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Uncoupled hydrogen and volatile fatty acids generation in a two-step biotechnological anaerobic process fed with actual site wastewater

Research Paper

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Among agro-wastes, olive mill wastewater (OMW) truly qualifies as a high impact organic residue due to its biochemical-rich composition and high annual production. In the present investigation, dephenolized OMW (OMW_{deph}) was employed as the feedstock for a biotechnological two-stage anaerobic process dedicated to the production of biohydrogen and volatile fatty acids (VFAs), respectively. To this end, two identically configured packed-bed biofilm reactors were operated sequentially. In the first, the hydraulic retention time was set to 1 day, whereas in the second it was equal to 5 days. The rationale was to decouple the hydrolysis of the organic macronutrients held by the OMW_{deph}, so as to quantitatively generate a biogas enriched in H_2 (first stage aim), for the acidogenesis of the residual components left after hydrolysis, to then produce a highly concentrated mixture of VFAs (second stage aim). Results showed that the generation of H_2 and VFAs was effectively split, with carbohydrates and lipids, respectively, being the main substrates of the two processes. About 250 ml H_2 L^{-1} day^{-1} was produced, corresponding to a yield of 0.36 mol mol^{-1} of consumed carbohydrates (expressed as glucose equivalents). The overall concentration of VFAs in the acidogenic process was 13.80 g COD L^{-1}, so that 2.76 g COD L^{-1} day^{-1} was obtained. Second generation biorefineries use a selected fraction of an organic waste to conduct a microbiologically-driven pathway towards the generation of one target molecule. With the proposed approach, a greater value of the waste was attained, since the multi-purpose two-stage process did not entail competition for substrates between the first and the second steps.

Introduction

Agricultural, industrial, forestry, fishery and municipal organic leftovers can be used as renewable resources in the development of second generation biorefinery processes. The design of multi-purpose biorefinery schemes has been found to be fundamental to meeting the overall economic sustainability of the process at the large scale [1–6]. Due to the considerable versatility of anaerobic mixed microbial consortia, dark fermentative anaerobic processes dedicated to the production of H_2, volatile fatty acids (VFAs) or CH_4, can be easily adapted and integrated in biorefinery chains fed with renewable complex organic matrices. H_2 and VFAs have been obtained by processing several biowastes under hydrolytic [7,8] and acidogenic [9] conditions, respectively. Both approaches may represent the first step of two steps processes, the second of which being dedicated to the production of biomethane. Despite the higher complexity, hydrolytic processes were often reported as more efficient, because of the possibility of separating microbial populations responsible for the different digestion activities, thus optimizing related process parameters [10].

Among the most investigated agro-wastes for second generation biorefineries, Olive Mill Wastewater (OMW) is one of the most interesting, due to its high availability (more than 10 Mt in 2011) as well as its rich and biochemically-diverse composition (for a review see [11]). OMW was tested as the raw material for the

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production of H_2 [12–14] and VFAs [15,16]. Recently, the possibility of processing dephenolized OMWs (OMW_{deph}), under dark fermentative anaerobic conditions in packed bed biofilm reactors (PBBRs), for the production of biofuel and bio-based chemicals, depending on the applied hydraulic retention time (HRT), was demonstrated [17].

In the present investigation, the possibility of simultaneously obtaining both products has been tested employing the same kind of feedstock, by sequentially operating, in a two-step process, two independent PBBRs dedicated to these target products. Since the biological anaerobic consumption of the organic macronutrients, carbohydrates, proteins and lipids follow different kinetics, a first reactor with an HRT of 1 day was operated to produce an H_2-rich biogas, while the effluent of this reactor was fed to another identically configured PBBR operated for 5 days HRT to generate VFAs. Thus, the main rationale was to decouple the degradation of organic macronutrients carried by OMW_{deph}, by physically controlling, in different reactors, the processes leading to these target compounds.

Materials and methods

Dephenolized olive mill wastewater (OMW_{deph})

The OMW employed in this study was provided by the Sant'Agata d'Oneglia (Imperia, Italy) three-phase olive mill. Polyphenols were removed according to a solid phase extraction (SPE) procedure [29] developed and applied according to previous investigations [18]. The main OMW features after removal of polyphenols were: pH, 4.5; density, 0.9 g cm^3; chemical oxygen demand (COD), 51.9 ± 9.9 g L^{-1}; phenols, 0.93 ± 0.19 g L^{-1}; VFAs, 4.70 ± 1.3 g_{COD} L^{-1}; carbohydrates, 7.14 ± 0.6 g L^{-1}; proteins, 0.34 ± 0.04 g L^{-1}; lipids, 5.35 ± 0.6 g L^{-1}.

Microbial consortium

The acidogenic microbial consortium employed as the inoculum was obtained and microbiologically characterized within a previous investigation dedicated to the development of an acidogenic process fed with OMW_{deph} [15]. The inoculum was stored at 4°C before being used in this study.

Packed bed biofilm reactors (PBBRs)

The two PBBRs used in the present investigation had the same configuration. They consisted of a hermetically closed glass column (40 cm in height, 5 cm outer diameter) with an empty volume of about 0.8 L, operated under continuous anaerobic conditions. Columns were packed with ceramic cubes of Vukopor S10® (Lanik, Boskovice, CZ) whose dimensions, porosity and density were 25 mm × 25 mm × 18 mm, 10 ppi and 2.38 g mL^{-1}, 1, respectively. This support was selected according to previous investigations since it favoured acidogenic activity [16]. Reactors were equipped with a recycle line: the recycling ratio, expressed as the ratio between the recycled broth flow and the whole flow entering the column, was about 0.95. PBBRs were fed according to an up-flow scheme with HRTs equal to 1 and 5 days (HRT1 and HRT5, respectively), corresponding to Organic Loading Rates (OLRs) of 38.79 and 7.76 g L^{-1} day^{-1}, respectively. Both systems were initially provided with a mixture of OMW_{deph} and microbial inoculum (10%, v:v). Such a mixture was flushed with nitrogen and pumped up in the columns. Nitrogen gas was also sparged into the reactor's head space during inoculation. Reactors were continuously operated for 53 and 41 days (HRT1 and HRT5, respectively). However, both reactors needed 26 and 14 days, respectively, before biogas or VFA production was stable, that is, oscillating around a value ±20%. In particular, the process was considered stable when such productivity was maintained for 5 full liquid retention times (equal to 5 and 25 days in HRT1 and HRT5, respectively). Finally, the average performance and the confidence interval were calculated as the mean and standard deviation observed during the last 27 days. The process temperature was maintained at 35°C with serpentine silicon tubing continuously recycling temperature controlled water; the pH was corrected to 7 on a daily basis by manual dropwise addition of a 10 M NaOH solution. The two processes were monitored daily for biogas production and composition, VFA and COD concentrations.

Analytical procedures

VFA concentration was monitored with a GC-7890A (Agilent Technologies, Milano, Italy) with a Flame Ionization Detector (FID) under the following conditions: column temperature 170°C, inlet temperature 250°C, detector temperature 280°C, pressure 5 psi, gas carrier nitrogen. Before analyses, samples were diluted with a 60 mM oxalic acid solution. VFA concentration was reported as COD equivalents (g_{COD} L^{-1}) by using stoichiometric conversions. Phenols were evaluated as reported in [18] according to the conventional Folin-Ciocalteu procedure. Soluble COD concentration was determined spectrophotometrically on centrifuged samples (14,000 rpm, 10 min) according to the potassium dichromate colorimetric oxidation method using COD Vario Tube Test (Aqualytic, Dortmund, Germany) following the manufacturer's instructions, while total carbohydrates were determined according to [19] using glucose as a standard (Sigma–Aldrich, Milano, Italy). Total lipids were evaluated as reported in [20] using as a standard the olive oil produced at the industrial plant to which the wastewater belongs. Total soluble proteins were determined with the Bradford method [21], by using the commercial protein assay dye reagent concentrate from BioRad (Milano, Italy). In particular, in order to avoid interference with biomass, samples for soluble proteins were first placed at −20°C for at least 24 h, then brought to room temperature and centrifuged at 14,000 rpm for 5 min.

The amount of biogas was measured as reported elsewhere [22] by using a Mariotte flask, which was hydraulically connected to the reactor head spaces. The biogas composition was determined by gas chromatography using a μGC, model 3000A (Agilent Technologies, Milano, Italy), under the conditions described in [15]. Bioconversion of OMW_{deph} organic matter into VFAs (COD = >VFAs) was calculated as the ratio between produced VFAs and influent net COD excluding its VFA fraction. Analytical measurements always showed a standard deviation below 5%.

Results

Dephenolized three-phase olive mill wastewater (OMW_{deph}) was fed to a PBBR operated with a HRT equal to 1 day. The main activity observed within this reactor (HRT1) was a sustained production of a biogas enriched in H_2, producing H_2 at a rate of 252 ± 38 mL L^{-1} day^{-1}. The biogas had a relative content of $12.7 \pm 2.5\%$ H_2 (Fig. 1a). However, a concomitant small accumulation of VFAs was also observed (total VFA concentration was

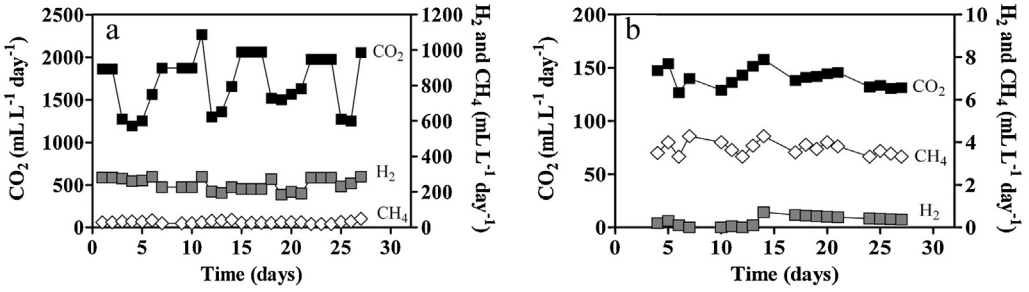

FIGURE 1

CO_2 (black squares), CH_4 (white diamonts) and H_2 (grey squares) profiles of productions as a function of time in HRT1 **(a)** and HRT5 **(b)** reactors.

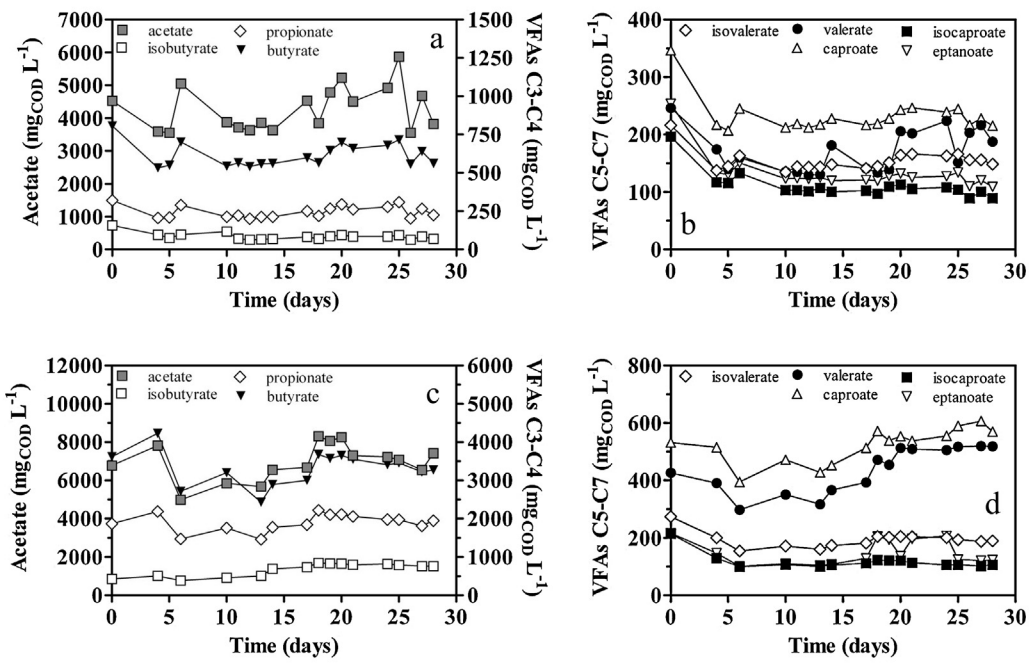

FIGURE 2

VFAs profiles of concentrations as a function of the time in HRT1 **(a,b)** and HRT5 **(c,d)** reactors.

$6.02 \pm 0.86\ g_{COD}\ L^{-1}$, equal to $1.32\ g_{COD}\ L^{-1}$ higher than that occurring in OMW_{deph}), and in the effluent the final pH was equal to 4.94 ± 0.27. Single VFA concentrations as a function of the experimental time in HRT1 effluent are shown in Fig. 2a,b. Acetic acid represented the main component of the VFA mixture. The process resulted in a small COD decrease (from 51.9 ± 9.9 to $45.7 \pm 3.5\ g\ L^{-1}$) and a consistent reduction of carbohydrates (from 7.14 ± 0.6 to $1.57 \pm 0.07\ g\ L^{-1}$), which were reduced by almost 80%. On the other hand, no significant change was noted in the protein and lipid content (0.45 ± 07 and $5.46 \pm 0.93\ g\ L^{-1}$, respectively), or the polyphenol content ($0.91 \pm 0.15\ g\ L^{-1}$).

The resulting effluent was then fed to a second PBBR, which was identically configured and operated with respect to the previous one except for the HRT, which was set to 5 days. Thus, the inlet for this second reactor (HRT5) was represented by the outlet of HRT1 ($HRT1_{OUT} = HRT5_{IN}$). An opposite pattern was observed in HRT5 compared to HRT1, since VFA production was consistent (from 6.02 to $13.80 \pm 2.3\ g_{COD}\ L^{-1}$). In particular, acetate, propionate and butyrate mainly occurred in HRT5 effluent (Fig. 2c,d); considering the concentration of VFAs in HRT1 effluent, the same acids were the most produced VFAs by the acidogenic process (6.7 ± 1.3, 1.9 ± 0.3 and $3.2 \pm 0.6\ g_{COD}\ L^{-1}$, respectively, Fig. 3). The final pH in the effluent ($HRT5_{OUT}$) was 5.89 ± 0.37. On the other hand, total biogas production in this reactor (i.e., $200 \pm 35\ mL\ L^{-1}\ day^{-1}$) was lower than H_2 production alone in HRT1; H_2 was only released in traces (Fig. 1b). This also applied to CH_4, which accounted for less than 3% of the total biogas production. COD decreased further from 45.7 to $31.4\ g\ L^{-1}$, and in particular, the degradation of carbohydrates was small (from 1.57 ± 0.07 to $0.88 \pm 0.06\ g\ L^{-1}$), while lipid degradation was enhanced (from 5.46 ± 0.9 to $3.17 \pm 0.3\ g\ L^{-1}$). Interestingly, a small yet significant accumulation of total soluble proteins was noted (from 0.45 ± 0.07 to $1.56 \pm 0.1\ g\ L^{-1}$). Since interference

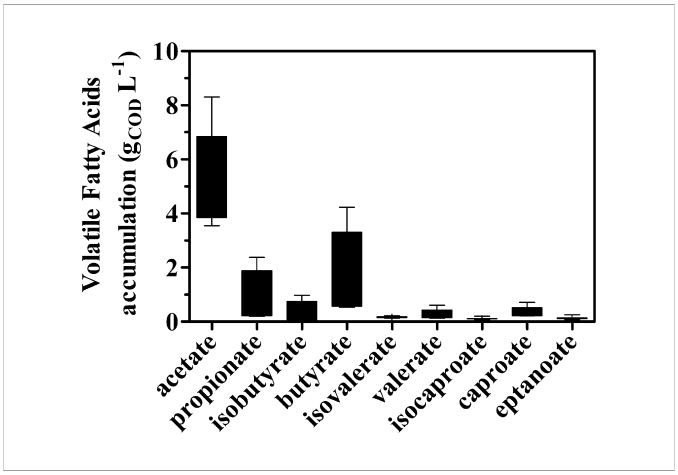

FIGURE 3

Accumulation of single VFAs in the HRT5 reactor.

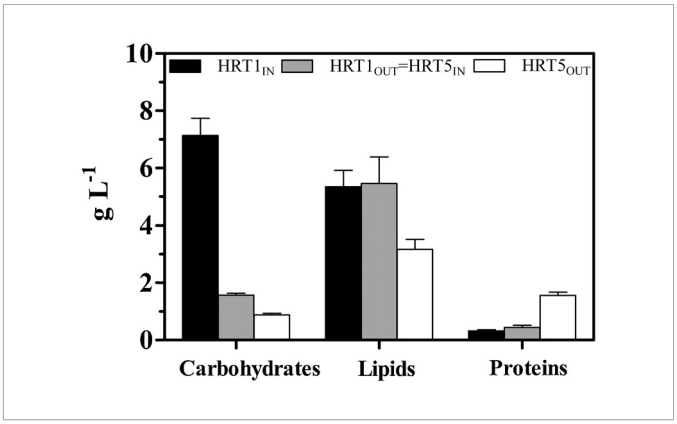

FIGURE 4

Concentration of carbohydrates, lipids and proteins in process feeding (OMW_{deph}) ($HRT1_{IN}$), HRT1 reactor effluent ($HRT1_{OUT}$ = $HRT5_{IN}$) and HRT5 reactor effluent ($HRT5_{OUT}$).

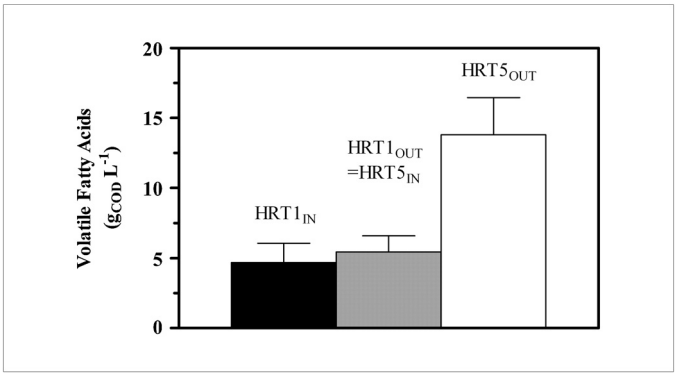

FIGURE 5

Total VFAs concentration in process feeding (OMW_{deph}) ($HRT1_{IN}$), HRT1 reactor effluent ($HRT1_{OUT}$ = $HRT5_{IN}$) and HRT5 reactor effluent ($HRT5_{OUT}$).

of biomass was prevented, particularly in samples analysed for proteins, such an increase may probably be due to soluble proteins released during the fermentation process. Finally, the polyphenolic content decreased slightly to 0.81 ± 0.14 g L^{-1}.

A summary of the organic macronutrient consumption and VFA accumulation after both PBBRs is reported in Figs. 4 and 5, while process yields of these reactors are shown in Table 1. In particular, it should be noted that COD conversion into VFAs increased from about 3 to 20% between HRT1 and HRT5. The final VFA content in HRT5 effluent represented almost 44% of the COD. However, it should also be noted that when summing HRT1 and HRT5 processes, about 40% of the initial OMW_{deph} COD was consumed (Table 1).

As a whole, carbohydrate consumption within HRT1 was observed in conjunction with significant H_2 production. Similarly, VFAs were produced to a large extent within HRT5 concurrent with a reduction of lipids. However, the processes were operated with very different HRTs, and when considering daily production/consumption activity, some interesting observations could be made. The total VFA production rate between PBBRs was comparable (1.32 vs. 1.56 g_{COD} L^{-1} day^{-1} in HRT1 and HRT5, respectively, Table 2), as was soluble protein release (0.13 vs. 0.22 g L^{-1} day^{-1} in HRT1 and HRT5, respectively, Table 2). However, the consumption of carbohydrates was sustained during HRT1, while in HRT5 this activity was severely down-regulated by 40 times (5.57 vs. 0.14 g L^{-1}), probably as a consequence of the fact that few carbohydrates were left after HRT1 operation. These considerations also apply to the lipid content in the wastewater, since its consumption was only observed in HRT5.

Similarly, when applying a per day comparison with respect to the net production of VFAs in HRT1 and HRT5, the rates of production were similar with the only exception being propionate production, which was absent in HRT1 but consistent in HRT5 (0.01 vs. 0.32 g COD L^{-1} day^{-1}, Table 3).

Discussion

Theoretically, in dark fermentative anaerobic digestion processes, hydrolysis, acidogenesis, acetogenesis and methanogenesis occur sequentially. Nevertheless, in practice, development of microbial communities that perform all of these pathways concomitantly up to methanogenesis is observed. However, some operational parameters can be adopted as tools to severely affect, on a quantitative basis, the concomitant occurrence of these phenomena. For

TABLE 1

Process yields of acidogenic anaerobic processes operated in series with different hydraulic retention times.

	VFA_{OUT}/COD_{IN} %	VFA_{OUT}/COD_{OUT} %	COD = >VFAs %	COD removed %
OMW_{deph}	–	9.1	–	–
HRT1	11.6	13.2	2.8	11.9
HRT5	30.2	43.9	19.6	31.2
HRT1 + 5	26.6	43.9	19.6	39.4

TABLE 2

Comparison of acidogenic digestion processing parameters in reactors operated in series with 1 (HRT1) and 5 days (HRT5) hydraulic retention time.

Processing parameters	HRT1	HRT5	
	g L^{-1} (= g L^{-1} day^{-1})	g L^{-1}	g L^{-1} day^{-1}
COD[a]	−6.18	−14.26	−2.85
VFAs[a]	+1.32	+7.78	+1.56
Total sugars	−5.57	−0.68	−0.14
Total proteins	+0.13	+1.11	+0.22
Total lipids	±0.00	−2.29	−0.46

[a] COD and VFAs are expressed in g_{COD} L^{-1} equivalent.

TABLE 3

Net volatile fatty acids (VFAs) productions during acidogenic digestion of dephenolized Olive Mill Wastewater (OMW_{deph}) at different hydraulic retention times.

Net VFAs production (g_{COD} L^{-1} day^{-1})	Acetate	Propionate	Isobutyrate	Butyrate	Isovalerate	Valerate	Isocaproate	Caproate	Eptanoate	Total
HRT1	0.40	0.01	0.08	0.33	0.09	0.13	0.09	0.12	0.08	1.32
HRT5	0.48	0.32	0.12	0.52	0.01	0.05	0.00	0.06	0.00	1.56

instance, in continuously operated reactors, one of the most typical parameters known to act this way is the HRT. Reduction of HRTs typically prevents processes which occur at a later stage, such as acetogenesis and methanogenesis [23]. In particular, full suppression of methanogenesis has been observed in granular biomass [24], that is, in a system where biomass retention is significantly higher than with the biofilm reactors adopted in this study. The concept of a two-phase anaerobic digestion system is not new, though it was initially proposed [25] and then later widely applied (reviewed in [26]) to separate hydrolytic/acidogenic activity from acetogenic/methanogenic activity. In the present investigation, this approach has been adopted to decouple hydrolysis and acidogenesis, with the final aim of achieving both a biofuel (H_2) and a biobased chemical (VFA) production, according to a modular (cascade) approach. In particular, provided that biodegradability of organic macronutrients increases with HRT in the order carbohydrates, proteins and lipids [27], the rationale of the present work was to link (within a biochemically-homogeneous waste organic fraction) one specific substrate to one target product, namely, carbohydrates to H_2 and lipids to VFAs.

Indeed, the main product of hydrolysis (i.e., H_2) was released at a high rate in the reactor operated with an HRT of 1 day, apparently as a result of intense consumption of carbohydrates (Fig. 4), provided that lipids were not consumed, and soluble proteins were substantially absent in the dephenolized OMW (OMW_{deph}) fed to the reactor (Fig. 4). This evidence allowed the yields of H_2 produced per feed or consumed carbohydrates (expressed as glucose equivalents) to be calculated, 0.36 and 0.25 (mol mol^{-1}), respectively. The yields obtained are an order of magnitude lower than those of processes fed with pure glucose or monosaccharide-rich organic matrices, such as molasses or cheese whey [8]; however, they were comparable with respect to the performance of a few reported processes which were fed with OMWs [12–14,17]. Among them, those carried out under continuous conditions, including our previous study [17], gave rise to very similar productivities (namely, 330 [14] and 146 ml L^{-1} day^{-1} [17]) when reactors were fed with the same HRT of 1 day; however, diluted OMW was employed in the latter experiment [14]. The relative H_2 content in the produced biogas was quite low. Normally this would be a concern, in the event of a subsequent step dedicated to biogas purification. However, this study represents a proof of concept and processes were not optimized. When the effluent of the first PBBR was supplied to a second one operated with a longer HRT (i.e., 5 days), the product of acidogenesis (i.e., VFAs) accumulated significantly (Fig. 5), while no H_2 was produced (Fig. 1b). Herein, given that proteins were absent and that not many carbohydrates were left in the HRT1 effluent, lipids were apparently used as the main substrate (Fig. 3). The metabolic route accounting for VFAs production from lipids is already known, and has been described in the Anaerobic Digestion Model proposed in [28]: lipids are degraded into monomers, which then follow the same pattern of sugars derived monomers. The fact that lipids are consumed at longer HRT has also been reported, although only for the purpose of studying methanogenic activity in OMWs [29,30] and other wastewaters and sludges [31–34].

However, although the acidogenesis yields were significantly different between the two PBBRs (Table 1, Fig. 5), yields on a per day basis concerning the performance of the two reactors were fully comparable, with the only exception being the propionate production, which was absent with short HRTs (Table 3). Nevertheless, when comparing these results with those achieved in identically configured PBBRs operated independently (i.e., not sequentially as in this study) [17], COD conversion into VFAs (COD = >VFAs) was higher, and COD removal rates were lower with respect to that found in this investigation. This means that generating two products (i.e., H_2 and VFAs) by setting up two separate reactors may require an extra energy source, which eventually affects the yields of the second process.

As a consequence, the amount of total VFAs in the final effluent was lower than the highest VFA concentration obtained in the previous study when an HRT of 5 days was applied (13.80 vs. 19.67 g COD L^{-1}) [17].

Conclusions

In the present study, the feasibility of decoupling a sustained hydrolysis, aimed at the production of H_2 by consuming carbohydrates, from acidogenic activity to generate VFA mixtures by using the remaining macronutrients (e.g., lipids) has been tested. The significance of this work lies with second generation biorefinery approaches, whose main goal is to maximise the value of biowastes through development of non-competitive processes, in order to gain multiple useful products (multi-purpose biorefinery schemes). Furthermore, together with a biofuel (H_2) and building blocks such as VFAs, high added-value polyphenols were also previously obtained in a viable solvent [35].

Despite the loss of efficiency compared to single-purpose processes [17], the present approach is a promising proof of concept, whose sustainability should be further evaluated with respect to seasonality of the residue, different bioresources, and scaled-up systems. Indeed, updated techno-economic analyses indicate that multi-purpose biorefinery schemes are the most sustainable refining hypotheses at large scale [1–6]. In this perspective, further implementation of the present approach may be achieved by efficient separation technologies (applied to both H_2 and VFAs) and water recovery.

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