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Boosting butyrate and hydrogen production in acidogenic fermentation of food waste and sewage sludge mixture: a pilot scale demonstration

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ABSTRACT

Within the urban scenario, the application of a biorefinery technology value chain can foster the conversion of different organic substrates into marketable and added-value products. In this work, a pilot-scale dark fermentation (DF) process has been carried out as a key step for volatile fatty acids (VFA) and hydrogen production from the liquid fraction of sewage sludge and food waste mixture. Six operating conditions have been monitored in terms of yield and process stability, by changing the hydraulic retention time (HRT) from 4 to 6 days and applying a short-term hyper-thermophilic hydrolysis (70 °C, 8 h) on the same feedstock mixture. A tubular centrifugation was utilized to remove part of the biosolids (driven to biogas production) before the DF step, which was applied on the soluble and/or colloidal organic matter only. The hydrolysis step favored the following acidification process, in which a fermentation yield up to 0.42 g COD_{VFA}/g VS_0 was achieved at 5 days as HRT. Hydrogen production (up to 34.4% v/v and 0.046 m^3 H_2/kg VS_0) was positively affected by the hydrolysis application and by the decrease of the HRT, highlighting the possibility to produce biohythane in the modeled two-phases anaerobic bioprocess. On the other hand, without the application of the hydrolysis, a selective production of butyric acid (up to 75% COD basin) was achieved, furnishing a different valorization route for the chosen urban organic feedstock. Changes in operating conditions and performances were also reflected by the adaptation of the microbial community, whose characterization highlighted the occurrence of several fermentative microorganisms (e.g., *Clostridiaceae*, *Ruminococcaceae*).

1. Introduction

In the perspective of the reduction of the greenhouse gas emissions, the generation of clean energy in a sustainable way represents a priority, in combination with a conscientious utilization of the bioresources (ec.europa.eu). As one of the most established technologies at industrial scale, the anaerobic digestion (AD) allows to obtain energy, and in some cases fertilizers from organic waste feedstock. However, because of the involvement of different microorganisms with specific biochemical pathways, many other valuable products can be obtained via dark fermentation (DF). The DF process includes the first metabolic pathways of the AD process, where the organic matter degradation occurs along with the accumulation of volatile fatty acids (VFA) and hydrogen, both precursors of methanogenic bacteria in the following pathways of the AD process (Chorukova et al., 2022). In this frame, the production of volatile fatty acids (VFA) and hydrogen from waste organic feedstock through the DF process has recently aroused high attention due to the exploitation of renewable bioresources at relatively low-cost (Lui et al., 2020; Varghese et al., 2022).

Compared to the traditional technologies based on fossil fuel sources, the interest in the hydrogen is substantially driven by its high energy yield (142.35 kJ/g) and the zero carbon emissions (Paudel et al., 2017).

Abbreviations: BMP, Biochemical methane potential; CSTR, continuous Stirred Tank Reactor; DF, dark fermentation; FID, flame ionization detector; GC, gas chromatograph; HRT, hydraulic retention time; OLR, organic loading rate; COD_{SOL}, soluble chemical oxygen demand; VFA, volatile fatty acids; TKN, total Kjeldahl nitrogen; TS, total solids; VS, volatile solids; WWTP, wastewater treatment plant.

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However, despite of these realistic advantages, its production from waste in a sustainable way is still not established. Concerning the DF, the biggest limit is represented by the low rate of hydrogen production, which is due to the fast hydrogen consumption by CO_2-reducing methanogens (Liu et al., 2020), and to the low degradation rate of some waste feedstock, especially when they are not subjected to any pretreatment step (Wang and Yin, 2018). To overcome these issues, the operating conditions of the biological processes have to be regulated and adapted to the type of waste feedstock to increase the hydrogen yield and its content in the generated gas mixture (Mozhiarasi et al., 2023). Among others, sewage sludge could be an excellent raw material for hydrogen recovery since it is characterized by a high concentration of organic matter and huge production in the municipalities. Apart from hydrogen, the produced VFA are also interesting molecules, being valuable building blocks for the chemical industry. To sustain the fermentability of sewage sludge, many authors recommend mixing this bioresource with other co-substrates for an improved nutritional balance (C/N ratio) and putrescence characteristics (Vidal-Antich et al., 2021) as well as hydrogen and VFA yield (Alemahdi et al., 2015).

A recent laboratory scale study demonstrated that food waste (FW) was a beneficial co-substrate for the acidogenic fermentation of sewage sludge (Vidal-Antich et al., 2021). Features like the high concentration of organics and C/N ratio substantially enhanced the sewage sludge and FW mixture fermentability. In addition, both waste streams are among the most attractive feedstock for the waste-based biorefineries, given their large and constant production in urban areas and municipalities. In fact, almost 45 million tons of dry sewage sludge production was reported on a global scale (Gao et al., 2020); this number is expected to grow with the implementation of urban wastewater treatment plants (WWTPs). In addition, more than 1.3 billion tons FW are globally produced each year (Du et al., 2021).

In a perspective of a sustainable waste-based biorefinery development, the DF of sewage sludge and FW can be seen as a key step to produce VFA and hydrogen. According to circular economy development and strategy, the mixing and the co-treatment of sewage sludge and FW may solve the related environmental concerns by obtaining bioproducts with high added value (chemicals, fuels and power) and by reducing the secondary waste to be disposed (Vidal-Antich et al., 2021). Within this scenario, the DF process can be seen as the first step of the two-phases AD (Anukam et al., 2019), which overcomes the limit of the single stage AD, where VFA accumulation was seen as a problem based on the inhibition on methanogenic microorganisms at high VFA concentration or low pH. With the two reactors connected in series (fermentation and digestion), the whole process increases its stability, with benefits in VFA and hydrogen production and, more in general, renewable energy production (Tena et al., 2021).

This study was focused on the investigation and on the evolution of a pilot scale DF process to define the optimal operating conditions for the production of VFA and hydrogen, proposing FW as a co-substrate of sewage sludge. The scope was to utilize two local sources for their joint valorization, contributing to the achievement of the fundamentals of the circular economy model, where the production of a second-generation biofuel named biohythane (a mixed gas comprising hydrogen, methane, and carbon dioxide) was also foreseen (Bertasini et al., 2023). The application of a mild hydrolysis on the feedstock and the variation of the hydraulic retention time (HRT) in the DF reactor were chosen as conditions to be investigated. In addition, a coaxial centrifugation unit has been placed before the DF process to evaluate the fermentability of the liquid fraction, more enriched in organics' content when the pre-hydrolysis step was adopted. The solid-rich fraction was instead tested for biogas production in parallel biochemical methane potential (BMP) tests to evaluate the quality of the potential produced biohythane in terms of hydrogen and methane content. High-throughput sequencing of 16S rRNA gene was performed as essential tool to describe the microbial community composition and dynamics in response to the adopted operating conditions.

2. Materials and methods

2.1. Feedstock

The sewage sludge was produced in the municipal WWTP of Treviso (northeast Italy). The plant was not equipped with the primary sedimentation and the sludge was taken from the static thickener connected to the secondary settler of the biological nutrient removal (BNR) water line. The co-substrate FW was recovered from the source separate collection of 53 districts in the Treviso province. After its collection, the FW was subjected to a mechanical trituration, inert removal, squeezing and homogenization in a dedicated external full-scale plant (Moretto et al., 2019). For this work, the squeezed FW was regularly taken in the Treviso WWTP facility, where the pilot experimental area was placed. Both streams have been characterized in terms of total and volatile solids (TS and VS), soluble chemical oxygen demand (COD_{SOL}), VFA, ammonia ($N–NH_4^+$), phosphate ($P\text{-}PO_4^{3-}$), Total Kjeldahl Nitrogen (TKN) and organic phosphorus (P) (Table 1).

2.2. Rationale of the approach and operating conditions of the processes

The DF process was carried out in three thermophilic stainless steel CSTR reactors (AISI 304) of 200 L each, equipped with a mechanical stirrer. The reactors were heated by a hot water recirculation system and maintained at 55 °C with an electrical heater controlled by a PT100-based thermostatic probe. Compared to mesophilic conditions, hydrogen production by DF at thermophilic temperature has been identified as thermodynamically favorable and minimally affected by the partial pressure of hydrogen in the liquid media (Mozhiarasi et al., 2023). The feeding system was semi-continuous, arranged once per day. The reactors were maintained in parallel at three different HRT of 4.0, 5.0 and 6.0 days, according to the values reported in a recent review (Dangol et al., 2022) (Table 2). The pH was regularly monitored in the effluent; no pH-control strategy was adopted since sewage sludge was utilized at 50% v/v ratio to furnish the required buffering capacity (Moretto et al., 2019).

For each CSTR, two periods could be distinguished: in phase "a", the hyper-thermophylic hydrolysis (70 °C, 8 h) was applied to the waste mixture; in phase "b", the hydrolysis pretreatment was not adopted, aiming to assess its impact on hydrogen and VFA production (in terms of yield and content/concentration). The hydrolysis was carried out in a separate stainless steel batch reactor of 380 L, also equipped with a mechanical stirrer and maintained at 70 °C by a hot water recirculation system. Independently from the application of the pre-hydrolysis step, the feedstock mixture was subjected to a solid/liquid separation stage, consisting in a coaxial centrifuge equipped with 100 μm porosity nylon filter bag for a partial removal of the solids, before the DF process, as depicted in Fig. 1. The feeding/emptying of the hydrolysis reactor, the transfer of the feedstock to the centrifugation unit and to the DF reactors were manual operations. The OLR values reported in Table 2 were dependent upon the VS content in the centrifuged waste mixture (3.1–6.0 kg VS/m^3 d) and, in turn, upon the variability in VS content of the feedstock (especially FW). The high-solid streams (both hydrolyzed and not hydrolyzed) were exploited for biogas production through

Table 1
Summary of the physical-chemical features of the waste stream.

Parameter	Sewage Sludge	Squeezed FW
TS (g/L)	28.4 ± 0.4	52 ± 9
VS (g/L)	22.4 ± 0.2	47 ± 8
COD_{SOL} (g/L)	0.45 ± 0.03	17 ± 8
COD_{VFA} (g/L)	–	3.1 ± 0.9
Ammonia (g $N–NH_4^+$/L)	0.35 ± 0.02	0.10 ± 0.06
Phosphate (g $P\text{-}PO_4^{3-}$/L)	0.11 ± 0.04	0.054 ± 0.004
TKN (g N/kg TS)	32 ± 2	18 ± 5
P (g P/kg TS)	5 ± 1	1.3 ± 0.6

Table 2

Operating parameters of the CSTR run DF processes.

CSTR run	phase	Temperature (°C)	HRT (days)	OLR (kg VS/m^3 d)	Hyper-thermophilic hydrolysis (°C, h)
1	a	55	4.0	4.6	70 °C, 8 h
	b		4.0	6.0	–
2	a	55	5.0	3.7	70 °C, 8 h
	b		5.0	4.8	–
3	a	55	6.0	3.1	70 °C, 8 h
	b		6.0	4.0	–

mesophilic BMP tests (Holliger et al., 2016).

2.3. Analytical methods

The effluent of the three reactors were analyzed for COD_{SOL}, VFA (both concentration and distribution) and pH measurements. The COD was quantified in accordance with the Standard Methods (APHA, 1998). For VFA analysis, the liquid samples were initially centrifuged and filtered at 0.20 μm filter porosity; the filtered sample was then injected in the Agilent 6890N gas chromatograph (GC), which was also utilized for hydrogen and methane quantification, according to the methods described elsewhere (Valentino et al., 2021). The gas production in the pilot reactors was quantified by a portable flow meter (Ritter CompanyTM).

2.4. Calculations and statistical analysis

Since COD_{SOL} and VFA were the main parameters monitored in the liquid phase, their ratio (COD_{VFA}/COD_{SOL}) was considered as an important indication of the acidification degree. In addition to the COD_{VFA}/COD_{SOL} ratio, the VFA yield (Y_{VFA}; COD basin) was also quantified as the ratio between the produced VFA, as net concentration subtracted to the initial VFA (COD_{VFA0}) and the initial VS (VS_0) (Moretto et al., 2019):

(1) $Y_{VFA} = (g\ COD_{VFA} - g\ COD_{VFA0})/g\ VS_0$

Independently from the application of the hyper-thermophilic hydrolysis, the VFA yield was calculated by considering the VS content of the feedstock mixture as starting point, before hydrolysis (when applied) and the centrifugation step.

The hydrogen content as percentage (v/v) in the gas mixture was periodically quantified in addition to the hydrogen yield, which was measured as specific hydrogen production (SHP):

(2) $SHP = (m^3\ H_2)/(kg\ VS_0)$.

The specific biogas production (SGP) in the BMP tests was quantified according to the model reported in Mata-Alvarez et al. (1990). The composition of the biohythane potentially achievable in a two-phases anaerobic process was evaluated with the mass balance assessment (Supplementary Material).

All the parameters characterizing the reactors' performances were calculated after the steady states were reached. The Analysis of Variance (ANOVA) and Tukey HSD post-hoc tests were conducted to evaluate the significance of the results. The null hypothesis (H_0) was rejected when the calculated p–values were less than or equal to level of significance (0.05). Statistical analyses were performed using software R (The R Foundation for Statistical Computing, version 4.2.2 – Innocent and Trusting).

2.5. Microbial community analysis

The sampling for microbial community analysis was carried out after the achievement of the steady state in each condition investigated (with both hydrolyzed and not hydrolyzed waste mixture). The DNA extraction was performed on 10 ml of sample, by using DNeasy PowerSoil Pro Kit (QIAGEN, Germantown, MD) and utilized as the template for the amplification of the V1–V3 region of 16S rRNA gene of *Bacteria* (27F 50-AGAGTTTGATCCTGGCTCAG-3'; 534R 50-ATTACCGCGGCTGCTGG-30), following the known procedure for the library preparation and sequencing (Crognale et al., 2019). Bioinformatics analyses were performed with QIIME2 v. 2018.2 (Bolyen et al., 2019), following the procedure described elsewhere (Crognale et al., 2021). High-throughput sequencing yielded a total of 97,832 sequence reads after quality control and bioinformatics processing that resolved into 977 ASVs. Datasets were available through the Sequence Read Archive (SRA) under accession PRJNA894822.

Based on the correlation matrix, the principal component analysis (PCA) was performed by comprising the relative abundances of the microbial taxa in all the CSTR runs. Only genera ≥1% of total reads were considered. Correlations between the microbiota, VFA and hydrogen were identified using Spearman's correlation. The statistical analyses related to the microbial community were performed by using PAST software package (Palaeontological Statistics, ver. 4.04) (Hammer et al., 2001).

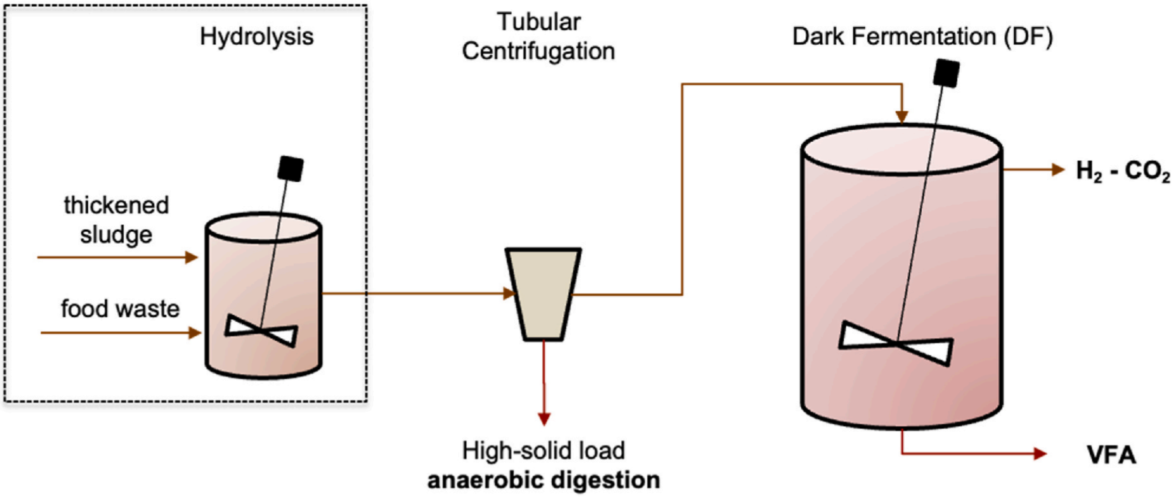

Fig. 1. Process scheme adopted at pilot scale (the hydrolysis step in the square frame was carried out only in Run 1a, 2a and 3a).

3. Results and discussion

3.1. Impact of HRT and hydrolysis on VFA production

Fig. 2 shows the trends of VFA and COD_{SOL} concentration in the different CSTRs operated at an HRT of 4.0, 5.0 and 6.0 days. The three CSTRs were initially fed with the hydrolyzed feedstock, which was used approximately for the first 30 days; afterwards, the operating conditions were switched by using a not hydrolyzed feedstock until the end. The modification of the process conditions did not affect the pH, which remained constantly in the range 4.8–5.3, without any correlation with the adopted HRT (Table 3).

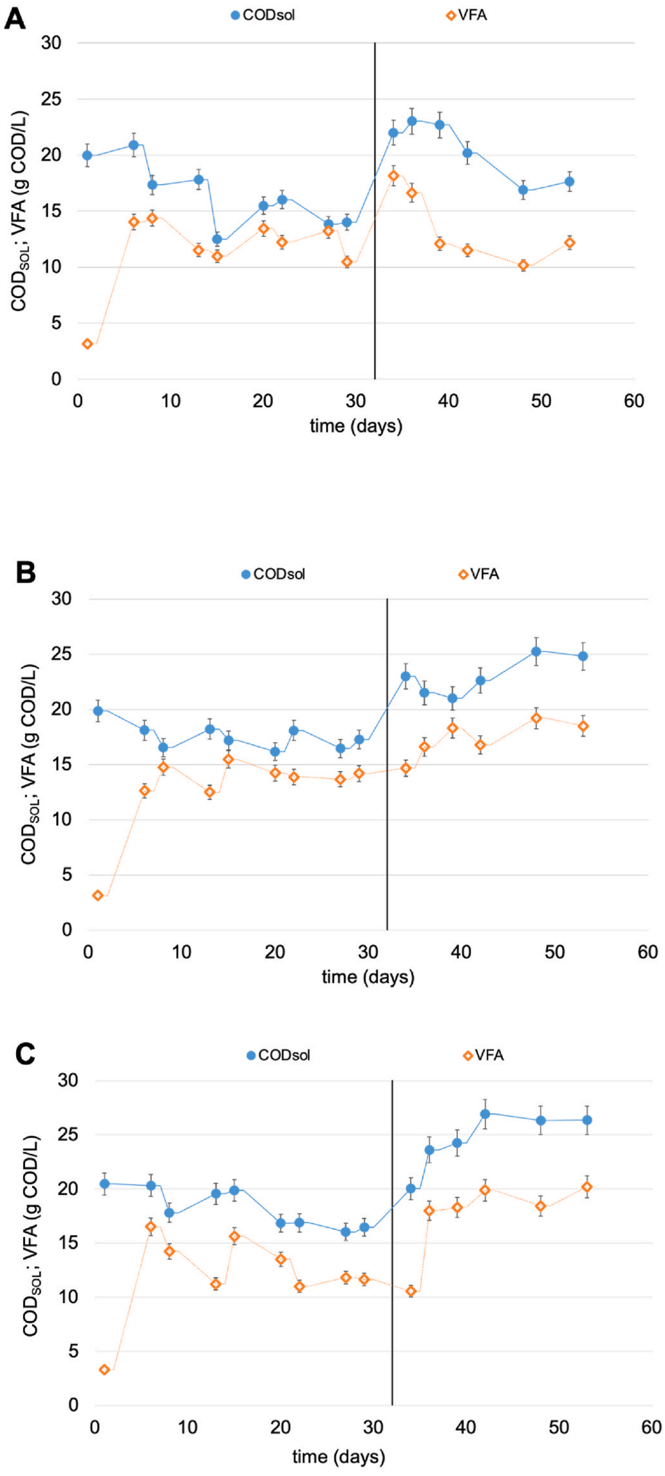

Fig. 2. VFA and COD_{SOL} evolution in the pilot CSTR runs conducted at the following HRTs: 4.0 (A), 5.0 (B) and 6.0 (C) days. (the solid line indicates the change from Phase a to Phase b).

After an initial lag phase, which was common for all the three reactors, the achieved VFA values were in the range 10–15 g COD_{VFA}/L, with no substantial fluctuations until the end of the tested conditions (day 32). The COD_{SOL} values were close to VFA concentration, meaning that the acidification process was effective in all the three runs. The average VFA concentrations were 12.5 ± 0.5, 13.9 ± 0.4 and 13.2 ± 0.7 g COD_{VFA}/L respectively in Run 1a, 2a and 3a, whereas the average COD_{SOL} concentration were 17 ± 1, 17.3 ± 0.3 and 18.0 ± 0.6 g COD/L. Therefore, the average COD_{VFA}/COD_{SOL} ratio was higher in Run 2a (0.80 ± 0.03) compared to Run 1a (0.75 ± 0.04) and Run 3a (0.73 ± 0.03) (Table 3). Hence, considering the COD_{VFA}/COD_{SOL} ratio, the HRT of 5.0 days was the best in terms of acidification of soluble organic matter. When the hydrolysis step was not applied to the feedstock mixture (phase b), the efficiency of the process was partially reduced, especially for the lowest HRT adopted (Run 1b). The increase in COD_{SOL} was related to the change of the substrate's characteristics (FW in particular). The higher COD_{SOL} in the three runs conducted with not hydrolyzed feedstock (Run 1-2-3b) was differently acidified at the three investigated HRT. Run 1b was heavily affected by the change of the feedstock, showing both COD_{VFA}/COD_{SOL} ratio and Y_{VFA} lower than counterpart values of Run 1a (Table 3). By increasing the HRT to 5.0 and 6.0 days, the acidification performances was more similar between the two approaches (hydrolyzed and not-hydrolyzed feedstock). Run 2b and 3b exhibited a COD_{VFA}/COD_{SOL} ratio of 0.74 ± 0.03 and 0.71 ± 0.04, respectively, together with a Y_{VFA} equal to 0.41 ± 0.02 and 0.38 ± 0.04 g COD_{VFA}/g VS_0, respectively. Therefore, both runs appeared to be preferred for the DF process performed with no hydrolyzed feedstock.

By considering the whole experimental plan, the intermediate HRT of 5.0 days was the best option for the enrichment of a stable and efficient anaerobic fermentative culture. The unconverted soluble matter roughly accounted for a 20% of the COD_{SOL}, being the rest represented by VFA. The Y_{VFA} was above 0.40 g COD_{VFA}/g VS_0 in both Run 2a-b; even if these values were sometimes lower than previous data collected elsewhere (Battista et al., 2022), it should be noted that a remarkable part of the VS_0 was subtracted to the DF process via centrifugation, to be ideally allocated for a parallel anaerobic digestion process (simulated by BMP tests).

Table 3 summarizes the main parameters with average data and standard deviation quantified during the steady state of all the CSTR runs.

Table 3
Main parameters with average data and standard deviations monitored and quantified in the CSTR runs.

Parameter	Run 1a	Run 1b	Run 2a	Run 2b	Run 3a	Run 3b
COD_{SOL} (g COD/L)	17.0 ± 1.0	20.0 ± 1.1	17.3 ± 0.3	23.0 ± 0.7	18.0 ± 0.6	25.0 ± 1.2
VFA (g COD_{VFA}/L)	12.5 ± 0.5	13.0 ± 1.3	13.9 ± 0.4	17.0 ± 1.0	13.2 ± 0.7	18.0 ± 1.1
COD_{VFA}/COD_{SOL} (g/g)	0.75 ± 0.04	0.66 ± 0.04	0.80 ± 0.03	0.74 ± 0.03	0.73 ± 0.03	0.71 ± 0.04
Y_{VFA} (g COD_{VFA}/g VS_0)	0.37 ± 0.02	0.32 ± 0.03	0.42 ± 0.01	0.41 ± 0.02	0.41 ± 0.02	0.38 ± 0.04
pH	4.9 ± 0.2	4.8 ± 0.3	5.2 ± 0.1	5.0 ± 0.1	5.3 ± 0.1	5.1 ± 0.1
SHP (Nm^3/kg VS_0)	0.046 ± 0.002	0.032 ± 0.002	0.042 ± 0.002	0.033 ± 0.003	0.035 ± 0.002	0.024 ± 0.001
H_2 content (%; v/v)	34.4 ± 0.9	25.0 ± 0.8	32.5 ± 0.7	25.0 ± 1.1	28.0 ± 1.2	21.0 ± 0.6

3.2. VFA composition

The applied HRT did not have a significant impact on VFA composition, with the only exception of Run 1a (HRT 4.0 days), where butyric acid was the most abundant at 46% $COD_{Butyric}/COD_{VFA}$, compared to Run 2a (HRT 5.0 days) and 3a (HRT 6.0 days) where butyric acid was roughly 20% lower, in favor of higher accumulation of acetic acid. In general, the DF process of urban waste, in particular the sewage sludge and FW mixture, is characterized by the production of a fragmented fermentation liquid in terms of VFA distribution (Ramos-Suarez et al., 2021), independently from the type of operating condition. An accumulation of a single VFA above 40% COD basin is difficult to be observed if some chain elongation processes are not applied (Roghair et al., 2018). In this study, the withdrawal of a substantial VS via tubular centrifugation before the DF probably limited the solid's solubilization (usually occurring under thermophilic conditions), affecting the final VFA spectrum. The latter was still fragmented but remarkably enriched in butyric (Run 1a) or acetic (Run 2a and 3a) acids.

When the hydrolysis was removed from the process outline, the solids' solubilization occurred only in the thermophilic DF. All the three runs exhibited a heavy accumulation of butyric acid, equal to 72%, 67% and 75% $COD_{Butyric}/COD_{VFA}$ respectively in Run 1b, 2b and 3b, with a VFA spectrum substantially unchanged. Hence, the hyper-thermophilic hydrolysis had a significant effect on VFA composition and, only when coupled with low HRT (and presumably lower solubilization grade; Run 1a), it allowed to obtain a certain accumulation of butyric acid. Without hydrolysis, the limited organics' solubilization process probably favored a selective butyric acid accumulation, at a high level (roughly 70% COD basin) never observed in the literature with urban organic waste (to the best authors' knowledge). Fig. 3 shows the distribution of each VFA in the different CSTRs (the concentration of each quantified VFA is reported in Table S1, Supplementary Material).

It is also important to highlight that butyric acid is widely used in food, chemical, animal feed and pharmaceutical industries, with a market demand close to 100,000 tons/year (Wang et al., 2016). Up to now, its current production is only limited to fossil-fuel feedstock, and it became unsustainable due to the related environmental concerns (Yang et al., 2013). Hence, the use of renewable resources for butyric acid production via DF can strongly limit the environmental impacts related to its market request.

3.3. Impact of HRT and hydrolysis on hydrogen production

Beside HRT, several authors stated that hydrogen production is also dependent on pH (Mozhiarasi et al., 2023). All the CSTR exhibited slightly acidic pH values which generally favored the accumulation of hydrogen in the gas phase (Gottardo et al., 2017). Fig. 4 shows the effect of the HRT and hydrolysis on the hydrogen production in the DF processes. By using a hydrolyzed feedstock, the highest hydrogen production (as SHP) was observed at the lowest investigated HRT of 4.0 d (Run 1a; 0.046 m^3/kg VS_0, ANOVA test, Tukey HSD post-hoc test). With not hydrolyzed feedstock, Run 1b and 2b exhibited not statistically different SHP values (0.032–0.033 m^3/kg VS_0, ANOVA test, Tukey HSD post-hoc test), but higher than hydrogen production reached in Run 3b (0.024 m^3/kg VS_0, ANOVA test, Tukey HSD post-hoc test).

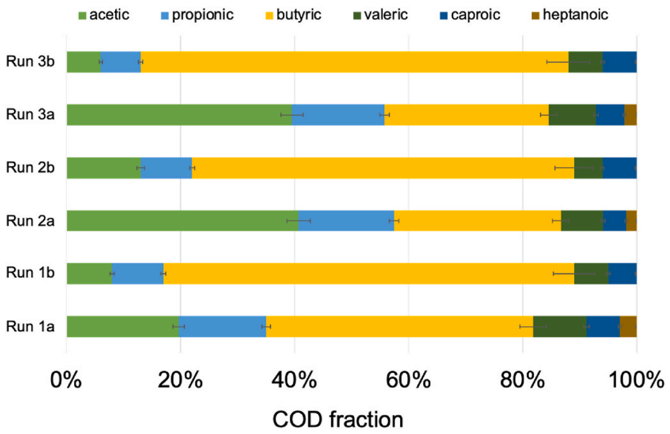

Fig. 3. Distribution of VFA in the steady-state of CSTR runs conducted with hydrolyzed feedstock (Run 1-2-3a) and not hydrolyzed feedstock (Run 1-2-3b).

Similar considerations can be made on the hydrogen percentage in the produced gas flow: the highest hydrogen content was reached with HRT of 4.0 days and thermally hydrolyzed feedstock mixture (Run 1a; 34.4% v/v, ANOVA test, Tukey HSD post-hoc test). The utilization of a not hydrolyzed feedstock decreased the hydrogen content in all the HRT investigated (as observed for the SHP); under this condition, the highest hydrogen content was obtained with HRT lower than 6.0 d, with not statistically different values between Run 1b (HRT 4.0 d) and Run 2b (HRT 5.0 d) (25% v/v, ANOVA test, Tukey HSD post-hoc test).

These results are aligned with prior investigation that showed how the hydrogen production via DF process increased as the HRT decreased (Sillero et al., 2022). Generally, this observation is due to the higher growth rates of hydrogen-producer consortia, compared to the growth rates of hydrogen-consumers. The optimum HRT for hydrogen production in DF also depends on the type of substrate since the HRT may impact the substrate's hydrolysis. This fact could explain why a statistically different hydrogen production between the runs with HRT of 4.0 d and 5.0 days was observed only with hydrolyzed feedstock. Literature

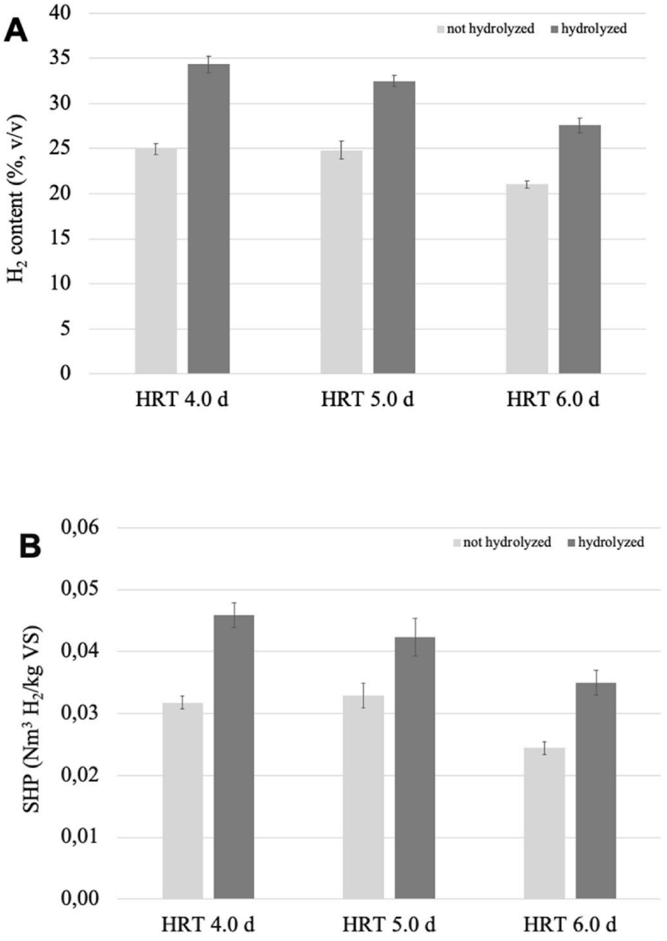

Fig. 4. Steady-state values of hydrogen content in the gas flow (A) and specific hydrogen production (SHP) (B) as a function of the three tested HRT with hydrolyzed and not-hydrolyzed feedstock.

data reported an optimal HRT of 3.0 days for hydrogen accumulation (up to 45% v/v) in the gas flow of thermophilic DF reactors fed with FW only (Micolucci et al., 2020). In this study, the presence of sewage sludge in the feedstock mixture decreased the hydrogen production compared to what has been observed in the literature example cited above. However, the adopted mild hydrolysis improved the biodegradability of the feedstock and the hydrogen production, as demonstrated by the greater hydrogen production in all CSTR runs where hydrolyzed feedstock was fed (ANOVA test, Tukey HSD post-hoc test). Firstly, this observation confirmed the importance of the pretreatment of sewage sludge, a carbon source more difficult to be fermented compared to FW. In fact, the organic compounds in the sewage sludge need to be solubilized by sludge disintegration process.

However, the rate of the sludge disintegration could be quite slow due to the presence of cell's membrane and/or extracellular polymeric substances. For this reason, the hydrolysis process before the DF improved the sludge biodegradability and the consequent hydrogen production. In addition, considering the chemical reactions associated with organic matter acidification (especially carbohydrate-rich feedstock), acetic acid production is stoichiometrically associated to higher hydrogen accumulation compared to butyric acid (Mozhiarasi et al., 2023). Accordingly, by considering all the CSTR runs and the average VFA values reported in Table S1, the decrease of the butyrate/acetate ratio (COD/COD) was reflected by an increase of both hydrogen content (%) and SHP as illustrated in Fig. S1 and Fig. S2 (Supplementary Materials).

3.4. *Simulated anaerobic digestion with BMP of the solid-rich streams*

The BMP tests were implemented with both hydrolyzed and not hydrolyzed solid-rich fractions to quantify biogas and the potential biohythane production in the two-phases anaerobic process. The cumulative methane production and the modeled SGP of each BMP test are reported in Fig. 5(A-B). The data showed a negative effect of the hydrolysis pre-treatment on the on the methanogenic bacteria since both cumulative CH_4 (mL) and SGP were lower with the hydrolyzed solid-rich feedstock. It must be underlined that the hydrolysis step enhanced the organics' solubilization in favor of the following acidification; hence, it is reasonable to assume that the residual solid fraction contributed less efficiently to CH_4 production. On the other hand, without the pre-hydrolysis, the CH_4 production increased by almost 25% compared to hydrolyzed solid-rich fraction. No remarkable differences of CH_4 content (52–53% v/v) in the biogas were observed. The modeled SGP, analyzed as a function of the hypothetical HRT to be adopted in the anaerobic digestion process (according to the methodology described in Mata-Alvarez et al., 1990) provided some noteworthy information. With 20 days as HRT, the untreated solid-rich fraction was characterized by an SGP of 0.54 m^3/kg VS, corresponding to 90% (roughly) of the maximum SGP achievable under mesophilic condition. Differently, hydrolyzed residual solids showed an SGP of 0.43 m^3/kg VS (at 20 days as HRT). This difference had a certain impact on the biohythane composition, potentially characterized by higher hydrogen content (13–14% v/v) only if the hydrolysis step was included in the whole mass balance assessment (Fig. S3 and Table S2; Supplementary Materials).

3.5. *Microbiological characterization*

The 16S rRNA amplicon sequencing performed on the biomass taken in the steady state of CSTRs revealed a microbiome mainly composed by fermentative bacteria highly involved in VFA and hydrogen production. Most important, strong differences in terms of the microbial community composition were observed between CSTRs conducted with hydrolyzed and not hydrolyzed feedstock (Fig. 6). In particular, the samples taken in the runs conducted with hydrolyzed feedstock were mainly characterized by genera *Clostridium sensu stricto* 7 (5.6–9.1%), *Defluviitoga* (13.0–37.7% of total reads), *Tepidimicrobium* (10.9–15.4%), and *Tepidiphilus* (11.4–43.6%). Differently, the microbial communities in the CSTRs operated without a previous hydrolysis step were mainly composed by genera *Caproiciproducens* (12.4–28.5%), *Clostridium sensu stricto* 7 (1.06–58.4%), *Clostridium sensu stricto* 12 (0–32.9%), *Fonticella* (up to 16.4%), and *Paraclostridium* (5.3–8.0%).

In line with process performances, the occurrence of these microorganisms can be generally related to the continuous and stable production of VFA (mainly acetate and butyrate) and hydrogen in all CSTRs (Stamatopoulou et al., 2020). Besides, the different microbiome observed in the CSTRs can be due to the different availability of soluble sugars in the feedstock. Presumably, the use of a pre-treatment on the feedstock favored the solubilization of a greater fraction of promptly biodegradable sugars in Runs 1a, 2a and 3a, promoting the selection of specific microorganisms compared to the tests conducted by feeding a raw feedstock (Runs 1b, 2b and 3b) (Kucharska et al., 2018).

Likewise, PCA analysis confirmed that the effect of hydrolyzed/not hydrolyzed feedstock on microbial selection was stronger than that exerted by the HRT (Fig. 7), in agreement with the observation for VFA production and distribution. Specific bacterial genera were significantly correlated with x-axis in the PCA and most responsible for the obtained clustering between Runs 1a, 2a, 3a (i.e., *Acetomicrobium*, *Caldicoprobacter*, *Defluviitoga*, *Tepidimicrobium*, *Tepidiphilus*, and unidentified members of *Ruminococcaceae* and *Firmicutes*) and Runs 1b, 2b, 3b (i.e., *Caproiciproducens*, *Paraclostridium*, *Romboutsia*, and *Turcibacter*). Additionally, significant positive correlation ($p < 0.05$, Fig. S4, Supplementary Materials) was observed between the relative abundance of *Clostridium sensu stricto* 1 and the hydrogen production. This genus showed the highest abundance in the biomass selected during Run 1a (hydrolyzed feedstock and HRT 4 days) in line with the highest hydrogen concentration detected. Indeed, *Clostridia*-belonging genera have been widely considered among the most important microorganisms involved in the fermentative hydrogen production (Wang and Yin, 2021). Moreover, regarding the VFA production, the genus *Paraclostridium* mainly present in CSTRs operated with not hydrolyzed feedstock, showed significant positive correlation ($p < 0.05$, Fig. S4, Supplementary Materials) with the concentration of butyric acid. Genus *Paraclostridium* was previously described as butyrate-producer at pH range of 5.0–6.0 (Detman et al., 2021), and the abundance of this

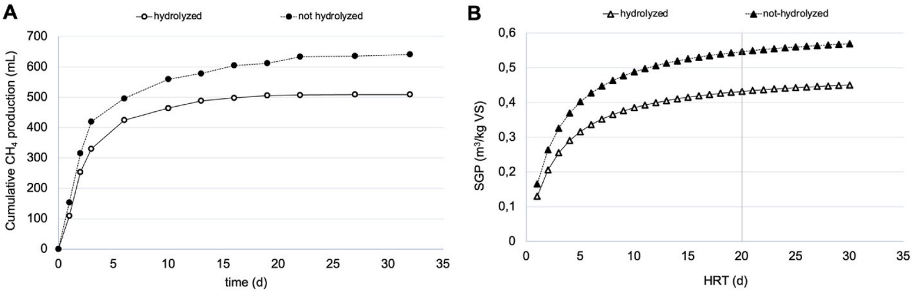

Fig. 5. Evolution of produced biogas (A) and theoretical SGP values (B) modeled in the BMP.

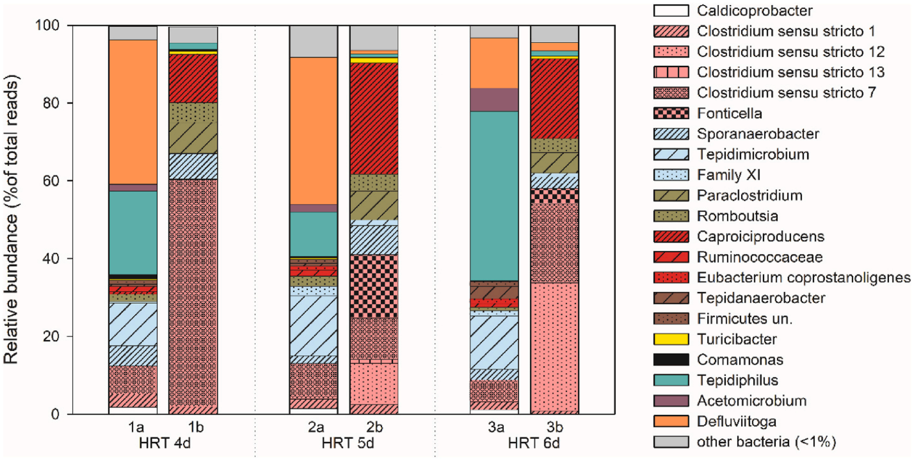

Fig. 6. Microbial community composition (genera ≥1% in at least one sample) in the steady-state of CSTRs runs conducted with hydrolyzed (Run 1a-2a-3a) and not hydrolyzed feedstock (Run 1b-2b–3b).

microorganism in CSTRs operated with non-hydrolyzed waste was in agreement with the dominance of this acid among the fermentation products in Runs 1b, 2b, 3b (72%, 67% and 75% $COD_{Butyric}/COD_{VFA}$, respectively). At the same time, butyric acid was negatively correlated ($p < 0.05$, Fig. S4, Supplementary Materials) with genera *Acetomicrobium*, *Defluviitoga*, *Tepidimicrobium*, and unidentified members of *Firmicutes* and *Ruminococcaceae*. These genera were more abundant in the biomass developed during the experiments conducted with hydrolyzed feedstock, where the concentration of butyric acid was not predominant (Run 1a, 2a, 3a).

4. Discussions

The utilization of FW as co-substrate of sewage sludge was evaluated in a continuous pilot-scale DF process as the key step in a possible biorefinery value-chain. To link the fermentation products and performances on the soluble and/or colloidal organic matter utilization, the solid-rich fraction of the mixture was excluded from the DF process by using an intermediate centrifugation step. According to the chosen operating parameters, the outcomes indicated different possibilities for the technology scale up and realization. Even though technically feasible in all the conditions tested, VFA production was optimal at intermediate HRT (5 days), independently from the hydrolysis application on the feedstock. Lower HRT (4 days) considerably increased the hydrogen production and, in case of the two-phases process implementation, positively affected the quality of the biohythane potentially recovered. In the best case examined (Run 1a) the hydrogen content in the biohythane could be equal to 14% v/v after the upgrade for CO_2 removal. Most of the literature studies revealed that the biohythane characterized by hydrogen level beyond 10% v/v can be burnt directly in internal combustion engines (Mozhiarasi et al., 2023). This application can be sustained only if an effective pretreatment is applied together with adequate operating conditions of the DF reactor. If the DF process is carried out according to the conditions described for Run 1a and 2a, a hydrogen content of 14% and 13% can be respectively achieved in the hypothetical two-phases process. In particular, Run 2a could represent a good compromise, based on the maximization of the VFA yield and the sufficiently high hydrogen content in the final biohythane. Hence, the pretreatment adopted in this study allowed to use the sewage sludge as exploitable substrate for biohythane production, usually considered more technically feasible with other kinds of municipal solid waste such as fruit and vegetable wastes or FW (Deheri and Acharya, 2022). FW alone has been often utilized for this purpose, but the low alkalinity associated to this waste made the two-phases process technically unfeasible without the digestate recirculation in the DF reactor (Micolucci et al., 2020). The use of sewage sludge, with its buffering capacity, allowed to maintain the required stability in the DF reactor (pH around 5.0) without any flux recirculation or chemicals addition.

On the other hand, without hydrolysis, in the face of an increased biogas production, the gas mixture potentially obtainable from the two anaerobic reactors cannot be recognized as biohythane according to its composition in CH_4–H_2 (being hydrogen content between 6 and 8% v/v) (Dangol et al., 2022). However, the accumulation of butyric acid above 70% $COD_{Butyric}/COD_{VFA}$ could lead to a different market perspective. Downstream processes to concentrate and/or separate VFA in the fermentation liquor to improve their marketability need to be considered. Recently, a wide variety of these processes have been reviewed: among others adsoption, extraction and distillation (Atasoy et al., 2018) as well as membrane-based technologies (Aktij et al., 2020). It has been outlined the necessity to dedicate more efforts on these technologies to improve their cost-effectiveness. With innovative and energy-efficient strategies for its recovery from fermentation broths (Chun et al., 2018), butyric acid can be used in the manufacturing of pharmaceuticals, plasticizers, and fuels, as well as in the production of esters or converted into fine chemicals (butanol and butyl butyrate) (Qureshi et al., 2022). Alternatively, without the need of any separation/purification technology, a butyrate-rich fermented stream can be utilized for the microbial synthesis of polyhydroxyalkanoates (PHA). A previous study indicated that for the synthesis of waste-based PHA, the DF has to be directed towards the production of butyric acid, which was recognized as the preferred VFA for an optimal PHA accumulation (Marang et al., 2013).

5. Conclusions

Acidification process via DF of the liquid fraction of sewage sludge and FW was technically feasible under different process conditions and generally favored by the mild hydrolysis of the feedstock mixture. The highest values of acidification yield (0.41–0.42 g $COD_{VFA}/g\ VS_0$) and COD_{VFA}/COD_{SOL} ratio (0.74–0.80) were obtained at 5 days as HRT in a thermophilic CSTR. The VFA spectrum was strongly affected by the hydrolysis step, independently from the HRT adopted. Butyric acid was accumulated up to 72%, 67% and 75% $COD_{Butyric}/COD_{VFA}$ at HRT 4, 5 and 6 days, respectively, when the pre-hydrolysis step was not applied. On the contrary, a larger heterogeneity in the VFA composition was observed without the hydrolysis pre-treatment. Microbiological characterization highlighted the occurrence of several fermentative microorganisms (e.g., *Clostridiaceae*, *Ruminococcaceae*) related to the high production of VFA (mainly acetate and butyrate) and hydrogen in all CSTRs, with differences in terms of relative abundance due to the

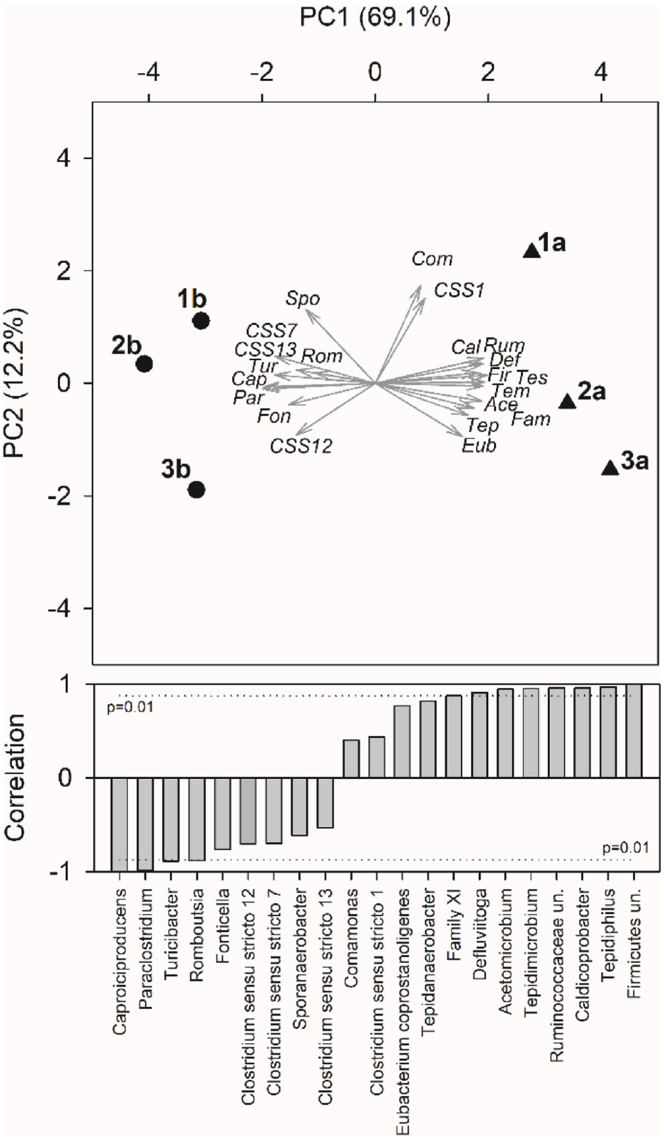

Fig. 7. Main components analysis biplot of microbial composition at genus level (≥1% in at least one sample) in the steady-state of the six runs (1a, 2a, 3a, 1b, 2b, 3b); bar plot shows the contribution of each variable (vector projection values) expressed as the correlation with the x- and y-axis. Ace, *Acetomicrobium*; Cal, *Caldicoprobacter*; Cap, *Caproiciproducens*; CSS1, *Clostridium sensu stricto* 1; CSS7, *Clostridium sensu stricto* 7; CSS12, *Clostridium sensu stricto* 12; CSS13, *Clostridium sensu stricto* 13; Com, *Comamonas*; Def, *Defluviitoga*; Eub, *Eubacterium coprostanoligenes*; Fam, Family XI; Fir, *Firmicutes* un.; Fon, *Fonticella*; Par, *Paraclostridium*; Rom, *Romboutsia*; Rum, *Ruminococcaceae* un.; Spo, *Sporanaerobacter*; Tem, *Tepidimicrobium*; Tep, *Tepidanaerobacter*; Tes, *Tepidiphilus*; Tur, *Turicibacter*.

different applied operating conditions.

In terms of hydrogen production, both hydrolysis and low HRT substantially improved the hydrogen accumulation, up to 34.4% v/v and 0.046 m^3 H_2/kg VS_0 at 4 days as HRT. This condition was the most suitable to sustain the production of biohythane as biofuel in a two-phases thermophilic-mesophilic process, where the solid-rich fraction can be used in the second anaerobic phase. However, in light of the highest VFA yield and the sufficiently high hydrogen accumulation, Run 2a (HRT 5 days and hydrolyzed feedstock) could also represent a good option for the future technology scale-up for VFA and biohythane production.

CRediT authorship contribution statement

Marco Gottardo: Investigation, Formal analysis, Data curation, Writing – original draft, Writing – review & editing, Supervision. **Joan Dosta:** Conceptualization, Data curation. **Cristina Cavinato:** Visualization. **Simona Crognale:** Conceptualization, Formal analysis, Data curation, Writing – review & editing. **Barbara Tonanzi:** Investigation, Data curation, Visualization. **Simona Rossetti:** Conceptualization. **David Bolzonella:** Conceptualization. **Paolo Pavan:** Visualization. **Francesco Valentino:** Supervision, Funding acquisition.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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