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BioH$_2$ from Dark Fermentation of OFMSW: Effect of the Hydraulic Retention Time and Organic Loading Rate

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Featured Application: This work provides important insights into the effect of the hydraulic retention time (HRT) and organic loading rate (OLR) in the production of bioH$_2$ by the dark fermentation (DF) of the organic fraction of municipal solid wastes (OFMSW). The experimental data reported in this work are useful in the design and optimization of full-scale DF bioreactors used in the dark fermentation of OFMSW for bioH$_2$ production.

Abstract: Food wastes represent one third of all food produced worldwide. It is crucial to both prevent the production of food waste and recover the wasted fraction with the aim to valorizing it. In this context, the conversion of the organic fraction of municipal solid wastes (OFMSW) into bioH$_2$ by dark fermentation (DF) is an important technology to valorize these wastes into renewable fuel. Nevertheless, the DF of OFMSW needs to be optimized for critical operational parameters. The main purposes of this study were to investigate (i) the effect of HRT during continuous bioH$_2$ production through DF and (ii) the effect of organic loading rate (OLR) ruled by HRT. In this work, three HRTs (4, 5, and 6 d) were tested in a mesophilic continuous stirred-tank reactor (CSTR). The HRTs of 4, 5, and 6 days, corresponding to OLRs of 23.6, 18.0, and 10.6 g volatile solids (VS)·L^{-1}·d^{-1}, respectively, showed bioH$_2$ yields of 8.48, 18.2, and 1.64 L·kg^{-1} VS$_{influent}$ with an H$_2$ content of approximately 25, 32, and 5% *v*/*v*, respectively. An accumulation of volatile fatty acids (VFAs) was registered with the decrease in HRT, causing a decrease in bioH$_2$ production. The 5 d HRT was the most favorable condition.

Keywords: bioH$_2$; dark fermentation; hydraulic retention time; microbial consortium; organic fraction of municipal solid wastes; organic loading rate

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1. Introduction

According to the Food and Agriculture Organization (FAO), an estimated one third of all food produced worldwide is lost or wasted every year [1]. Food loss and food waste represent an amount of approximately 1.3 billion tons of food resources wasted per year [2,3] throughout the food chain. Despite the need for more sustainable food management systems [4–6], food waste is always expected to be produced, at least at both the distribution and consumption levels. Food wastes are a major fraction of municipal solid wastes (MSWs). Since they are composed of different organic wastes, they are referred to as the organic fraction of municipal solid wastes (OFMSW) [7].

Currently, the OFMSW is mainly landfilled, thermally treated with heat recovery, composted, or anaerobically digested in high- to medium-income countries. In low-income countries, this fraction is dumped with no significant environmental control [8]. In Europe, the most effective and sustainable valorization routes for OFMSW are being pursued as the

European Directive 2018/851 [9] will impose the segregated collection of biowastes from 31 December 2023 onwards with the aim of valorizing them.

Biohydrogen (bioH_2) (H_2 produced from biological processes) is a renewable, fossil carbon-free fuel and has received considerable attention as an alternative energy carrier to fossil fuels in recent years. Effectively, H_2 is the fuel with the highest energy content per mass unit of all known fuels (142 MJ.kg^{-1}). The only final by-product of its combustion is water, which can be collected and further used, and consequently, no greenhouse gases (GHGs) are released. H_2 can be directly burnt in internal combustion engines or in fuel cells to produce different types of energy [10]. The major challenges related to the use of H_2 lie in its sustainable production and safety storage [11,12]. Currently, in industrial applications, H_2 is mainly being produced from fossil fuels and through highly energy-intensive processes, rendering it less attractive from an environmental point of view [13]. However, H_2 can be also obtained from sustainable technologies such as water electrolysis, the deployment of which is taking leverage around the world, and biological processes which need further study for sustainable up-grading. These technologies will generate a cleaner and more sustainable fuel (H_2) and avoid or minimize GHG emissions [14,15]. To date, the production rate, stability, and efficiency of H_2 from biological sources must be improved to make it commercially viable [16].

Dark fermentation (DF) has been proposed as a promising process to produce bioH_2 owing to its low chemical energy demand and more environmentally friendly performance when compared to thermochemical processes. During DF, carbohydrate-rich substrates are anaerobically fermented, in the absence of light by a consortium of microorganisms or pure cultures [17]. Their metabolic activity causes the oxidation of the organic substrate, generating an excess of electrons. In the presence of the hydrogenase enzyme, the protons (H^+) present in the anaerobic environment work as electron acceptors and produce molecular hydrogen [18,19]. The OFMSW, generally constituted of food wastes with a high biodegradable fraction and approximately 85–95% *w*/*w* volatile solids (VS) and 75–85% *w*/*w* moisture content, is one of the organic feedstocks that can be used in DF [20]. The OFMSW is rich in proteins, lipids, complex polysaccharides, nitrogen, phosphorus, and metal ions that work as micronutrients. This organic waste is abundant and can be used as a superior feedstock for the fermentative processes of hydrogen-producing microorganisms [21].

The production of bioH_2 from OFMSW by DF still presents several bottlenecks that must be solved, such as the variability of the feedstock, homogeneity inside the bioreactor, pH control, temperature control, and the inhibition of H_2-consumers supplied through the feedstock, among other variables that may diminish the bioH_2 yield [21,22]. The optimal temperature and pH values for DF are usually defined in the same way as for biogas production; namely, mesophilic (35–38 °C) and thermophilic (50–55 °C) temperatures are indicated as adequate for bioH_2 production, and a neutral pH is referred to as the one promoting the highest bioH_2 yield [3,10]. The temperature range selected depends on the bioH_2 yield required and the heat available to warm up the bioreactor. Concerning the pH, a neutral pH is very difficult to use in bioreactors working with mixed populations, as it favors the development of H_2-consuming methanogens. Thus, an acidic pH is preferable when the bioreactor is operated with such a complex microbial consortium to inhibit the development of H_2-consuming methanogenic Archaea.

A relevant operational parameter that can be used to optimize the bioH_2 yield is the hydraulic retention time (HRT), which corresponds to the time under which the substrate is in contact with the fermentative microbial population. As the HRT governs the contact time between the fermentative population and the organic substrate, its change also affects the organic loading rate (OLR) applied to the bioreactor. The OLR quantifies the mass of organic feedstock daily applied per unit volume of bioreactor. Hence, as the HRT increases, the OLR decreases and *vice versa*. The optimal HRT and OLR values are dependent on the feedstock composition, its biodegradability, and the ability of the fermentative population to degrade it and grow inside the bioreactor.

In this context, the main aims of this study were to investigate (i) the effect of HRT on the bioH_2 yield obtained in the DF of OFMSW, in a lab-scale bioreactor, and consequently (ii) the effect of the OLR applied to the bioreactor when the HRT is changed. The innovation of the present work lies in the use of mixed anaerobic cultures and real OFMSW both obtained from an industrial-scale biogas plant, instead of pure cultures and a synthetic substrate as commonly reported in the literature.

2. Materials and Methods

2.1. Organic Feedstock and Its Origin

Samples from the influent of the hydrolysis tank of a full-scale Anaerobic Digestion (AD) plant, located in Lisbon (Portugal), were collected twice a month and used as the organic feedstock of the DF experiments. This full-scale AD processes the OFMSW from canteens, restaurants, malls, and households with a separative collection system of organic wastes. The OFMSW samples were stored at 4 °C in Schott glass bottles until their use in the DF experiments. This procedure diminished the biological degradation of the organic substrates present in the OFMSW and guaranteed an approximately constant composition.

2.2. Feedstock Characterization

The OFMSW was characterized by the following parameters: total solids (TS), volatile solids (VS), and fixed solids (FS) (method 2540) [23]; total chemical oxygen demand (tCOD) and soluble chemical oxygen demand (sCOD) (method 5220 B) [23]; total Kjeldahl nitrogen (TKN) (method ISO 5663:1984), ammonium nitrogen (N-NH_4^+) (method ISO 5664:1984), and organic nitrogen (o-N) (difference between TKN and N-NH_4^+); total phosphorus (tP) (method ISO 6878:2004). Elemental analysis (EA) comprised the quantification of CHNS, which was performed in a Thermo Finnigan Elemental Analyzer (CE Instruments, model Flash EA 1112 CHNS series). Volatile fatty acids (VFAs) comprised the quantification of the acetic acid (AA), propionic acid (PA), and butyric acid (BA), which were analyzed with an HPLC system (Dionex ICS3000, Sunnyvale, CA, USA) equipped with a Biorad Aminex 87H column, at 30 °C, and a UV detector at 210 nm. The eluent used was H_2SO_4 10 mN, with a flow rate of 0.6 mL.min^{-1}, at 30 °C.

The average characterization of the OFMSW is reported in Table 1.

Table 1. Characterization of the OFMSW (average ± standard deviation; n = 10 samples).

Parameters	Units	Values
pH	Sørensen	4.63 ± 0.04
TS	g.L^{-1}	94.3 ± 13
VS	g.L^{-1}	82.7 ± 13.4
FS	g.L^{-1}	11.4 ± 1.7
tCOD	g O_2.L^{-1}	113 ± 7
sCOD	g O_2.L^{-1}	67 ± 9
tP	g N.L^{-1}	2.9 ± 0.2
TKN	g N.L^{-1}	1.8 ± 0.1
H-NH_4^+	g N.L^{-1}	1.1 ± 0.3
o-N	g N.L^{-1}	525 ± 92
C	(% w/v)	10.3 ± 0.2
N	(% w/v)	7.89 ± 0.05
H	(% w/v)	0.69 ± 0.07
S	(% w/v)	<0.01
O [(1)]	(% w/v)	79.9
C/N	-	15.0
C/P	-	159.1

[(1)] Calculated by the difference: O = 100−(C + H + N + S + FS).

2.3. Dark Fermentation Assays

The DF assays were carried out in a 2.1 L lab-scale continuous-stirred tank reactor (CSTR) (BIOFLO 1000, New Brunswick Scientific/Eppendorf UK Ltd., Stevenage, UK), equipped with control systems for temperature (thermocouple from New Brunswick Scientific/Eppendorf UK Ltd., Stevenage, UK), pH (epoxy electrode with temperature compensation from Hanna Instruments HI8711E), and redox potential (epoxy redox electrode with a reference solution from Thermo Scientific, Orion 97–78). The stirring system was constituted by a shaft equipped with two blade systems, which was kept at a low speed (10 rpm) to prevent the occurrence of major stress in the bacteria consortium.

The DF assays were carried out under mesophilic conditions (37 ± 1 °C) and an average pH of 5.0. The electrical conductivity was measured in the anaerobic effluent of the bioreactor using a conductivity meter (Orion Star A215, Thermo Scientific, Thermo Fisher Scientific Inc., Indonesia). The biogas produced was stored in two acrylic cylindrical columns with maximum volumes of 3437 and 3288 mL. These columns were filled up with acidified deionized water (pH = 2.0) to reduce the solubilization of CO_2 in the water. The experimental setup is shown in Figure 1.

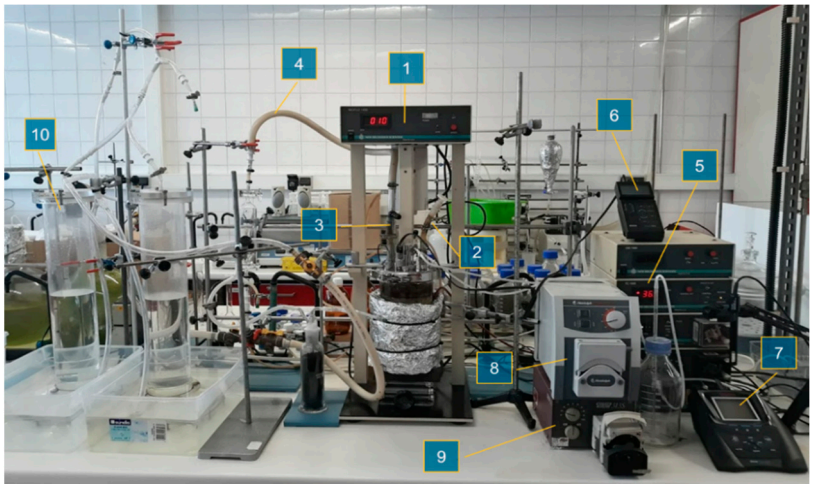

Figure 1. Experimental setup: (**1**) stirring system with an external engine and shaft; (**2**) feeding tube (influent); (**3**) outflow tube (effluent); (**4**) biogas outflow line; (**5**) temperature controlling system; (**6**) redox potential module; (**7**) pH and conductivity meter; (**8**) HCl pumping system; (**9**) influent pump; and (**10**) water columns for biogas storage.

According to Ghimire [19], an HRT between 2 and 6 d is appropriate for the DF of organic food wastes in completely mixed bioreactors. As such, the HRT of 4, 5, and 6 days were selected for the DF experimental assays. These assays were coded as HRT4, HRT5, and HRT6, respectively.

Throughout this work, the gas produced in the bioreactor is named "biogas" being mainly composed of H_2 and CO_2. The term "biogas" is more common for methanogenic bioreactors, in which the gas produced is composed of CH_4 and CO_2. In the absence of a better designation, the term "biogas" was adopted in this work, even if no CH_4 should be expected in the gas produced.

The bioreactor was inoculated with 700 mL of anaerobic sludge that was obtained from the effluent of the anaerobic tank of the aforementioned full-scale AD plant. The reason for using such high inoculum volume was to avoid a long lag phase due to biomass growth. The chemical characterization of the inoculum is shown in Table 2.

Table 2. Chemical characterization of the inoculum.

Parameters	Units	Values
Conductivity	$mS.cm^{-1}$	26.7
pH	Sørensen	8.3
TS	$g.L^{-1}$	23.1
VS	$g.L^{-1}$	13.8
Ashes	$g.L^{-1}$	9.3
tCOD	$g\ O_2.L^{-1}$	20.5
sCOD	$g\ O_2.L^{-1}$	13.6
TKN	$g.L^{-1}$	2.44
$N\text{-}NH_4^+$	$g.L^{-1}$	1.23
o-N	$g.L^{-1}$	1.21

After inoculation, the bioreactor was fed daily according to an impulse-feed mode with adequate volumes of OFMSW to accomplish the desired HRTs of 4, 5, and 6 days.

Figure 2 summarizes the experimental procedure followed in this work.

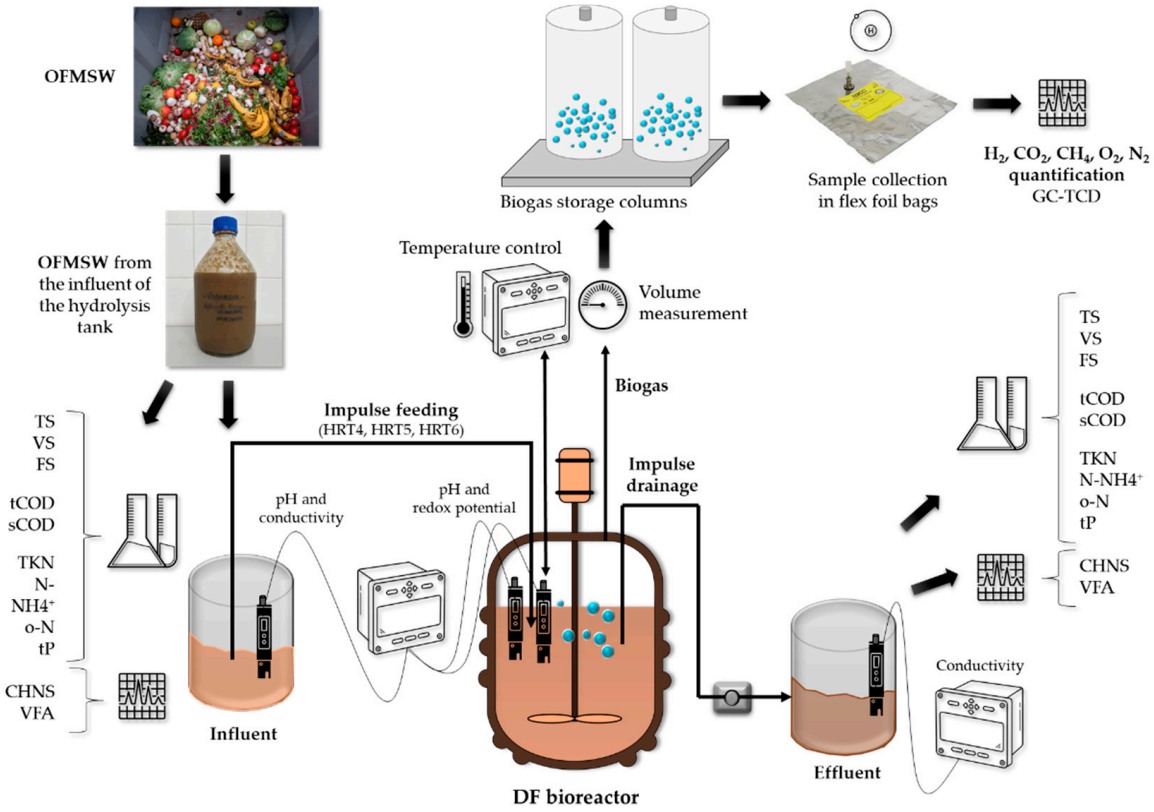

Figure 2. Synthesis of the experimental procedure.

2.4. Characterization of Biogas and Quantification of $BioH_2$

For each experiment with a different HRT and ORT, it was necessary to operate the bioreactor for 2 to 3 times the HRT before starting to collect data under steady-state conditions. The system was under steady-state conditions when the biogas production remained constant over 7 consecutive days.

As soon as the steady-state conditions has been reached, the volume of biogas accumulated throughout each experiment was measured by direct quantification of the gas collected in the two cylindrical storage columns. At the end of each assay, biogas samples were transferred to FlexFoil bags (SKC, 262) and analyzed by gas chromatography with a thermal conductivity detector (GC-TCD) for the following gases: H_2, CO_2, CH_4, O_2, and N_2. H_2 and CO_2 are the major components of DF biogas. CH_4 was analyzed to assess

the inhibition level of H_2-consuming methanogens. O_2 and N_2 were analyzed to assess the air-tightness level of the experimental setup, and in the case of N_2, the occurrence of denitrification inside the bioreactor was also assessed.

The accumulated volume of bioH_2 (Equation (1)) for each assay was calculated through the percentage of H_2, obtained by GC-TCD, and the accumulated biogas volume:

$$V_{bioH_2} = \frac{H_2 \times V_{biogas}}{100} \quad (1)$$

where V_{bioH_2} represents the accumulated bioH_2volume produced in each experimental trial (L), H_2 is the percentage of H_2 in the biogas (% v/v), and V_{biogas} is the accumulated biogas volume produced in each assay (L).

The bioH_2 production rate for each experimental trial was calculated by Equation (2):

$$F_{bioH_2} = \frac{V_{bioH_2}}{n} \quad (2)$$

where F_{bioH_2} is the bioH_2 production rate (L H_2.d^{-1}), n is the total days of bioreactor operation for each experimental trial (d), and V_{bioH_2} is as defined for Equation (1).

The volumetric $bioH_2$ production rate was calculated according to Equation (3):

$$VHP = \frac{F_{bioH_2}}{V_r} \quad (3)$$

where VHP is the volumetric bioH_2 production rate (L H_2.L$^{-1}_{\text{bioreactor}}$.d^{-1}), F_{bioH_2} is as defined for Equation (2), and V_r is the bioreactor working volume (L).

2.5. OLR, Removal Efficiencies, and BioH_2 Yield

The organic loading rate (OLR), expressed as TS, VS, or COD, was calculated by Equation (4):

$$OLR_{i,in} = \frac{c_{i,influent}}{V_r} \times F_{influent} \quad (4)$$

where $OLR_{i,in}$ is the OLR of the parameter i (TS, VS, or COD) applied to the bioreactor (g TS.L^{-1}.d^{-1} or g VS.L^{-1}.d^{-1} or g O_2.L^{-1}.d^{-1}), $c_{i,influent}$ is the concentration of the parameter i (TS, VS, or COD) (g TS.L^{-1}, g VS.L^{-1}, or g O_2.L^{-1}) in the influent, $F_{influent}$ is the daily volume of the influent (L.d^{-1}), and V_r is the bioreactor working volume (L).

The removal efficiencies of organic matter in the bioreactor, expressed as TS, VS, tCOD, sCOD, N, and P, were calculated according to Equation (5):

$$\eta_{i,removal} = \frac{c_{i,influent} - c_{i,effluent}}{c_{i,influent}} \times 100 \quad (5)$$

where $\eta_{i,removal}$ is the removal efficiencies of the parameter i (TS, VS, COD, N, or P) (%), $c_{i,influent}$ is the concentration of the parameter i (TS, VS, COD, N, or P) (g TS.L^{-1}, g VS.L^{-1}, g O_2.L^{-1}, mg N.L^{-1}, or mg P.L^{-1}) in the influent, and $c_{i,effluent}$ is the concentration of parameter i (TS, VS, COD, N, or P) (g TS.L^{-1}, g VS.L^{-1}, g O_2.L^{-1}, mg N.L^{-1}, or mg P.L^{-1}) in the effluent of the bioreactor. The 100 factor allows expressing the removal efficiency as a percentage.

The BioH_2 yield was calculated through Equation (6):

$$\eta_{bioH_2} = \frac{F_{bioH_2}}{c_{VS,influent} \times F_{influent}} \times 1000 \quad (6)$$

where η_{bioH_2} is the $bioH_2$ yield expressed as a function of the OLR for VS in the influent (L H_2.kg^{-1} VS_{influent}), F_{bioH_2} is the bioH_2 production rate (L H_2.d^{-1}), $c_{VS,influent}$ is the VS

concentration in the influent (g VS.L^{-1}), and $F_{influent}$ is the daily volume of the influent (L.d^{-1}). The 1000 factor allows the conversion of g into kg VS.

2.6. Calorific Value of Biogas

The calorific value of the produced biogas is calculated through Equation (7):

$$H_{u,act} = \frac{F_{bioH_2} \times \rho_{H_2} \times LHV_{H_2}}{c_{VS,influent} \times F_{influent}} \times 1000 \quad (7)$$

where $H_{u,act}$ is the calorific value of the produced biogas expressed as a function of the OLR for VS in the influent (kJ.kg^{-1} $VS_{influent}$) and excluding the moisture content, F_{bioH_2} is the bioH_2 production rate (L H_2.d^{-1}), ρ_{H_2} is the hydrogen density at standard temperature and pressure (8×10^{-5} kg.L^{-1}), LHV_{H_2} is the hydrogen lower heating value (120.7×10^3 kJ.kg^{-1}), $c_{VS,influent}$ is the concentration of VS in the influent of the bioreactor (g VS.L^{-1}), and $F_{influent}$ is the daily volume of the influent (L.d^{-1}). The 1000 factor allows the conversion of g into kg VS.

3. Results and Discussion

3.1. Operational Parameters

Figure 3 summarizes the operational parameters of the bioreactor.

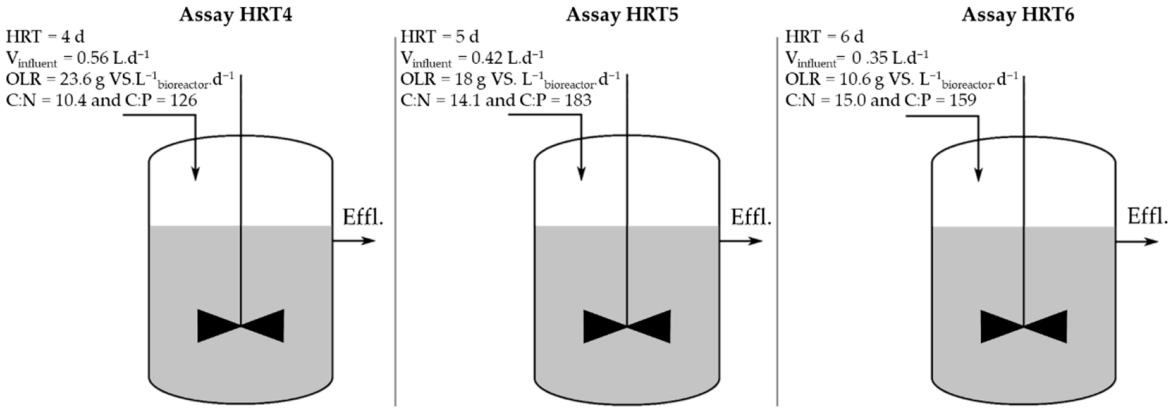

Figure 3. Global overview of the operational parameters in the HRT4, HRT5, and HRT6 assays.

The OLR applied to the bioreactor is defined as the ratio between the mass flow rate of biodegradable solids fed and the working volume of the bioreactor, expressed in g VS.L$^{-1}_{bioreactor}$.d^{-1}. Considering the fact that the volume of the bioreactor is constant, this parameter is dependent on the HRT and the concentration of VS present in the substrate, influencing the fermentation pathway, the substrate conversion efficiency, the composition of the microbial population, and consequently the production of bioH_2.

The change in HRT has a direct impact on OLR variation, namely as the decrease in HRT leads to an increase in OLR due to the increase in the daily mass of VS applied to the bioreactor. Increasing the VS load applied to the bioreactor is preferred for the energy efficiency of fermentation processes. However, excessively high VS loads can result in an unstable fermentation process due to the accumulation of VFAs and can lead to the inhibition in bioH_2 production.

3.2. Control Parameters

During DF assays, pH, temperature, redox potential, and conductivity were monitored as control parameters. Table 3 shows the average values of pH, redox potential, and conductivity during tests with different HRTs.

Table 3. Average values (n = 10 samples) of pH, redox potential, and conductivity in DF assays.

Parameters	Units	DF Assays		
		HRT4	**HRT5**	**HRT6**
HRT	d	4	5	6
$V_{bioreactor}$ (working volume)	L	2.1	2.1	2.1
$F_{influent}$	$L.d^{-1}$	0.53	0.42	0.35
$OLR_{TS,influent}$	$g\ TS.L^{-1}.d^{-1}$	25.9	20.6	12.6
$OLR_{VS,influent}$	$g\ VS.L^{-1}.d^{-1}$	23.6	18.0	10.6
Influent pH	Sørensen scale	4.7 ± 0.2	4.6 ± 0.2	4.6 ± 0.1
Effluent pH		5.0 ± 0.2	5.2 ± 0.1	5.1 ± 0.7
Influent conductivity	$mS.cm^{-1}$	21 ± 1	20 ± 2	18 ± 2
Effluent conductivity		25 ± 2	25 ± 1	22 ± 2
Redox potential inside the bioreactor	mV	-47 ± 38	-56 ± 26	-110 ± 12

The temperature in the bioreactor was kept constant at 37 ± 1 °C during all the assays. The maintenance of this parameter is crucial since the temperature at which DF occurs has a direct impact on the microbial metabolism and conversion efficiency of the substrate into $bioH_2$.

The pH provides information on the stability of the bioreactor as its variation depends on its buffer capacity. The variations in pH are related to the variations in the chemical species involved in the DF process, such as the accumulation of VFAs, ammonia, and CO_2. During the DF assays, the pH was adjusted to a value of 5 (if necessary, with the addition of 1 M HCl), to inhibit the activity of methanogenic hydrogenotrophic Archaea. It was observed that the pH remained relatively stable in all the assays at a pH value of approximately 5. The DF is considered stable for pH values between 4.5 and 5.5 since this is the pH interval in which the activity of hydrolytic and acidogenic bacteria is favored [24].

The redox potential is a significant parameter during DF as it causes interference in bacterial physiology and in processes as important as maintaining the pH of the interior of cells and the transmembrane potential [25]. Both the HRT4 and HRT5 assays showed higher values of -47 ± 38 and -56 ± 26 mV, respectively, compared to the value of -110 ± 12 mV in the HRT6 assay.

There are a limited number of bibliographic references in the literature on adequate redox potential for $bioH_2$ production. Some of them even express redox potential in terms of pH values [26], which makes the redox conditions more difficult to interpret. In any case, it is known that acidogenic bacteria participating in DF can produce $bioH_2$ under the same redox conditions as methanogens (between −200 and −350 mV) [27]. As shown in Table 3, only relatively low reduction conditions have been achieved in the DF assays as compared to [27], but $bioH_2$ was produced as will subsequently be shown in this work.

Concerning the electrical conductivity, relatively stable values of 25 ± 2, 25 ± 1, and $22 \pm 2\ mS.cm^{-1}$ were registered for the HRT4, HRT5, and HRT6 assays, respectively, which do not indicate an increase in the salt content inside the bioreactor.

3.3. Removal Efficiencies of Solids and COD

The characterization of the influent and effluent has the immediate purpose of analyzing the removal efficiency of the organic matter in the bioreactor, which can then be related to the conversion efficiency into $bioH_2$.

3.3.1. Total and Volatile Solids

Figures 4 and 5 show the average concentrations of TS and VS, respectively, in the influent and effluent of the bioreactor, as well as their removal efficiencies.

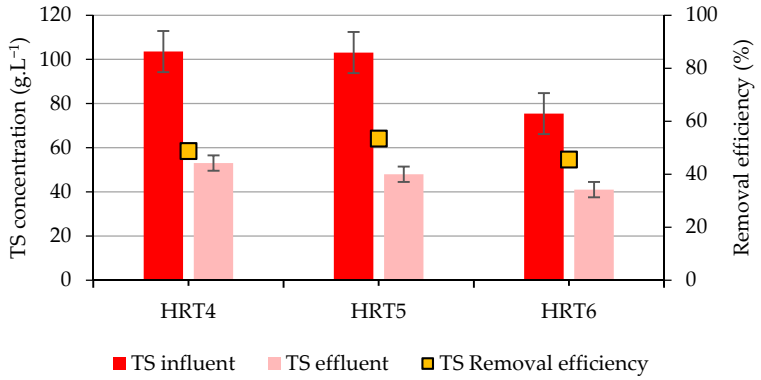

Figure 4. Average concentrations (n = 3 samples per HRT) of total solids in the influent (TS influent) and effluent (TS effluent) of the bioreactor, and respective removal efficiencies (error bars: standard deviation) (concentrations: on the left y axis; removal efficiencies: on the right y axis).

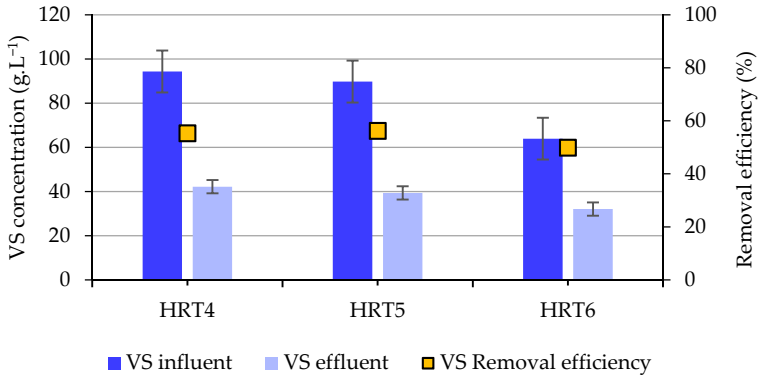

Figure 5. Average concentrations (n = 3 samples per HRT) of volatile solids in the influent (VS influent) and effluent (VS effluent) of the bioreactor, and respective removal efficiencies (error bars: standard deviation) (concentrations: on the left y axis; removal efficiencies: on the right y axis).

In the HRT4 and HRT5 assays, the TS concentrations in the influent were similar, corresponding to values of 104 and 103 g TS.L^{-1}, respectively. The HRT6 assay showed an average concentration in the influent of 75.5 g TS.L^{-1}, which was significantly lower than for the other assays. In turn, the average concentrations of VS in the influent during the HRT4, HRT5, and HRT6 were 94.4, 89.8, and 63.9 g VS.L^{-1}, respectively. The HRT5 assay achieved TS and VS removal efficiencies (53.5 and 56.2%, respectively) higher than those obtained for HRT4 (48.8 and 55.3%, respectively) and HRT6 (45.6 and 49.9%, respectively).

According to Algapani et al. [28], during the DF of OFMSW under thermophilic conditions, the removal efficiencies of TS and VS of 29.5 and 38.0%, respectively, were recorded with an HRT of 5 d and an OLR of 16.3 g VS.L^{-1}.d^{-1}, which demonstrates the better performance of the anaerobic consortium tested in the present work on the degradation of TS and VS. This is of particular relevance because these authors tested their biological system under thermophilic conditions which should theoretically present higher degradation rates of solids than in mesophilic systems, as tested in the present work.

In another study by Paudel et al. [29], the DF of OFMSW was tested under mesophilic conditions with an HRT of 2 d and an OLR of 17.6 g VS.L^{-1}.d^{-1}. A VS removal efficiency of 29.06 ± 2.76% was achieved. The removal efficiency of VS, with an HRT of 5 d, obtained in the present work, was again higher than the value reported by these authors.

Only the values reported by Redondas et al. [30] are similar to the removal efficiencies obtained in the present work. These authors simulated food wastes by using a synthetic substrate under thermophilic conditions with an OLR of 11.4 g VS.L^{-1}.d^{-1} and an HRT of 2 d. They reported a VS removal efficiency of 53.2 ± 6.5%.

Globally, the removal efficiencies of TS and VS registered for all three HRTs tested in this work were higher than the values reported in the literature, which can be attributed to the higher biodegradability of the OFMSW tested in the present work when compared to the synthetic organic substrates generically used in the previously published papers.

3.3.2. tCOD and sCOD

Figures 6 and 7 show the average concentrations and removal efficiencies of tCOD and sCOD in the influent and effluent of the bioreactor, for each tested HRT.

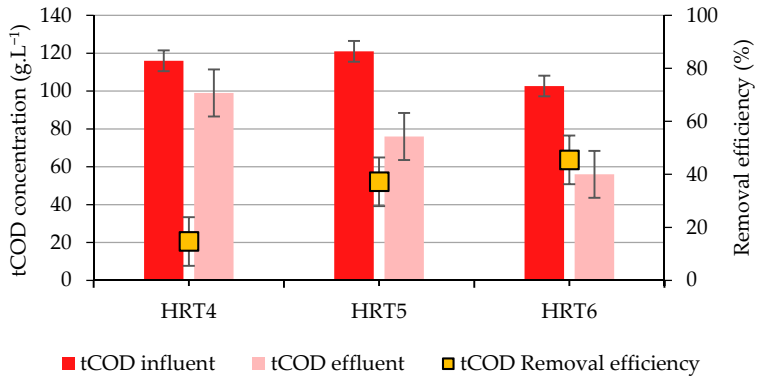

Figure 6. Average concentrations (n = 3 samples per HRT) of total COD in the influent (tCOD influent) and effluent (tCOD effluent) of the bioreactor, and respective removal efficiencies (error bars: standard deviation) (concentrations: on the left y axis; removal efficiencies: on the right y axis).

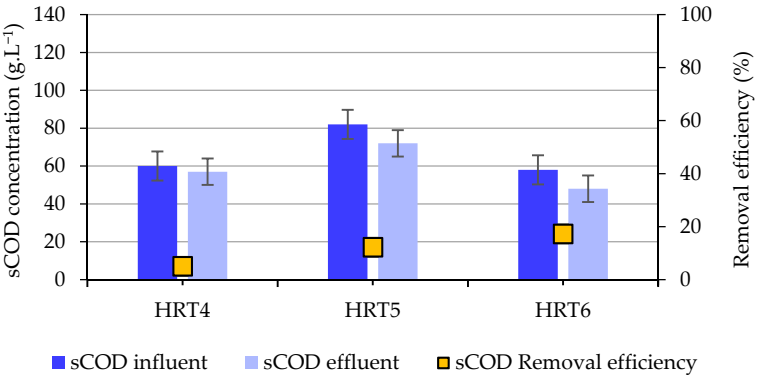

Figure 7. Average concentrations (n = 3 samples per HRT) of soluble COD in the influent (sCOD influent) and effluent (sCOD effluent) of the bioreactor, and respective removal efficiencies (error bars: standard deviation) (concentrations: on the left y axis; removal efficiencies: on the right y axis).

The HRT6 assay showed the highest removal efficiencies of tCOD and sCOD with values of 45.5% and 17.2%, respectively. The highest removal efficiencies registered for tCOD rather than for sCOD suggest that one significant part of the organic matter used by the DF consortium was present in the OFMSW in the form of particulate matter. The tCOD and sCOD removal efficiencies increased with the increase in HRT, which means that lower COD loads were more beneficial for the COD removal.

According to Algapani et al. [28], a removal rate of 12.86% was obtained during the DF of OFMSW, with an HRT of 5 d, at a pH of 5.5, and under thermophilic conditions. This value is much lower than that recorded in the present study under similar operational conditions (37.2% for an HRT of 5 d and pH of 5.2).

3.3.3. Nitrogen and Phosphorus

Table 4 shows the concentrations of TKN, N-NH_4^+, and o-N in the influent and effluent of the bioreactor, for each experimental assay.

Table 4. Concentrations of TKN, N-NH_4^+, o-N, and relative proportions of N-NH_4^+, o-N in the influent and effluent of the DF bioreactor for all experimental assays.

Assay	Sampling Point	TKN (mg N.L^{-1})	N-NH_4^+ (mg N.L^{-1})	o-N (mg N.L^{-1})	N-NH_4^+ (% TKN)	o-N (% TKN)
HRT4	Influent	2844	1969	875	69	31
	Effluent	2592	2406	186	93	7
HRT5	Influent	3017	1663	1354	55	45
	Effluent	2249	2129	120	95	5
HRT6	Influent	3134	1662	1472	53	47
	Effluent	2337	2159	177	92	8

The used influent showed high concentrations of TKN with an average concentration of 2998 mg N.L^{-1}. Compared with the literature for similar organic substrates [29,31,32], this can be considered an organic substrate containing a high concentration of TKN. High levels of TKN were also found in the effluent of all assays, revealing the intensive hydrolysis of proteins during the DF [32]. To support this observation, the organic nitrogen (o-N) showed a significant degradation in all assays, contributing to the increase in N-NH_4^+ from the influent to the effluent of the bioreactor.

Salerno et al. [33] investigated the mechanisms of DF inhibition by N-NH_4^+, using glucose as the organic substrate, under continuous and batch conditions. In the batch tests, the bioH_2 production rate was highly dependent on the medium pH and the presence of N-NH_4^+ higher than 2000 mg N.L^{-1}. At pH 6.2 and with an initial N-NH_4^+ concentration of 2000 mg N.L^{-1}, the bioH_2 production rate decreased from 56 to 16 mL.h^{-1} (71.4% decrease rate) and the N-NH_4^+ registered an increase to a concentration of 10000 mg N.L^{-1} during the DF process. In turn, at pH 5.2, the bioH_2 production rate decreased from 49 to 7 mL.h^{-1} (85.7% decrease rate) with a variation of N-NH_4^+ from 2000 to 16000 mg N.L^{-1}.

Therefore, the concentration of N-NH_4^+ of 2000 mg N.L^{-1} can adversely affect the performance of DF, especially under batch conditions and in an acidic medium.

Similar conclusions were registered by these authors [33] in the study developed with a continuously operated bioreactor. The bioreactor (V = 2 L) was initially acclimated to 800 mg N.L^{-1} of N-NH_4^+. BioH_2 production and yield were 170 mL.h^{-1} and 1.9 mol H_2.mol^{-1} glucose, respectively, for this N-NH_4^+ concentration, having decreased to 105 mL.h^{-1} and 1.1 mol H_2.mol^{-1} glucose (decrease rates of 38.2% and 42.1% for bioH_2 production and yield, respectively) with the increase in N-NH_4^+ to 7800 mg N.L^{-1}. Even if the DF consortium became less sensitive to N-NH_4^+ under continuous mode than in batch mode, the authors concluded that the production of bioH_2 was affected by high concentrations of this nitrogen species. In any case, it is important to notice that Salerno et al. [33] registered bioH_2 production for a concentration of N-NH_4^+ as high as 7800 mg N.L^{-1} which was 4.4-times higher than the average concentration determined in the influent of the present study (N-NH_4^+: 1765 mg N.L^{-1}) and 3.5-times higher than the average concentration determined in the effluent of the bioreactor for the three experimental assays (N-NH_4^+: 2231 mg N.L^{-1}). Thus, it is supposed that N-NH_4^+ did not work as an inhibitor during the DF experiments for all the tested HRTs in the present work.

Figure 8 shows the average concentrations and removal rates of total phosphorus in the bioreactor for each tested HRT.

In a completely mixed bioreactor, a significant removal rate of total phosphorus is theoretically not supposed to be registered as this nutrient is submitted to the conversion between the liquid phase to the solid phase and *vice versa*, and from inorganic species to organic species and vice versa; however, the total phosphorus remains globally constant [34]. In this context, the removal rates registered in this work may be explained by the retention of biomass inside the bioreactor due to the low mixing rate. The retention of solids occurs not only as biomass attached to the physical parts of the bioreactor (biofilms) but also in the upper part of the liquid (floating material and scums). The presence of solids in the floating material and scums turned the collection of homogeneous samples in the effluent a

big challenge. Additionally, they worked as another compartment of solid material for the retention of phosphorus inside the bioreactor. No values were found in the literature on total phosphorus with which the data obtained in the present work could be compared.

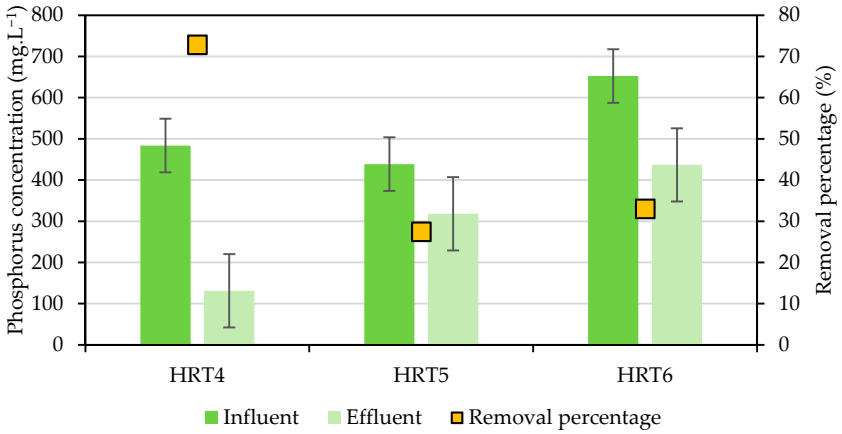

Figure 8. Average concentrations (n = 3 samples per HRT) of total phosphorus in the influent (tP influent) and effluent (tP effluent) of the bioreactor, and the respective removal rates (error bars: standard deviation) (concentrations: on the left y axis; removal percentages: on the right y axis).

3.4. Volatile Fatty Acids

Figure 9 shows the concentrations of VFAs, namely acetic acid, propionic acid, and butyric acid, in the influent and effluent of the bioreactor, for each of the tested HRTs.

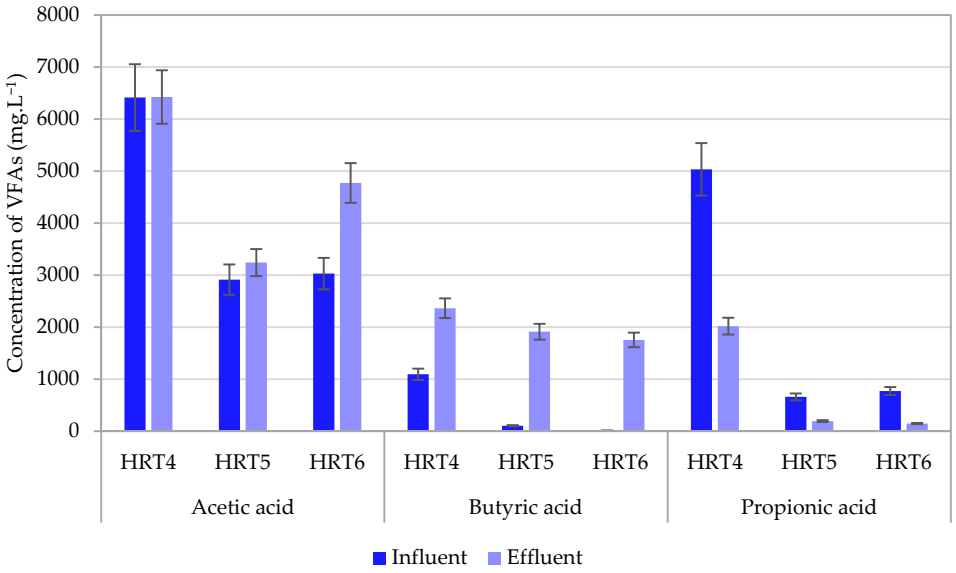

Figure 9. Average concentrations (n = 3 samples per HRT) of VFAs in the influent and effluent of the DF bioreactor for each of the tested HRTs (error bars: standard deviation).

The VFA contents present in the substrate were different during the three experimental assays. This may have been due to the residence times of the OFMSW inside the pre-processing unit (waste sorting and classification; pulping; mixture with anaerobic sludge; mixture with process water) in the full-scale AD plant. This may lead to the fermentation of the organic substrates and increase in VFAs in the feedstock when the residence times are longer in the pre-processing unit.

The main VFAs present in the effluent of the DF bioreactor were acetic acid and butyric acid. These VFAs are related to the most efficient pathways for the production of bioH_2, especially by *Clostridium* sp. [25,35]. As regards propionic acid, this organic acid was

detected in very low concentrations in the HRTs of 5 and 6 d, in comparison with the HRT of 4 d.

The OFMSW used in the HRT4 assay was characterized by a high content of VFAs, especially acetic acid (6.413 $g.L^{-1}$), which may indicate that this OFMSW sample was already in an advanced stage of fermentation, probably due to a high retention time in the pre-processing unit at the full-scale AD plant. Consequently, it may not have offered favorable conditions for $bioH_2$ production. Additionally, the concentrations of acetic acid were much higher than those reported in the literature (1.7 $g.L^{-1}$ [35]; 1.4 $g.L^{-1}$ [30]) for food wastes.

The molar ratio between butyric acid and acetic acid (BA/AA) is a useful quantitative indicator of the stability of metabolic pathways associated with the production of $bioH_2$ [36,37]. Table 5 shows the BA/AA molar ratios obtained for the different tested HRTs as well as the optimum molar ratio defined by Kim et al. [37].

Table 5. Butyric acid to acetic acid (BA/AA) molar ratio for the three HRTs.

Molar Ratio	HRT4	HRT5	HRT6	Optimum Value
BA/AA	0.26	0.40	0.26	>2.6

According to Kim et al. [37], the BA/AA molar ratio is directly proportional to the $bioH_2$ yield. The authors established this direct relation during the DF of sucrose, under mesophilic conditions, with an OLR in the interval of 10–60 g $COD.L^{-1}.d^{-1}$, at pH 5.5 and an HRT of 12 h. These authors [37] also indicated that a BA/AA molar ratio greater than 2.6 presents an efficient production of $bioH_2$, despite the fact that other authors have stated that more work is needed to support this optimum value.

The BA/AA molar ratios obtained in the present work were much lower than the optimum value indicated in the literature [38,39], demonstrating that the high concentration of acetic acid may have limited the production of $bioH_2$, not contributing to the maximization of the $bioH_2$ yield. In another study by Angeriz-Campoy et al. [24], BA/AA molar ratios between 0.9 and 1.2 were recorded during the co-digestion of OFMSW under thermophilic conditions. These values are lower than the optimum value defined by Kim et al. [37], however, even here, the BA/AA molar ratios obtained in the present work were lower than the ratios obtained by Angeriz-Campoy et al. [24].

As shown in Figure 9, the increase in OLR produced a significant increase in the concentration of VFAs, particularly when the HRT decreased from 5 to 4 d. The VFA content substantially increased as the OLR increased from 12.58 g $TS.L^{-1}.d^{-1}$ (VFAs = 6.7 $g.L^{-1}$) (HRT of 6 d) to 26 g $TS.L^{-1}.d^{-1}$ (VFAs = 10.8 $g.L^{-1}$) (HRT of 4 d). However, the VFAs produced in the HRT4 assay were composed of 81% acetic acid and butyric acid.

It was also found that the HRT4 trial showed the highest increase from the influent to the effluent in the total concentration of acetic acid and butyric acid compared to the other trials (HRT4: increase of 21.4%; HRT5: increase of 14.3%; HRT6: increase of 18.1%). The increase in the OLR in the HRT4 assay may have led to an unstable DF process through the accumulation of VFAs, namely acetic acid, with an inhibition of $bioH_2$ production.

Jiang et al. [39] studied the effect of pH, OLR, and temperature in the DF of food wastes under batch conditions. Their results revealed that the maximum production of VFAs occurred at pH 6 and the acetic and butyric acids corresponded to approximately 77% of the overall VFAs produced. These authors observed that the concentration of VFAs increased as the OLR increased from 5 g $TS.L^{-1}.d^{-1}$ (VFAs = 13.27 $g.L^{-1}$) to 16 g $TS.L^{-1}.d^{-1}$ (VFAs = 24.93 $g.L^{-1}$). At the highest OLR, 65% of the produced VFAs were constituted by acetic and butyric acids. The produced VFAs in the current work, with HRTs of 4, 5, and 6 d were not as high as in the study by Jiang et al. [39] because the feeding conditions were different. Jiang et al. used batch conditions, while the current work was performed under continuous feeding. The latter conditions tendentially present lower concentrations of the final products as the HRT tested are shorter than under batch conditions.

3.5. Production and Composition of Biogas

Table 6 shows the accumulated biogas production and composition, as well as the bioH_2 volumetric production (VHP) rate, bioH_2 yield (η_{bioH2}), and available energy.

Table 6. Production and composition of biogas, bioH_2 volumetric production (VHP) rate, bioH_2 yield (η_{bioH2}), calorific value ($H_{u,act}$), and thermal energy in biogas.

Parameters	Units	DF Assays		
		HRT4	HRT5	HRT6
HRT	d	4	5	6
n (total days of operation)	d	38.4	30.3	18.8
$OLR_{VS,in}$	g $VS.L^{-1}.d^{-1}$	23.6	18.0	10.6
V_{biogas} accumulated	L	64.5	62.4	15.0
H_2	% v/v	25	32	5
CO_2	% v/v	75	68	77
CH_4	% v/v	<0.1	<0.1	18
O_2	% v/v	<0.1	<0.1	<0.1
N_2	% v/v	<0.1	<0.1	<0.1
V_{bioH2} accumulated	L H_2	16.13	19.97	0.75
F_{bioH2}	L $H_2.d^{-1}$	0.42	0.66	0.04
VHP rate	L $H_2.L^{-1}_{bioreactor}.d^{-1}$	0.20	0.32	0.02
η_{bioH2}	L $H_2.kg^{-1}$ $VS_{influent}$	8.48	18.2	1.64
$H_{u,act}$	kJ.kg^{-1} $VS_{influent}$	123	175	17.3
Thermal energy in biogas	kWh.t^{-1} $VS_{influent}$	34.2	48.6	4.81

The HRT6 (OLR: 10.6 g $VS.L^{-1}.d^{-1}$) was not effective in the production of bioH_2 (H_2: 5% v/v; η_{bioH2}: 1.64 L $H_2.kg^{-1}$ $VS_{influent}$; VHP rate: 0.02 L $H_2.L^{-1}_{bioreactor}.d^{-1}$). This can be explained by the development of methanogenic bacteria as CH_4 was detected (18% v/v) in the biogas collected during this experimental trial.

Although the pH was kept at 5.1 ± 0.7, the HRT of 6 days was high enough to allow the development of hydrogenotrophic methanogens which contribute to bioH_2 consumption and CH_4 production.

Additionally, it should be noted that the redox potential during the HRT6 assay (−110 ± 12 mV) was significantly lower compared to the HRT5 (−56 ± 26 mV) and HRT4 (−47 ± 38 mV) assays, which may have contributed to the use of CO_2 as the final electron acceptor instead of H^+, providing the necessary conditions for CH_4 production and H_2 consumption.

It is also important to notice that the anaerobic sludge used as inoculum in the present work was composed of biological anaerobic flocs. The floc structure protects the cells located in the inner part of the flocs. Therefore, even if the medium pH is not favorable to methanogens, the floc structure allows them to remain active inside the flocs. When the HRT is sufficiently high to allow the methanogens' growth inside the biological flocs, then significant H_2 consumption may occur and CH_4 will be detected in biogas.

For all these reasons, the results obtained in the HRT6 assay were very different from those reported by Zahedi et al. [35]. These authors investigated the effect of different OLRs in the DF of OFMSW under thermophilic conditions. At an OLR of 13 g $VS.L^{-1}.d^{-1}$ and an HRT of 6 d, these authors reported an H_2 content of 36 ± 5% v/v and a VHP rate of 0.27 L $H_2.L^{-1}.d^{-1}$.

The trial with the HRT of 4 d and OLR of 23.6 g $VS.L^{-1}.d^{-1}$ showed much better results (H_2 content: 25% v/v; η_{bioH2}: 8.48 L $H_2.kg^{-1}$ $VS_{influent}$; VHP rate: 0.20 L $H_2.L^{-1}_{bioreactor}.d^{-1}$) than with the HRT of 6 d. The biogas calorific value was 123 kJ.kg^{-1} $VS_{influent}$ which is equivalent to a thermal energy potential of 34.2 kWh.t^{-1} $VS_{influent}$. This experimental trial did not reach the best results, maybe due to the excessive organic load applied to the bioreactor and the high concentration of acetic acid that was present in the feedstock.

Cisneros-Pérez et al. [40] performed a study on the impact of OLR in the DF of glucose. The following conditions were tested: fluidized-bed reactor; temperature of 37 °C; OLR of

24−60 g COD.L^{-1}.d^{-1} supplied by glucose; HRT of 2−10 h; pH 5.7; different pre-treatments of the inoculum were tested. The best results (7 L H_2.L^{-1}.d^{-1}, 3.5 mol H_2.mol^{-1} glucose) were obtained with the highest load (60 g COD.L^{-1}.d^{-1}) and an HRT of 6 h. A lower HRT caused a decrease in the bioH_2 production rate, possibly caused by the cell washout from the bioreactor.

The HRT of 5 d (OLR of 18.0 g VS.L^{-1}.d^{-1}) showed the best results in the present study: an H_2 content of 32% v/v; η_{bioH2}: 18.2 L H_2.kg^{-1} $VS_{influent}$; and VHP rate of 0.32 L H_2.$L^{-1}_{bioreactor}$.d^{-1}. The biogas calorific value was 175 kJ.kg^{-1} $VS_{influent}$, corresponding to a thermal energy potential of 48.6 kWh.t^{-1} $VS_{influent}$. Among the three experimental trials, the HRT of 5 d and an OLR of 18.0 g VS.L^{-1}.d^{-1} provided the optimal conditions tested for the DF of a real OFMSW feedstock under mesophilic conditions and at a pH of approximately 5.

Table 7 compares the main results of the present work with some of the data available in the literature.

Table 7. Comparison of the main experimental data obtained in the present work with the data available in some bibliographic references.

Organic Substrate	Inoculum	T (°C)	pH	HRT (h or d)	Bioreactor (Volume | Type)	OLR (g VS.L^{-1}.d^{-1})	H_2 (%v/v)	η_{bioH2} (L H_2.kg^{-1} $VS_{influent}$)	VHP (L H_2.L^{-1}.d^{-1})	Reference
OFMSW	Mixed culture	37	5.2	5 d	2.1 L | CSTR	18	32	18.2	0.32	Present study
OFMSW	Mixed culture	55	5.5	5 d	4.5 L | CSTR	16	62 ± 13.3	104.5 ± 54.9	2.7	[28]
OFMSW	Mixed culture	55	5.7	3.3 d	200 L | CSTR	16.8	38.5 ± 9.7	80	275	[31]
Synthetic OFMSW	Mixed culture	34	5.5	0.5 h	3 L | CSTR	45.4	30.4 ± 0.7	13.13 ± 1.04	0.60	[30]
OFMSW	Mixed culture	55	5.3	6.6 d	5.5 L | CSTR	13	36 ± 5	20 ± 5	0.27	[35]
			5.7	4.4 d	5.5 L | CSTR	19	39 ± 6	31 ± 7	0.42	
			5.8	3 d	5.5 L | CSTR	28	41 ± 2	23 ± 4	0.93	
OFMSW	Mixed culture	35	5.5	24 h	1.7 L | CSTR	19	39	1.23	0.37	[41]
OFMSW	Mixed culture	35	5.0	8 h	10 L | CSTR	106	32	99.8	1.35	[29]

The studies carried out under similar conditions to those of the present work (type of substrate, OLR, and HRT) but under a thermophilic regime produced better results in terms of bioH_2 production rate and yields [29,36], except when an HRT of 6.6 d was used [35]. The thermophilic regime can be more interesting for bioH_2 production, provided that heat is available in the plant to heat the bioreactor and an ulterior high added-value valorization process of the VFAs is performed, aiming to increase the financial return of the plant.

Additionally, considering the data obtained in the present work, it is possible to conclude that HRT values higher than 6 d seem not to be advantageous for the production of bioH_2 in the DF of OFMSW under both mesophilic and thermophilic conditions.

The tests performed in bioreactors with bigger volumes revealed that although the percentages of bioH_2 are similar to that obtained in the present study, they achieve much higher bioH_2 production rates and yields, showing that scaling up this process may contribute to its sustainability with a negligible loss of productivity. However, the mass and energy balances and economic assessment must be considered in future works to support the argument on DF sustainability at a larger scale.

4. Conclusions

The HRT of 5 days, corresponding to an OLR of 18.0 g VS.L^{-1}.d^{-1}, presented the best results on the DF of OFMSW. The bioH_2 content in biogas (H_2: 32% v/v), the bioH_2 yield (η_{bioH2}: 18.2 L H_2.kg^{-1} $VS_{influent}$), and the volumetric production rate (VHP: 0.32 L H_2.$L^{-1}_{bioreactor}$.d^{-1}) for this HRT were comparable or even higher than some experimental data reported in the literature. Additionally, these results are better than the experimental data obtained for the HRTs of 4 and 6 days. For the former HRT, an overload of organic matter may have occurred. For the latter HRT, H_2-consuming methanogens gained importance in the microbial consortium, contributing to the appearance of CH_4 in

the biogas and to lower bioH_2 production, eventually due to its consumption for CH_4 production. The overall conclusion is that the optimal conditions for bioH_2 production through the DF of OFMSW are significantly affected by the HRT and OLR, as these parameters are fundamental to establishing the metabolic pathway able to maximize bioH_2.

By using the real samples of OFMSW characterized by high chemical variability, it is also possible to conclude that the characteristics of the substrate can drive the process of VFA accumulation, thus leading to some instability. Thus, the substrate variability becomes a parameter that must be controlled in the aim of maintaining optimized conditions for bioH_2 production.

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