated based on the hydrogen production yield (HY) and the maximum volumetric hydrogen production rate (VHPR). The cumulative hydrogen production was also modeled by the modified Gompertz kinetic model (Eq. (1), where *H* is the cumulative hydrogen production (NmL), *Hmax* is the maximum cumulative

Table 1

Physicochemical characteristics of the different feedstocks (mixed sewage sludge, OFMSW leachates, and OFMSW) used in this study.

Parameter	Mixed sludge	Leachate-1	Leachate-2	OFMSW-L1	OFMSW-L2	OFMSW-L3
pH	6.73 ± 0.01	6.11 ± 0.01	7.21 ± 0.04	5.57 ± 0.04	5.19 ± 0.12	5.09 ± 0.01
Total alkalinity (g $CaCO_3$/L)	3.62 ± 0.50	17.40 ± 0.28	18.75 ± 0.35	1.54 ± 0.03	2.00 ± 0.14	1.38 ± 0.00
Total COD (g/L)	42.39 ± 2.92	113.19 ± 0.00	92.74 ± 3.69	192.67 ± 4.71	141.67 ± 3.73	178.23 ± 59.93
Soluble COD (g/L)	1.78 ± 0.03	N.D.	N.D.	116.76 ± 1.55	63.31 ± 2.39	145.19 ± 0.04
Soluble TOC (g/L)	1.31 ± 0.05	41.99 ± 0.58	24.01 ± 0.06	29.06 ± 0.65	23.91 ± 1.34	59.81 ± 0.99
Total carbohydrates (g/L)	3.14 ± 0.44	2.43 ± 0.15	1.34 ± 0.14	114.32 ± 12.34	56.79 ± 3.26	171.90 ± 22.08
Soluble TN (g/L)	0.13 ± 0.00	N.D.	4.36 ± 0.00	0.76 ± 0.01	1.34 ± 0.04	1.44 ± 0.01
TKN (g/L)	1.49 ± 0.07	5.62 ± 0.06	4.63 ± 0.03	2.88 ± 0.16	3.04 ± 0.10	7.73 ± 0.15
[a] C/N	28.45	20.14	20.03	66.90	46.60	23.06
Total solids (g/L)	32.95 ± 0.17	63.03 ± 0.95	69.87 ± 2.31	163.66 ± 1.71	166.75 ± 0.16	264.53 ± 4.07
Volatile solids (g/L)	21.04 ± 0.19	45.45 ± 0.96	45.84 ± 2.15	150.06 ± 1.71	138.07 ± 2.45	249.19 ± 4.99
VS/TS	0.64	0.72	0.66	0.92	0.83	0.94
Lactate (g/L)	0.00 ± 0.00	0.00 ± 0.00	1.42 ± 0.20	3.78 ± 0.08	6.54 ± 0.07	4.09 ± 0.01
Formate (g/L)	0.00 ± 0.00	0.12 ± 0.01	0.62 ± 0.01	0.06 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Acetate (g/L)	0.79 ± 0.01	12.78 ± 0.07	6.95 ± 0.57	0.81 ± 0.19	2.61 ± 0.05	5.77 ± 0.53
Propionate (g/L)	0.18 ± 0.00	9.20 ± 0.08	3.59 ± 0.23	0.29 ± 0.01	0.24 ± 0.00	0.02 ± 0.03
Iso-butyrate (g/L)	0.02 ± 0.00	0.62 ± 0.08	0.08 ± 0.01	0.49 ± 0.01	0.83 ± 0.20	0.06 ± 0.04
Butyrate (g/L)	0.07 ± 0.00	8.43 ± 0.37	3.33 ± 0.24	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Iso-valerate (g/L)	0.03 ± 0.00	0.64 ± 0.03	0.00 ± 0.00	0.19 ± 0.00	0.31 ± 0.09	0.00 ± 0.00

N.D.: Not determined; COD: chemical oxygen demand; TOC: total organic carbon; TN: total nitrogen; TKN: total Kjeldahl nitrogen.

[a] C/N ratios were roughly estimated as total COD divided by TKN.

hydrogen production (NmL), *Rmax* is the maximum hydrogen production rate (NmL/d), and λ is the duration of the lag phase (days). VHPR was calculated as *Rmax* normalized to the working volume. Additionally, ANOVA analysis ($p < 0.05$) was performed to evaluate significant differences for HY results from the pH and TS tests.

$$H = H_{max} \bullet exp\left\{ - exp\left[\frac{R_{max} \bullet e}{H_{max}} \bullet (\lambda - t) + 1 \right] \right\} \tag{1}$$

2.4. Analytical procedures

The concentrations of COD, solids, total Kjeldahl nitrogen (TKN) and pH were determined using standard methods (APHA, 2005). Total carbohydrates were measured by the phenol–sulfuric method (Dubois et al., 1951). Total organic carbon (TOC) and total nitrogen (TN) concentrations were analyzed in prefiltered samples (0.7 μm) using a TOC analyzer (TOC-L series, Shimadzu, Japan), which was equipped with a chemiluminescence module TNM-L for TN analysis. Organic acids, including lactate, formate, acetate, propionate, butyrate, *iso*-butyrate, valerate, *iso*-valerate, *iso*-caproate, and hexanoate, were analyzed by high-performance liquid chromatography (HPLC). The HPLC (model LC-2050, Shimadzu, Japan) was equipped with a UV–VIS detector (model UV-2600i, Shimadzu, Japan) and a HyperREZ XP H + column (Thermo Fisher Scientific Inc., EE.UU.). The column temperature was maintained at 75 °C, while the flow rate of the mobile phase (25 mM H_2SO_4) was kept at 0.6 mL/min. Sodium L-lactate (Sigma-Aldrich part number 71718, USA) and a mixture of VFAs (Sigma-Aldrich CRM46975, USA) were used as standards. Finally, the biogas composition was determined by gas chromatography (GC-TCD) in an Agilent 8860 GC (California, EEUU) according to the procedure described elsewhere (García-Depraect et al., 2022).

3. Results and discussion

3.1. Hydrogen production potential via lactate-driven dark fermentation

As shown in Fig. 2 and Table 2, only small amounts of hydrogen (4–14.5 NmL H_2/L) were produced when evaluating the hydrogen production potential of OFMSW leachates. Similarly, hydrogen production from the raw mixed sewage sludge was completely inhibited and only 49.1 ± 22.3 NmL H_2/L (1.3 ± 0.6 NmL H_2/g COD added) was recorded from the sterilized mixed sludge (Fig. 1b and Table 2). The hydrogen content peaked at 13 % and 2 % for the mixed sewage sludge and the OFMSW leachates, respectively. In contrast, OFMSW-L1 supported a cumulative biogas production of 7452.0 ± 734.8 NmL/L, with a maximum hydrogen content of 48.7 ± 6.6 %. Thus, the corresponding hydrogen production for OFMSW-L1 was 3211.6 ± 353.4 NmL/L, with an associated HY of 70.1 ± 7.7 NmL H_2/g VS added or 54.7 ± 6.0 NmL H_2/g COD added. It is worth mentioning that the methanogenic activity was effectively suppressed, since no methane was detected in any of the feedstocks tested. Regarding the kinetics of hydrogen production from OFMSW-L1, according to the kinetic modeling performed ($R^2 = 0.9968$; Fig. 2a), the lag phase was estimated as 10.8 h while the maximum VHPR was 3399.5 ± 227.3 NmL H_2/L-d. More consistent results were obtained for the maximum hydrogen production potential, which was estimated to be 3254.9 ± 127.1 NmL H_2/L. Thus, of the four different feedstocks tested, only OFMSW was found to be suitable for hydrogen production under the experimental conditions applied.

Table 2
Comparison of the fermentative hydrogen production performance obtained from real organic feedstocks.

Feedstock	Cumulative biogas (NmL/L)	Peak H_2 content (%)	Cumulative H_2 (NmL/L)	HY (NmL/g COD added)	HY (NmL/g VS added)
[a] Mixed sludge	341.5 ± 53.3	13.3 ± 1.5	49.1 ± 22.4	1.3 ± 0.6	2.9 ± 0.8
Leachate-1	200.7 ± 53.5	2.1 ± 0.9	14.5 ± 8.8	0.13 ± 0.08	0.32 ± 0.19
Leachate-2	281.4 ± 27.9	2.9 ± 0.5	4.0 ± 0.8	0.04 ± 0.01	0.09 ± 0.02
OFMSW-L1	7452.0 ± 734.8	48.7 ± 6.6	3211.6 ± 353.4	54.7 ± 6.0	70.1 ± 7.7

HY: Hydrogen yield. [a] Pretreated mixed sludge.

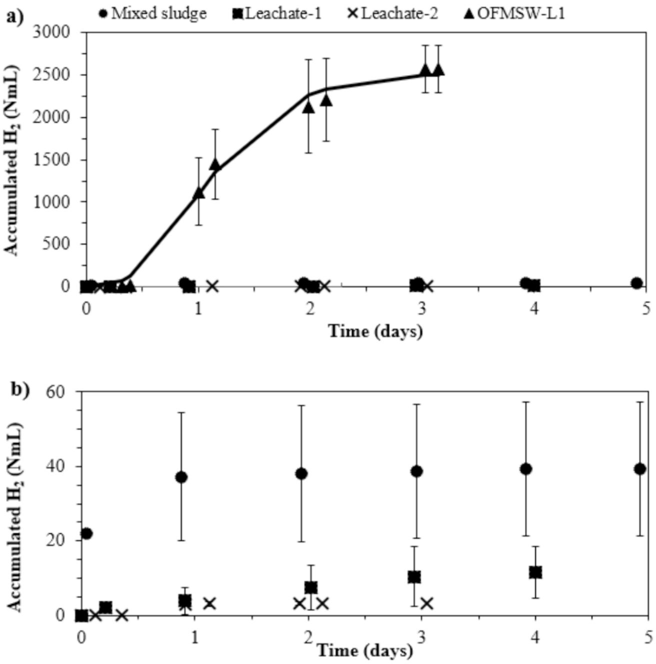

Fig. 1. Assessment of the hydrogen production potential via LD-DF of real organic wastes at a fixed pH of 6.0 (a). Close-up of the cumulative hydrogen production for leachate-1, leachate-2, and mixed sewage sludge (b). The continuous line represents the predicted data obtained from kinetic modeling using the modified Gompertz model.

The low hydrogen production observed from the pretreated mixed sludge was in partly attributed to its low carbohydrate content (3.1 ± 0.4 g/L) and slow biodegradability (0.04 soluble COD/total COD) (Table 1). Indeed, from a technical point of view, it is challenging to produce fermentative hydrogen from mixed sewage sludge. The main limitations of mixed sludge are its low C/N ratio, estimated here at 28.45 based on the ratio of total COD to TKN, and poor hydrolysis associated with the complex structure of the sludge (Yang and Wang, 2017). The lack of hydrogen production in the unsterilized mixed sludge test, coupled with its production in the sterilized test, suggests the presence of microorganisms that inhibit hydrogen production in the mixed sludge. Furthermore, the sterilization step may have acted as a pretreatment, possibly contributing to the slight improvement in hydrogen production observed in the sterilized mixed sludge. In the case of the OFMSW leachates, both leachates tested were characterized by a low carbohydrate content (1.3–2.4 g/L) and high concentration of VFAs (20.6–57.1 g COD equiv./L, corresponding to a pre-acidification degree of 50–70 %) (Table 1). In addition, both leachates had a similar C/N ratio of approximately 20, which was the lowest C/N ratio of all the feedstocks tested. The pre-fermentation that urban organic wastes typically undergo during storage and transportation prior to processing could explain such characteristics (Nie et al., 2022). It has been reported in the literature that the accumulation of VFAs can have a toxic effect on the metabolic activity of hydrogen producers (Elbeshbishy et al., 2017; Noblecourt et al., 2018). Wang et al. (2008) investigated the batchwise effect of ethanol, acetate, butyrate, and propionate on the fermentative hydrogen production from glucose by mixed culture. The authors

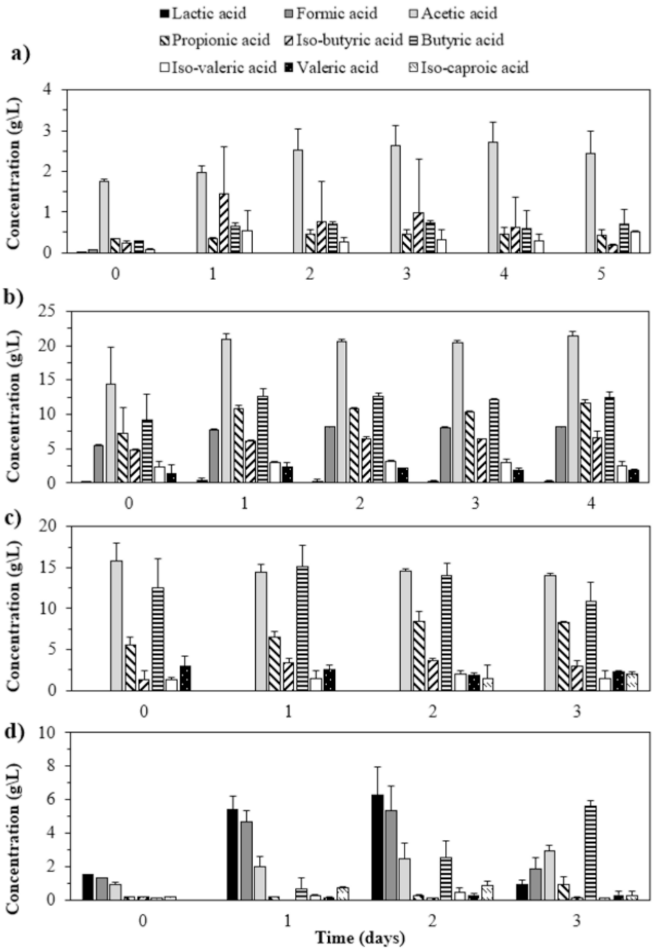

Fig. 2. Time course of organic acids during the LD-DF of mixed sludge (a), leachate-1 (b), leachate-2 (c) and OFMSW-L1 (d).

concluded that the VFAs exerted a grater inhibitory effect on hydrogen production compared to ethanol; the inhibition threshold for acetate, butyrate and propionate was 0.6, 0.9 and 0.7 g/L respectively, while hydrogen was completely inhibited at 1.8, 2.6 and 2.2 g/L, respectively. On the contrary, the OFMSW-L1 feedstock was highly biodegradable and had a higher carbohydrate content (114.3 ± 12.3 g/L) and a lower degree of pre-acidification of 5.7 % compared to the mixed sewage sludge and the leachates (Table 1). Thus, the content of fermentable carbohydrates and the degree of pre-acidification in the initial substrate were found to be important features for an efficient fermentative hydrogen production.

Overall, the HY obtained here for the mixed sewage sludge corresponded well with the values reported in the literature for sewage sludge, which ranged from 0.13 to 18.6 NmL H_2/g VS added or from 1.4 to 13.8 mL NmL H_2/g COD added (Yang and Wang, 2017). On the other hand, only few studies in the literature have reported the use of OFMSW leachate for hydrogen production via DF. Different types of leachates originated from landfills, composting, and food waste treatment facilities have been found to be unsuitable as DF feedstock alone, but good nutrient supplements for co-fermentation (Liu et al., 2011; Qi et al., 2018). For example, Liu et al. (2011) evaluated the continuous fermentative hydrogen production from fresh compost leachate supplemented with phosphate using an expanded granular sludge bed reactor, achieving the highest hydrogen productivity (2.2 NL H_2/L-d with approximately 30 % hydrogen content) at 12 h HRT and with a liquid upward velocity of 3.7 m/h. The plausible reason for the discrepancy between the low hydrogen production observed for both leachates tested in the present study and the results reported by Liu and co-workers (2011) may be attributed to the different characteristics of the leachate used herein, in which the degree of pre-acidification and the content of fermentable carbohydrates must be considered. Finally, the HY obtained herein for the OFMSW-L1 was in agreement with those obtained for OFMSW, which have been reported to range from 29 to 127 NmL H_2/g VS added (Salazar-Batres et al., 2022).

Although the type and concentration of organic acids depended on the type of feedstock, the mixed sewage sludge and both types of OFMSW leachates showed minimal variations in the organic acid profile over time. As shown in Fig. 3, acetate, propionate, *iso*-butyrate, and butyrate were the major acids found in these feedstocks. Leachate-1 also showed significant amounts of formate over time. Additionally, lactate was absent throughout the fermentation with the mixed sewage sludge and the leachates. It should be noted that the acid profile observed in both the mixed sewage sludge and the leachates at the beginning of the fermentation differed from that initially recorded in the feedstock characterization (Table 1). Such a difference can be explained by the pre-fermentation of the feedstocks, which seems reasonable considering that the mixed sewage sludge and the leachates were stored at 4 °C.

On the contrary, the profile of organic acids observed during the DF of OFMSW-L1 exhibited a completely different behavior. OFMSW-L1 showed an accumulation of lactate during the first two days of fermentation, reaching an average titer of up to 6.3 ± 1.7 g/L. Formate also accumulated in parallel with lactate, indicating an active electron flux downstream. At the end of the fermentation, the concentrations of lactate and formate were 0.9 ± 0.3 and 1.9 ± 0.6 g/L, respectively. Residual lactate may be an indication of an imbalance in the batch fermentation process, indicating the need to further improve hydrogen production. In contrast to lactate, an accumulation of acetate and butyrate in the fermentation broth was observed as the fermentation time progressed, reaching maximum concentrations of 2.9 ± 0.4 g/L and 5.6 ± 0.4 g/L, respectively. The accumulation and further consumption of lactate coupled with the simultaneous production of hydrogen and butyrate observed in the fermentation of OFMSW-L1, along with the use of a mixed culture inoculum with the metabolic capacity to perform the LD-DF, strongly suggests that lactate acted as a hydrogen precursor. In other words, lactate was a cross-feeding metabolite, resulting in the production of hydrogen and butyrate (Detman et al., 2019; Diez-Gonzalez et al., 1995; García-Depraect et al., 2019; Kim et al., 2022; Tao et al., 2016). Such behavior of organic acids and hydrogen production has been well documented during the LD-DF of other substrates. For instance, Martínez-Mendoza et al. (2022) reported a pronounced lactate accumulation trend (up to 33.0 ± 4.5 g/L) followed by lactate consumption during the DF of simulated fruit and vegetable waste at 5 % initial TS and a fixed pH of 7. In another study, Asunis et al. (2019) studied the batch DF process at different pH values using cheese whey substrate. In that study, lactate peaked during the

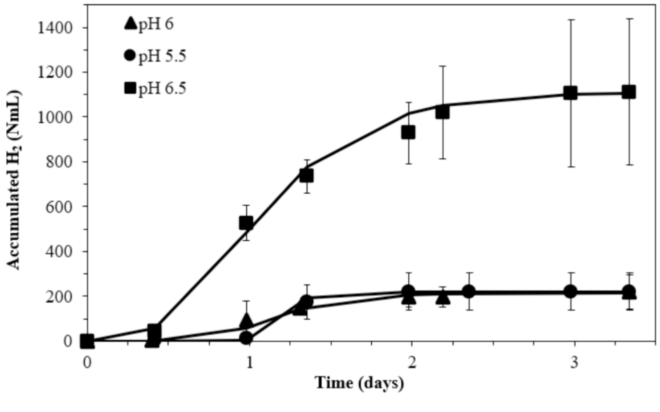

Fig. 3. Effect of pH on the cumulative hydrogen production recorded batchwise from the LD-DF of OFMSW-L2. The solid lines represent the data predicted by the modified Gompertz model.

first 50 h of fermentation but was then consumed while acetate and butyrate concentrations increased (Asunis et al., 2019). The two-phase lactate fermentation has also been reported in the DF of tequila vinasse (García-Depraect et al., 2019). The similarities between this and previous experimental studies on LD-DF support the reproducibility of the batch tests performed here. It is important to note that although analogous results have been reported for different substrates, the nature of the inoculum and the organic quality of the substrates could lead to significant differences in the hydrogen production efficiency. Common substrates that support LD-DF, such as fruit and vegetable waste, food waste, cheese whey, vinasse, and others, are characterized by high levels of fermentable carbohydrates. In addition, these substrates often serve as a reservoir for LAB. (García-Depraect et al., 2021). In the LD-DF process, microorganisms with the ability to produce hydrogen from lactate are crucial. When this criterion is met, LAB present in the substrate or inoculum ferment carbohydrates to lactate. Consequently, the lactate produced can be metabolized to produce hydrogen. It is worth noting that future research efforts should include additional omics analyses, such as metagenomic and metatranscriptomic analyses, to provide deeper insights into the LD-DF process. From a practical point of view, it is expected that superior amounts of hydrogen production would be produced from OFMSW by favoring the LD-DF process, as this pathway takes advantage of the syntrophic interaction between LAB and LO-HPB, thus utilizing the intrinsic presence of LAB. In this context, the results obtained in this study could provide valuable insights for the design and operation of future continuous LD-DF processes, which could provide data for techno-economic analysis.

3.2. Effect of pH on the lactate-driven dark fermentation of the organic fraction of municipal solid waste

A series of batches with OFMSW-L2 was carried out with a fixed TS concentration of 5 % and under different operating pH values ranging from 5.5 to 6.5. It was found that the selected pH affected the amount of biogas produced, which was in the range of 1888.8 to 2806.0 NmL/L, with the lowest and highest production achieved at pH 5.5 and pH 6.5, respectively. The hydrogen content, ranging between 24.3 % and 44.7 % (*v*/*v*), was also influenced by pH. Notably, a more neutral pH resulted in off-gas with higher hydrogen content (Table 3). As shown in Fig. 4, the cumulative production of hydrogen was 1389.7 ± 212.5 NmL/L at pH 6.5, 270.2 ± 67.5 NmL/L at pH 6.0, and 275.9 ± 104.8 NmL/L at pH 5.5. The highest HY of 33.7 ± 5.2 NmL H_2/g VS added (45.2 ± 6.9 NmL H_2/g COD added) was indeed recorded at pH 6.5, while similar and lower HY values (c.a. 7 NmL H_2/g VS added or 9 NmL H_2/g COD added) were achieved at pH 6.0 and pH 5.5. As expected, the modified Gompertz model predicted the experimental hydrogen production behavior very well (R^2 of 0.97–0.99). The maximum VHPR was estimated to be (in NmL H_2/L-d) 1143.1 ± 232.2, 382.4 ± 193.32 and 362.5 ± 128.56 for pH 6.5, 6.0 and 5.5, respectively. The lag phase (λ) ranged from 3.1 h at pH 6.5 to 9.6 h at pH 5.5. Thus, the operating pH significantly influenced the hydrogen production efficiency, highlighting the importance of pH control in the LD-DF process.

Table 3
Comparison of the LD-DF performance for OFMSW-L2 at different operating pH values.

pH	Cumulative biogas (NmL/L)	Peak H_2 content (%)	Cumulative H_2 (NmL/L)	HY (NmL/g COD added)	HY (NmL/g VS added)
5.5	1888.8 ± 97.1	29.2 ± 6.6	275.9 ± 104.8	9.0 ± 3.4 ^{a}	6.7 ± 2.5 ^{a}
6.0	2649.2 ± 502.3	24.3 ± 7.4	270.2 ± 67.5	8.8 ± 2.2 ^{a}	6.6 ± 1.6 ^{a}
6.5	2806.0 ± 92.2	44.7 ± 9.6	1389.7 ± 212.5	45.2 ± 6.9^b	33.7 ± 5.2^b

HY: Hydrogen yield. Different letters indicate that results within the same column are significantly different at a 95% confidence level, as determined by ANOVA statistical comparison.

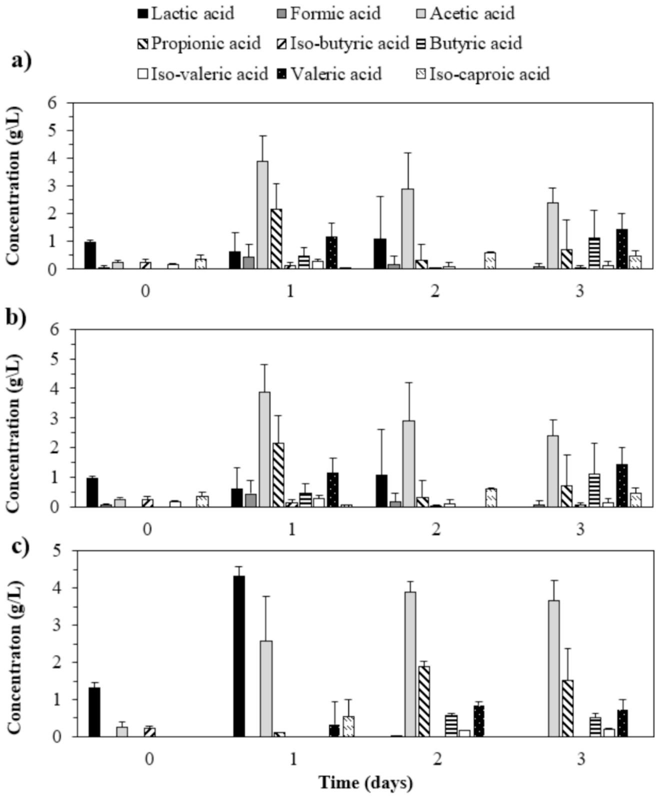

Fig. 4. Time course of organic acids recorded during the LD-DF of OFMSW-L2 at pH 5.5 (a), 6.0 (b), and 6.5 (c).

On the other hand, lactate was identified as an important DF cross-feeding metabolite, especially at pH 6.5, where it accumulated during the first 24 h of cultivation, reaching concentrations of up to 4.3 ± 2.5 g/L, but was completely consumed by 47 h (Fig. 5). Although carbohydrate concentrations were not monitored, it is reasonable to assume that some fermentable carbohydrates were consumed along with lactate production. A fraction of the total amount of hydrogen recorded could also be produced (especially in the early production phase) from direct carbohydrate metabolism, as recently demonstrated by Fuentes-Santiago et al.

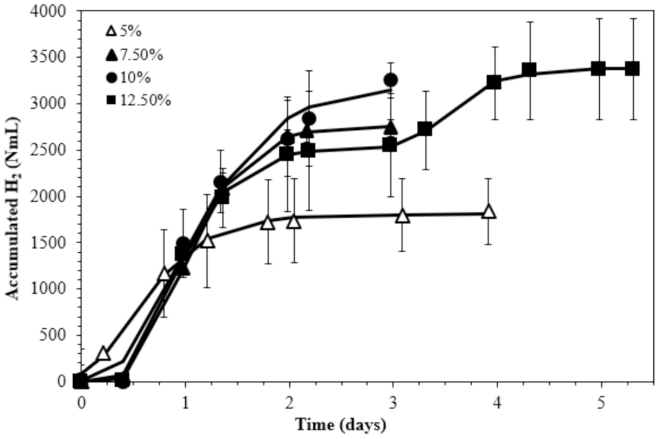

Fig. 5. Effect of the initial total solids content on the cumulative hydrogen production observed batchwise during the LD-DF of OFMSW-L3. The predicted hydrogen production by the modified Gompertz model is shown by continuous lines.

(2023). Carbohydrates were found to be essential (as concluded in section 3.1) because they are precursors of lactate and acetate, which in turn are the reactants for hydrogen production via LD-DF. Recently, fermentable carbohydrates have been associated with a positive response in the expression of the metabolic machinery required for the lactate-consuming, acetate-dependent, hydrogen-producing pathway (Fuentes-Santiago et al., 2023). Increasing concentrations of acetate and butyrate were also detected during fermentation, reaching maximum concentrations of 6.7 ± 1.7 g/L and 2.1 ± 0.9 g/L, respectively at pH 6.5. Butyrate is a known end product of LD-DF, while the presence of acetate is essential for the production of hydrogen via LD-DF, as it acts as an electron acceptor (García-Depraect et al., 2021). Eq. 2 illustrates the utilization of lactate and acetate and the production of butyrate and hydrogen, with a Gibbs free energy of − 156.6 kJ/mol (Matsumoto and Nishimura, 2007). Most *Clostridium* spp. seem to use the via pyruvate-ferredoxin oxidoreductase. In the LD-DF, an FAD-dependent lactate dehydrogenase (LDH), which forms a stable complex with an electron transfer flavoprotein (EtfA/B), facilitates the energetically favorable oxidation of lactate using NAD^+ as the oxidant. This process is accompanied by the oxidation of reduced ferredoxin. Then, pyruvate is oxidized to acetyl-coenzyme A (acetyl-CoA), which is further metabolized to acetate (via acetate kinase) and butyrate (via phosphotransbutyrylase and butyrate kinase or butyryl-CoA:acetate CoA transferase) (Detman et al., 2019). Lower acetate and butyrate titers were found at pH 6.0 and pH 5.5, which may explain the lower hydrogen production. Other end products such as *iso*-butyrate, *iso*-valerate and *iso*-caproate were also detected at lower concentrations.

$$2\,CH_3CH(OH)COOH + CH_3COOH \rightarrow \frac{3}{2}CH_3CH_2CH_2COOH + H_2 + 2CO_2 + H_2O \tag{2}$$

The results of this study highlight the critical role of pH as a key operational parameter that affects the efficiency of hydrogen production through several mechanisms. These mechanisms may include the modulation of microbial communities, regulation of enzymatic activity and metabolic fluxes, and influence on substrate solubilization and transformation (García-Depraect et al., 2021; Ghimire et al., 2015; Regueira-Marcos et al., 2023). Previous comprehensive reviews have compiled works evaluating the influence of pH on fermentative hydrogen production over the pH range of 4.5 to 7.5 (García-Depraect et al., 2021; Li and Fang, 2007), indicating that the optimal pH for hydrogen production is highly dependent on the nature of the feedstock and the microbial community structure involved (Delavar and Wang, 2021). In the experiments conducted, pH values ranging from 5.5 to 6.5 did not inhibit hydrogen production. However, the exact metabolic mechanism behind the improvement in hydrogen production at pH 6.5 remains unclear. Lower pH values resulted in reduced hydrogen production, which is consistent with findings from other LD-DF studies suggesting that a pH of 5.5 promotes lactate accumulation but inhibits hydrogen production (Regueira-Marcos et al., 2023; Asunis et al., 2019). Furthermore, in a single-stage LD-DF process, it is believed that operating at a near-neutral pH enhances both the conversion of carbohydrates to lactate and the subsequent conversion of lactate to hydrogen (Martínez-Mendoza et al., 2022; Regueira-Marcos et al., 2023; García-Depraect et al., 2021). In this regard, it has been reported that the LD-DF of cheese whey at pH 6.0 boosted not only the fermentation of carbohydrates to produce lactate, but also the oxidation of lactate to produce hydrogen (Asunis et al., 2019). Therefore, pH can significantly affect the balance between lactate producers and LO-HPB. The boosted activity of LO-HPB at near-neutral pH may therefore support the results observed here, a fact that deserves attention in future studies. Finally, it is worth discussing the effect of pH on the hydrolysis of particulate organics, especially when dealing with complex feedstocks such as OFMSW, since solid particles must be hydrolyzed before they can be taken up by bacteria. In this regard, additional research is warranted to investigate whether the operating pH in DF studies using OFMSW affects the extent of hydrolysis.

3.3. Effect of TS content on the lactate-driven dark fermentation of the organic fraction of municipal solid waste

The initial TS concentration of OFMSW was directly correlated with the amount of substrate availability at the start of fermentation. Therefore, the TS content will affect the substrate-to-biomass ratio which in turn could affect the microbial community structure and hydrogen production performance (García-Depraect et al., 2021; Jung et al., 2021). In this work, the influence of the TS content in the range of 5 to 12.5 % on hydrogen production was investigated in a third series of LD-DF tests conducted batchwise with OFMSW-L3. Both the hydrogen content and the total amount of biogas produced were affected by the initial TS concentration (Fig. 6 and Table 4). In particular, the hydrogen content in the exhaust gas reached maximum values of 34.7, 41.8, 37.9 and 30.1 % (*v*/*v*) at 5, 7.5, 10 and 12.5 % TS loads, respectively (Table 4). It was also observed that higher TS contents resulted in higher biogas productions; 9263.8 ± 700.9 NmL/L at 5 % TS and 17115.3 ± 2961.6 NmL/L at 12.5 % TS. However, it was found that the initial TS loading had no significant effect on HY. When the cumulative hydrogen production was related to the initial amount of VS as a proxy for organic matter, the final HY were (in NmL H_2/g VS added) 55.1 ± 10.7, 55.9 ± 1.1, 49.3 ± 2.8 and 40.9 ± 6.6 at 5, 7.5, 10 and 12.5 % TS, respectively. The corresponding HY values, expressed as NmLH_2/g COD added, are shown in Table 4. It is worth noting that the OFMSW batch that showed superior HY (on a VS basis) in this study was OFMSW-L1 (see Section 3.1). This batch had the highest carbohydrate content and the highest C/N ratio (66.9) of all OFMSW batches tested. It has been reported that the TS content could play a key role in the equilibrium between LAB and LO-HPB, where TS contents between 5 and 7.5 % have promoted hydrogen production, while a higher TS content has favored the overgrowth of LAB (Ghimire et al., 2018; Regueira-Marcos et al., 2023).

According to the estimated *Rmax* values from the modified Gompertz model (R^2 values of 0.97–0.99), the maximum VHPR (in NmL H_2/L-d) were 1806.9 ± 407.6, 3892.0 ± 872.1, 2974.0 ± 1007.5 and 3552.5 ± 385.0 (first fit) and 585.6 ± 131.2 (second fit) for fermentation tests at 5, 7.5, 10 and 12.5 % TS content, respectively. The fermentation test at an initial TS content of 12.5 % showed a diauxic hydrogen production behavior, which could be explained by the presence of different hydrogen-producing metabolic pathways, supported by the temporal dynamics in the production and utilization of hydrogen precursors such as lactate (Regueira-Marcos et al., 2023). The length of lag phase showed very little variation among the different TS contents tested, as λ values ranged between 9.6 and 14.4 h. In contrast, the total duration of fermentation differed significantly with the initial TS content, in particular, a higher TS content resulted in longer fermentations (up to 60 h in the 12.5 % TS test). At 10 % TS, it should be noted that the modeled data closely matched with the experimental results, indicating that most of the potential total hydrogen production occurred within the first three days of fermentation.

The evolution of organic acids at different TS concentrations is shown in Fig. 6. A clear tendency for lactate accumulation was observed during the first 1–2 days of fermentation, while lactate was consumed thereafter. The maximum lactate concentrations in the fermentation broth were higher as the initial TS content increased. Thus, the highest lactate titer of 18.14 g/L was found at 12.5 % TS, while the lowest concentration (3.83 g/L) was observed at 5 % initial TS content. It is worth noting that higher OFMSW loads induced greater lactate productions, which were translated into higher hydrogen production. The formation of butyrate and acetate accompanied by lactate consumption and hydrogen production were also observed regardless of the TS content. The maximum concentrations of acetate and butyrate in the fermentation broth were 5.2 g/L at 10 % TS and 9.9 g/L at 12.5 % TS, respectively. Other organic acids such as formate and propionate also

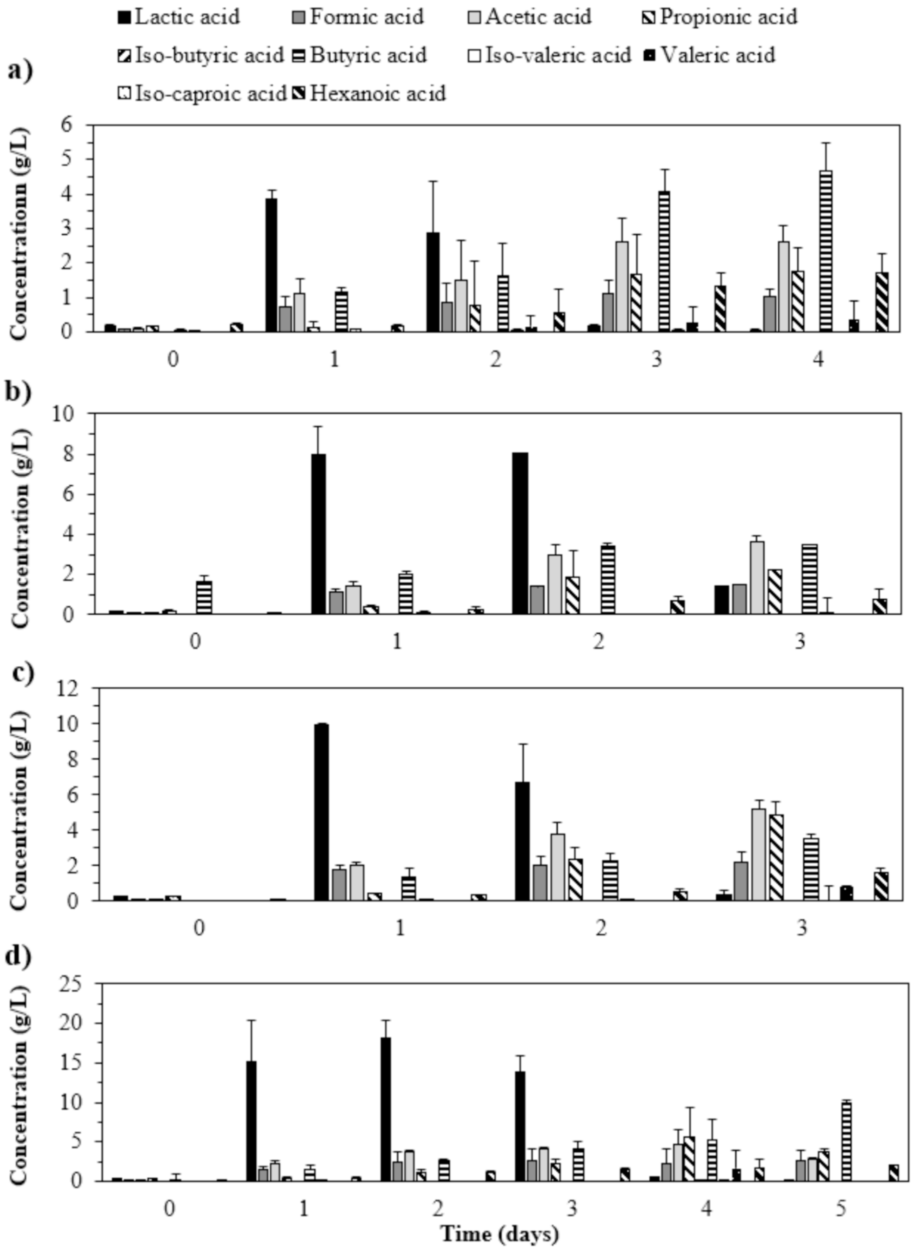

Fig. 6. Time course of organic acids recorded during the LD-DF of OFMSW-L3 at total solids loading (% *w/w*) of 5.0 (a), 7.5 (b), 10.0 (c), and 12.5 (d).

Table 4
Comparison of the LD-DF performance for OFMSW-L3 at different initial total solids (TS) contents.

Initial TS content (% *w/w*)	Cumulative biogas (NmL/L)	Peak H_2 content (%)	Cumulative H_2 (NmL/L)	HY (NmL/g COD added)	HY (NmL/g VS added)
5.0	9263.8 ± 700.9	34.7 ± 1.0	2272.4 ± 440.4	73.8 ± 14.3	55.1 ± 10.7
7.5	10171.6 ± 1683.0	41.8 ± 1.0	3456.2 ± 65.1	74.9 ± 1.4	55.9 ± 1.1
10.0	13399.7 ± 1271.3	37.9 ± 1.6	4068.4 ± 232.6	66.1 ± 3.8	49.3 ± 2.8
12.5	17115.3 ± 2961.6	30.1 ± 3.3	4216.9 ± 682.7	54.8 ± 8.9	40.9 ± 6.6

HY: Hydrogen yield.

appeared at lower concentrations, similar to what was observed in the pH tests. The use of a specialized microbial consortium capable of facilitating LD-DF, coupled with the observed behavior of lactate broth concentration throughout the fermentation, provides strong evidence to support the validation of the LD-DF pathway during the TS assessment test. The results obtained showed that the use a specialized microbial consortium capable of performing LD-DF effectively manages both the inherent heterogeneity of OFMSW and high TS concentrations of up to 12.5 %. The high reproducibility of the hydrogen production pathway observed here, despite the use of different OFMSW batches with different characteristics, is of paramount importance in the field of DF. In the future, it is imperative that further studies deepen the understanding of the microbiological aspects of the process and investigate optimization strategies, such as the implementation of two-stage LD-DF and the addition of enhancing agents such as magnetic nanoparticles.

4. Conclusions

This study evaluated mixed sewage sludge, OFMSW leachates, and OFMSW as potential substrates for hydrogen production via LD-DF. OFMSW emerged as the most competitive substrate due to its high fermentable carbohydrate content and low pre-acidification level. pH mainly influenced the LD-DF of OFMSW, with the process accommodating high TS contents up to 12.5 %. Optimal HY (55.9 ± 1.1 NmL H_2/g VS added) was achieved at pH 6.5 and 7.5 % TS with OFMSW-L3. Lactate served as a key cross-feeding metabolite for hydrogen production. Overall, LD-DF proved to be an effective fermentation pathway for hydrogen production using real and heterogeneous OFMSW.

CRediT authorship contribution statement

Cristina Martínez-Fraile: Writing – original draft, Methodology, Investigation. **Raúl Muñoz:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization. **María Teresa Simorte:** Writing – review & editing, Conceptualization. **Inmaculada Sanz:** Writing – review & editing, Conceptualization. **Octavio García-Depraect:** Writing – review & editing, Validation, Supervision, Methodology, Funding acquisition, Formal analysis, Conceptualization.

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 data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2024.130846.

References

Apha, 2005. Standard methods for the examination of water and wastewater, 20th ed. American Public Health Association/American Water Works Association/Water Environment Federation, Washington, DC.

Asunis, F., De Gioannis, G., Isipato, M., Muntoni, A., Polettini, A., Pomi, R., Rossi, A., Spiga, D., 2019. Control of fermentation duration and pH to orient biochemicals and biofuels production from cheese whey. Bioresour. Technol. 289, 121722.

Bernal, A.P., de Menezes, C.A., Silva, E.L., 2021. A new side-looking at the dark fermentation of sugarcane vinasse: Improving the carboxylates production in mesophilic EGSB by selection of the hydraulic retention time and substrate concentration. Int. J. Hydrogen Energy 46, 12758–12770.

Blanco, V.M.C., Oliveira, G.H.D., Zaiat, M., 2019. Dark fermentative biohydrogen production from synthetic cheese whey in an anaerobic structured-bed reactor: Performance evaluation and kinetic modeling. Renew. Energy 139, 1310–1319.

Cui, P., Wang, S., Su, H., 2022. Enhanced biohydrogen production of anaerobic fermentation by the Fe3O4 modified mycelial pellets-based anaerobic granular sludge. Bioresour. Technol. 366, 128144.

Delavar, M.A., Wang, J., 2021. Numerical investigation of pH control on dark fermentation and hydrogen production in a microbioreactor. Fuel 292, 120355.

Detman, A., Mielecki, D., Chojnacka, A., Salamon, A., Błaszczyk, M.K., Sikora, A., 2019. Cell factories converting lactate and acetate to butyrate: Clostridium butyricum and microbial communities from dark fermentation bioreactors. Microb. Cell Fact. 18, 36.

Detman, A., Laubitz, D., Chojnacka, A., Kiela, P.R., Salamon, A., Barberán, A., Chen, Y., Yang, F., Błaszczyk, M.K., Sikora, A., 2021. Dynamics of dark fermentation microbial communities in the light of lactate and butyrate production. Microbiome 9, 158.

Diaz-Cruces, V.F., García-Depraect, O., León-Becerril, E., 2020. Effect of lactate fermentation type on the biochemical methane potential of tequila vinasse. Bioenerg. Res. 13, 571–580.

Diez-Gonzalez, F., Russell, J.B., Hunter, J.B., 1995. The role of an NAD-independent lactate dehydrogenase and acetate in the utilization of lactate by Clostridium acetobutylicum strain P262. Arch. Microbiol. 164, 36–42.

Dubois, M., Gilles, K., Hamilton, J.K., Rebers, P.A., Smith, F., 1951. A colorimetric method for the determination of sugars. Nature 168, 167.

EBA, 2023. Biohydrogen: affordable green and yet overlooked. European Biogas Association, Brussels. Available at: https://www.europeanbiogas.eu/biohydrogen-affordable-green-and-yet-overlooked/. (accessed on 23 January 2024).

Elbeshbishy, E., Dhar, B.R., Nakhla, G., Lee, H.-S., 2017. A critical review on inhibition of dark biohydrogen fermentation. Renew. Sustain. Energy Rev. 79, 656–668.

European Commission, 2020. A hydrogen strategy for a climate-neutral Europe. Available at: https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A52020DC0301 (accessed on 16 December 2023).

Fuentes-Santiago, V., Valdez-Vazquez, I., Vital-Jácome, M., Zavala-Méndez, M., Razo-Flores, E., Carrillo-Reyes, J., 2023. Carbohydrates/acid ratios drives microbial communities and metabolic pathways during biohydrogen production from fermented agro-industrial wastewater. J. Environ. Chem. Eng. 11 (3), 110302.

Fuess, L.T., Ferraz, A.D.N., Machado, C.B., Zaiat, M., 2018. Temporal dynamics and metabolic correlation between lactate-producing and hydrogen-producing bacteria in sugarcane vinasse dark fermentation: The key role of lactate. Bioresour. Technol. 247, 426–433.

García-Depraect, O., Rene, E.R., Diaz-Cruces, V.F., León-Becerril, E., 2019. Effect of process parameters on enhanced biohydrogen production from tequila vinasse via the lactate-acetate pathway. Bioresour. Technol. 273, 618–626.

García-Depraect, O., Castro-Muñoz, R., Muñoz, R., Rene, E.R., León-Becerril, E., Valdez-Vazquez, I., Kumar, G., Reyes-Alvarado, L.C., Martínez-Mendoza, L.J., Carrillo-Reyes, J., Buitrón, G., 2021. A review on the factors influencing biohydrogen production from lactate: The key to unlocking enhanced dark fermentative processes. Bioresour. Technol. 324, 124595.

García-Depraect, O., Martínez-Mendoza, L.J., Diaz, I., Muñoz, R., 2022. Two-stage anaerobic digestion of food waste: Enhanced bioenergy production rate by steering lactate-type fermentation during hydrolysis-acidogenesis. Bioresour. Technol. 358, 127358.

Ghimire, A., Frunzo, L., Pirozzi, F., Trably, E., Escudie, R., Lens, P.N.L., Esposito, G., 2015. A review on dark fermentative biohydrogen production from organic biomass: Process parameters and use of by-products. Appl. Energy 144, 73–95.

Ghimire, A., Trably, E., Frunzo, L., Pirozzi, F., Lens, P.N.L., Esposito, G., Cazier, E.A., Escudié, R., 2018. Effect of total solids content on biohydrogen production and lactic acid accumulation during dark fermentation of organic waste biomass. Bioresour. Technol. 248, 180–186.

Gökçek, Ö.B., Baş, F., Muratçobanoğlu, H., Demirel, S., 2023. Investigation of the effects of magnetite addition on biohydrogen production from apple pulp waste. Fuel 339, 127475.

Gomez-Romero, J., Gonzalez-Garcia, A., Chairez, I., Torres, L., García-Peña, E.I., 2014. Selective adaptation of an anaerobic microbial community: Biohydrogen production by co-digestion of cheese whey and vegetables fruit waste. Int. J. Hydrogen Energy 39, 12541–12550.

Jung, J.-H., Sim, Y.-B., Park, J.-H., Pandey, A., Kim, S.-H., 2021. Novel dynamic membrane, metabolic flux balance and PICRUSt analysis for high-rate biohydrogen production at various substrate concentrations. Chem. Eng. J. 420, 127685.

Kim, D.-H., Yoon, J.-J., Kim, S.-H., Park, J.-H., 2021. Effect of conductive material for overcoming inhibitory conditions derived from red algae-based substrate on biohydrogen production. Fuel 285, 119059.

Kim, D.-H., Yoon, J.-J., Kim, S.-H., Park, J.-H., 2022. Acceleration of lactate-utilizing pathway for enhancing biohydrogen production by magnetite supplementation in Clostridium butyricum. Bioresour. Technol. 359, 127448.

Krishnan, S., Kamyab, H., Nasrullah, M., Wahid, Z.A., Yadav, K.K., Reungsang, A., Chaiprapat, S., 2023. Recent advances in process improvement of dark fermentative hydrogen production through metabolic engineering strategies. Fuel 343, 127980.

Li, C., Fang, H.H.P., 2007. Fermentative hydrogen production from wastewater and solid wastes by mixed cultures. Crit. Rev. Environ. Sci. and Technol. 37, 1–39.

Liu, Q., Zhang, X., Yu, L., Zhao, A., Tai, J., Liu, J., Qian, G., Xu, Z.P., 2011. Fermentative hydrogen production from fresh leachate in batch and continuous bioreactors. Bioresour. Technol. 102, 5411–5417.

Martínez-Mendoza, L.J., Lebrero, R., Muñoz, R., García-Depraect, O., 2022. Influence of key operational parameters on biohydrogen production from fruit and vegetable waste via lactate-driven dark fermentation. Bioresour. Technol. 364, 128070.

Matsumoto, M., Nishimura, Y., 2007. Hydrogen production by fermentation using acetic acid and lactic acid. J. Biosci. and Bioeng. 103 (3), 236–241.

Munier, E., Licandro, H., Beuvier, E., Cachon, R., 2023. Bioinformatics and metabolic flux analysis highlight a new mechanism involved in lactate oxidation in Clostridium tyrobutyricum. Int Microbiol. 26 (3), 501–511.

Nie, E., He, P., Zou, J., Zhang, H., Lü, F., 2022. Neglected effect of transportation on the property of municipal biowaste and the subsequent biomethane potential. J. Clean. Prod. 352, 131603.

Noblecourt, A., Christophe, G., Larroche, C., Fontanille, P., 2018. Hydrogen production by dark fermentation from pre-fermented depackaging food wastes. Bioresour. Technol. 247, 864–870.

Ohnishi, A., Hasegawa, Y., Fujimoto, N., Suzuki, M., 2022. Biohydrogen production by mixed culture of Megasphaera elsdenii with lactic acid bacteria as lactate-driven dark fermentation. Bioresour. Technol. 343, 126076.

Park, J.-H., Kim, D.-H., Kim, H.-S., Wells, G.F., Park, H.-D., 2019. Granular activated carbon supplementation alters the metabolic flux of Clostridium butyricum for enhanced biohydrogen production. Bioresour. Technol. 281, 318–325.

Park, J.-H., Kim, D.-H., Baik, J.-H., Park, J.-H., Yoon, J.-J., Lee, C.-Y., Kim, S.-H., 2021. Improvement in H2 production from Clostridium butyricum by co-culture with Sporolactobacillus vineae. Fuel 285, 119051.

Pérez-Rangel, M., Barboza-Corona, J.E., Navarro-Díaz, M., Escalante, A.E., Valdez-Vazquez, I., 2021. The duo Clostridium and Lactobacillus linked to hydrogen production from a lignocellulosic substrate. Water Sci. Technol. 83, 3033–3040.

Pugazhendhi, A., Anburajan, P., Park, J.-H., Kumar, G., Sivagurunathan, P., Kim, S.-H., 2017. Process performance of biohydrogen production using glucose at various HRTs and assessment of microbial dynamics variation via q-PCR. Int. J. Hydrogen Energy 42, 27550–27557.

Qi, C., Huang, J., Wang, B., Deng, S., Wang, Y., Yu, G., 2018. Contaminants of emerging concern in landfill leachate in China: A review. Emerg. Contam. 4, 1–10.

Rasmey, A.-H.-M., Abd-Alla, M.H., Tawfik, M.A., Bashandy, S.R., Salah, M., Liu, R., Sun, C., Hassan, E.A., 2023. Synergistic strategy for the enhancement of biohydrogen production from molasses through coculture of Lactobacillus brevis and Clostridium saccharobutylicum. Int. J. Hydrogen Energy 48 (2).

Regueira-Marcos, L., García-Depraect, O., Muñoz, R., 2023. Elucidating the role of pH and total solids content in the co-production of biohydrogen and carboxylic acids from food waste via lactate-driven dark fermentation. Fuel 338, 127238.

Sailer, G., Eichermüller, J., Poetsch, J., Paczkowski, S., Pelz, S., Oechsner, H., Müller, J., 2021. Characterization of the separately collected organic fraction of municipal solid waste (OFMSW) from rural and urban districts for a one-year period in Germany. Waste Manag. 131, 471–482.

Salazar-Batres, K.J., Quijano, G., Moreno-Andrade, I., 2022. Biohydrogen from the Organic Fraction of Municipal Solid Waste. In: Kuddus, M., Yunus, G., Ramteke, P. W., Molina, G. (Eds.), Organic Waste to Biohydrogen, Clean Energy Production Technologies. Springer Nature, Singapore, pp. 1–30.

Sivagurunathan, P., Kuppam, C., Mudhoo, A., Saratale, G.D., Kadier, A., Zhen, G., Chatellard, L., Trably, E., Kumar, G., 2018. A comprehensive review on two-stage integrative schemes for the valorization of dark fermentative effluents. Crit. Rev. Biotechnol. 38 (6), 868–882.

Sivagurunathan, P., Sahoo, P.C., Kumar, M., Prakash Gupta, R., Bhattacharyya, D., Ramakumar, S.S.V., 2023. Unrevealing the role of metal oxide nanoparticles on biohydrogen production by Lactobacillus delbrueckii. Bioresour. Technol. 367, 128260.

Tao, Y., Hu, X., Zhu, X., Jin, H., Xu, Z., Tang, Q., Li, X., 2016. Production of butyrate from lactate by a newly isolated Clostridium sp. BPY5. Appl. Biochem. Biotechnol. 179 (3), 361–374.

Wang, B., Wan, W., Wang, J., 2008. Inhibitory effect of ethanol, acetic acid, propionic acid and butyric acid on fermentative hydrogen production. Int. J. Hydrogen Energ. 33 (23), 7013–7019.

Yang, G., Wang, J., 2017. Fermentative hydrogen production from sewage sludge. Crit. Rev. Env. Sci. Tec. 47, 1219–1281.