Comparison of single-stage and temperature-phased anaerobic digestion of sugar beet by-products

K. Aboudi*, X. Gómez-Quiroga, C.J. Álvarez-Gallego and L.I. Romero-García

Chemical Engineering and Food Technology Department, University of Cádiz, Campus Río San Pedro, 11510 Puerto Real, Spain
*kaoutar.aboudi@uca.es

Abstract

This study investigated in single-stage and temperature-phased long term semi-continuous, the anaerobic digestion of sugar beet by-products (exhausted sugar beet cossettes). Comparison of different configurations of single stage (mesophilic and thermophilic conditions) with temperature phased (by coupling thermophilic or hyper-thermophilic with mesophilic conditions) have been studied. The mesophilic single stage significantly outperformed both single stage thermophilic condition and mesophilic stages of temperature-phased conditions. Therefore, specific methane production in the mesophilic single stage was 48% and 78% higher than the obtained in the temperature-phased conditions, respectively from thermophilic-mesophilic and hyper-thermophilic-mesophilic reactors. Volatile solids removal has been similar in both single stage and two-phased conditions. It has been concluded that for sugar beet by-products, the temperature increase had a detrimental effect on the biogas production and the process performance. Methanogenesis has been found to be the limiting step in the two-phased conditions. The high acetate accumulation resulted of low tolerance of methanogenic populations.

Keywords: anaerobic digestion, single stage, temperature-phased, Thermophilic, hyper-thermophilic, mesophilic

Introduction

Anaerobic digestion (AD) has demonstrated to be an effective technology to recover renewable energy from organic wastes [1]. The AD process implies a four sequential steps: hydrolysis, acidogenesis, acetogenesis and methanogenesis [2]. It is widely accepted that the two kind of microorganisms which are typically involved in the AD process are acidogenic bacteria and methanogens archaea [3]. However, the existence of each kind of microorganisms depends strictly to the environnemental conditions of the digester (i.e. temperature, pH, substrate characteristics, etc.).

Temperature is an important factor in the anaerobic process [4]. Therefore, temperature conditions influence the microbial population dynamics, affecting the kinetic microorganisms growth and, hence, both the degradation of the organic material and its conversion into biogas [5–9]. Thermophilic anaerobic systems (55°C) have become the more widespread choice due to the high hydrolysis rate reachable for treating wastes since those systems allow to handle higher organic loading rates and led to higher organic matter degradation efficiency [10]. However, thermophilic systems are more vulnerable to abrupt temperature changes which may generate high fractions of free ammonia and volatile fatty acids that inhibit the process [11].

Methane generation is also feasible under higher temperatures above 55°C at hyper-thermophilic conditions. Hyper-thermophilic temperatures have been usually chosen to treat wastes with high protein, lipids and solid matter, such as food wastes and manures [2,3,6,7,12]. Nevertheless, hyper-thermophilic conditions may present

the risk of unstable systems and high organic loading rates are difficult to be applied. Otherwise, mesophilic anaerobic systems (37°C) have commonly been used since they offer more stability [7,12]. Moreover, mesophilic conditions had shown higher net energy production than thermophilic conditions due to reduced heating costs [13].

Thermodynamic equilibrium in the AD process shows that endergonic reactions (i.e. degradation of propionate into acetate, CO_2, H_2) would be energetically more favorable at high temperatures, while exergonic reactions (i.e. hydrogenotrophic methanogenesis) are less favored at high temperatures [14].

The combination of the advantages of both thermophilic or hyper-thermophilic with mesophilic systems has been widely used in a temperature phased systems to favour both bio-hydrogen and bio-methane generation [15–17]. In this process, two physically separated reactors are used in which each reactor operates at a different temperature under controlled conditions. Both devices are microbiologically separated: in the first device the hydrogenic stage -hydrolysis and acidogenesis- is carried out, and in the second device, the methanogenesis stage - acetogenesis and methanogenesis- occurred [18,19]. However, it has been reported that TPAD systems are not always suitable and advantageous than single stage systems to ensure microbial communities efficiency and stability [17,20]. Therefore, depending on the substrate characteristics and environmental conditions, this process may have limitations [21]. In TPAD assays, along with substrate characteristics, the rates of the organic material hydrolysis and the amount of VFAs generated in the first step and its conversion into methane in the second stage, are crucial for an efficient process [4].

This study was thus conducted to investigate the single stage (SSAD) and temperature-phased (TPAD) of sugar beet by-products. In the single stage assays both thermophilic and mesophilic conditions have been studied. The TPAD processes used in this work were formed by coupling thermophilic or hyper-thermophilic reactors (stage I) with mesophilic reactors (stage II).

2. Material and methods

2.2. Substrate

The substrate used in this study consisted of sugar beet by-products (SBB) and it was collected from "Azucarera" company (AB-sugar group) situated in southern of Spain. SBB were in dry pellets form (8 mm diameter) composed of the exhausted sugar beet cossettes pulp after several extraction processes. Pellets were stored at -20 C before the feeding procedure and were rehydrated with distilled water 24 hours before the daily feeding (once a day).

2.1. Semi-continuous reactors start-up procedure (SS and TPAD)

Six semi-continuous stirred tank reactors (SCSTR), were used in this study at a total solids of 8% [22].The raw pellets had a TS content of 829.15 ± 2.43 g/kg which was diluted with deionised water to achieve the desired concentration of TS. The SCSTR consisted of a 2.5 L working volume. stainless steel reactors. The temperature condition of each reactor was maintained by a heating plate located at the base of each reactor which was covered by a metal jacket for better heat transfer. Temperature was continuously monitored by an inner temperature sensor and controlled by a PID (Proportional-Integral-Derivative) control system. Each reactor had an independent motor agitation and a stirring blade [23].

To facilitate the discussion of results, each operation phase was named according to system operation and temperature conditions. Table 1 Illustrate detailed conditions of each assay. Table 2 illustrate physical and chemical properties of SBB employed. The start-up for methanogenic reactors in both SS and TPAD started at

the HRT of 20 days according to [24]. [25] reported that in SCSTR, the HRT controls the microbial growth rate and therefore HRTs must be greater than the maximum growth rate of microorganism, because faster dilution rates cause washout. The same feedstock consisting on rehydrated pellets of exhausted sugar beet was added in both single stage at thermophilic and mesophilic digestion at HRT and OLR of 20 days and 3.4 ± 0.2 gVS/L_r^*d, respectively. HRT of 10 days and OLR 6.6 ± 0.4 gVS/L_r^*d was chosen for acidogenic reactors while a HRT of 20 days (OLR= 2.3-2.5 gVS/L_r^*d) was applied for methanogenic reactors in order to make mesophilic reactors comparable. The two stage mesophilic digesters were fed with effluent from acidogenic thermophilic or hyper-thermophilic reactor according to each condition. The single-stage and two-stage processes were operated for around 7 months.

Table 1. Operating conditions for single-stage (SSAD) and temperature-phased (TPAD) assays

Runs	Process / Temperature / Phase	HRT (days)	OLR (gVS/Lr* d)
RTA	SSAD Thermophilic Acidogenic	10	6.6 ± 0.4
RHA	SSAD Hyper-thermophylic Acidogenic		
RTM	SSAD Thermophilic Methanogenic	20	3.4 ± 0.2
RMM	SSAD Mesophilic Methanogenic		
R2TM	TPAD Thermophilic Acidogenic - Mesophilic Methanogenic	20	2.3 ± 0.1
R2HTM	TPAD Hyper-thermophilic Acidogenic - Mesophilic Methanogenic		2.5 ± 0.2

Table 2. Characteristics of SBB used in this study

Items	**Values**
pH	5.84 ± 0.47
TS (%)	82.91 ± 0.24
VS (%TS)	91.76 ± 2.48
T-COD (g O_2/kg) *	154.84 ± 13.57
S-COD (g O_2/kg) *	64.52 ± 2.48
DOC (g C/kg) *	41.82 ± 6.08
TVFA (g HAc/kg) *	5.27 ± 0.75
NH_4^+-N (g N/kg) *	0.33 ± 0.06
T-N (g N/kg) *	8.09 ± 1.54
Alkalinity (g$CaCO_3$/kg) *	0.11 ± 0.01
Carbohydrates (g/kg) *	972.90 ± 62.96
- Glucose (g/kg) *	268.45 ± 30.24
- Fructose (g/kg) *	204.40 ± 15.78
- Sucrose (g/kg) *	500.05 ± 16.94

*g/kg on wet basis.

2.4. Analytical parameters

Standard methods for the Examination of Water and Wastewater [26] were used to analyse Total solids (TS), volatile solids (VS), total and soluble chemical oxygen demands (T-COD, S-COD), dissolved organic carbon (DOC), pH, alkalinity and ammonium. Samples of soluble parameters (S-COD, DOC) and volatile fatty acids

(VFAs) were previously lixiviated for 2 h with deionized water and then filtered through a 0.47 mm filter [27]. Samples of VFAs were filtered again through a 0.22 μm Teflon® filter and analysed with a gas chromatograph (Shimadzu® GC-2010) equipped with a flame ionization detector (FID) and a capillary column filled with Nukol® (diameter of 0.25 μm and 30 m length). T-COD was measured directly from the diluted effluent without any previous filtration and total nitrogen (T-N) was directly analysed from the dried sample. The dissolved organic carbon (DOC) analysis was carried out in an Analytic-Jena® multi N/C 3100 carbon analyser with a chemiluminescence detector (CLD) according to the combustion-infrared method (5310B) of the Standard methods [26]. Biogas generated during the assays was collected in a 10 L Tedlar® gas bag (SKC) and its volume was measured daily using a high precision drum-type gas meter (Ritter® TG5). Composition of biogas (H_2, CH_4, CO_2) was determined by using a gas chromatograph (Shimadzu® GC-2010) with a stainless steel column packed with Carbosieve® SII (diameter of 3.2 mm and 3.0 m length) and a thermal conductivity detector (TCD).

3. Results and discussion

3.1 Single-stage mesophilic and thermophilic anaerobic digestion

Fig 1 shows the evolution of the operational parameters of the single stage reactors at both mesophilic and thermophilic conditions. The accumulated and the daily methane productions for the mesophilic and thermophilic single stage reactors are depicted in Fig 1 (a) and (b), respectively. As seen in the Fig 1 **(a),** the reached accumulated methane in the mesophilic reactor was 1.5 fold higher than the obtained at thermophilic conditions. The MPR in RMM started with higher daily productions with average values of 1.20 ± 0.38 LCH_4/Lr^*d and then decreased to lower, but stable values for a long operation time with an average MPR of 0.62 ± 0.21 LCH_4/L_r^*d. The SMP of this reactor was at 188.30 ± 31.62 $mlCH_4/gVS_{added}$ (Table 3). In the RTM, the MPR has shown fluctuations with low values in the start-up period before stabilising at values of 0.30 ± 0.12 LCH_4/Lr^*d for a long period (Fig 1**(b)**). As the operation conditions have been similar in both thermophilic and mesophilic temperatures (OLR and HRT), it has been expected that higher productions could be obtained from the thermophilic reactor [28]. However, this aspect is strictly related to the nature of the substrate used. Hence, for some substrates, the high hydrolysis rate of the organic matter content could imbalance the activity of microorganisms responsible to convert the hydrolysed material into methane [29,30]. In a previous study of Da Ros et al., [29], authors studied mesophilic and thermophilic anaerobic co-digestion of winery wastewater sludge and wine lees and observed that mesophilic rector operated in stable conditions while the thermophilic reactor failed after only one HRT (23 days). They reported that this was due to volatile fatty acids (VFAs) accumulation in the medium which stopped microorganism's activity. Similarly, in the present work, VFAs accumulation seemed to be the reason of the lower methane productions observed in the RTM. Fig 1 **(c)** and **(d)** shows the evolution of the VFAs generated, respectively in RMM and RTM.

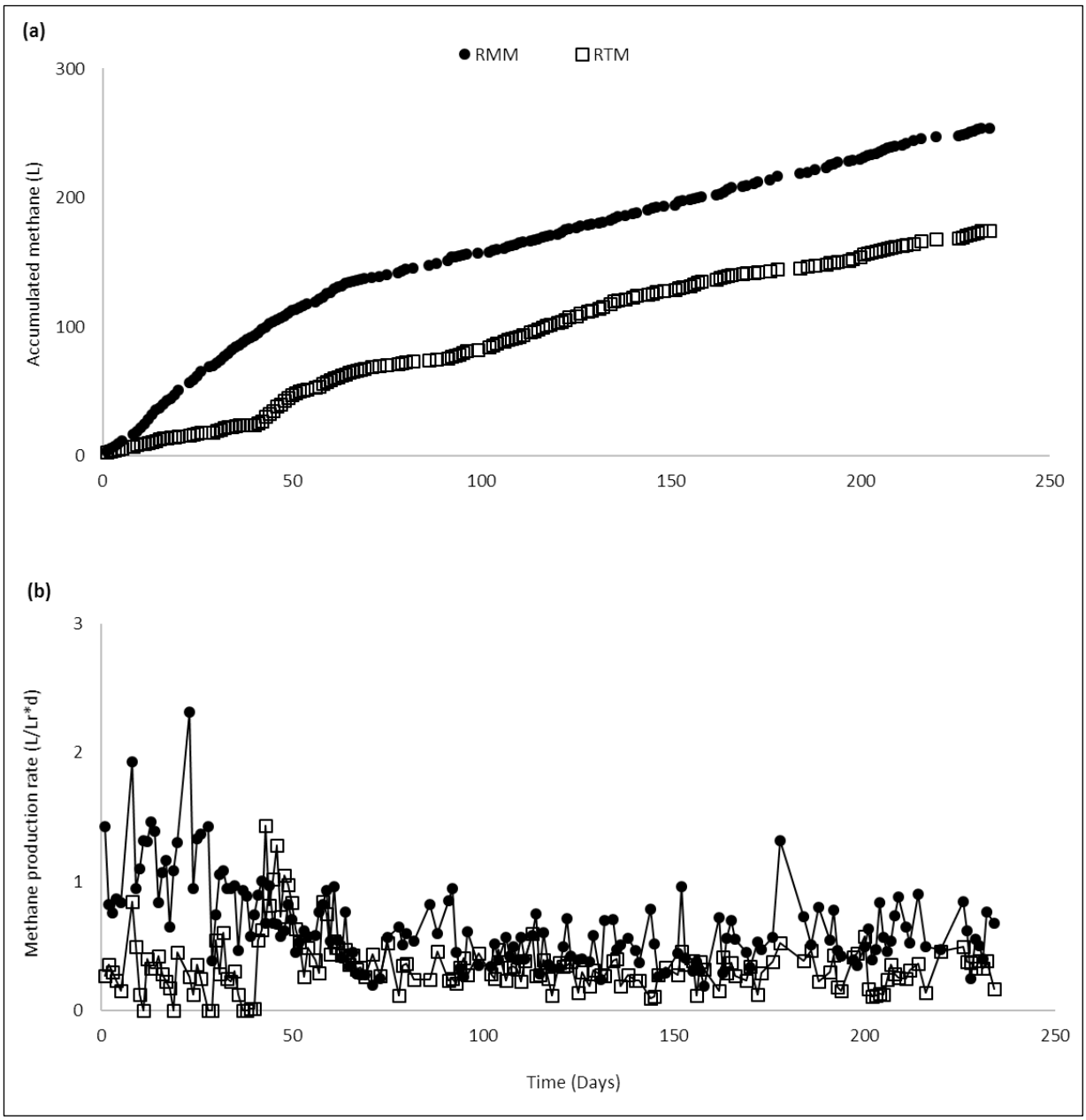

Fig. 1 Accumulated methane and methane production rates in the single stage mesophilic and thermophilic reactors

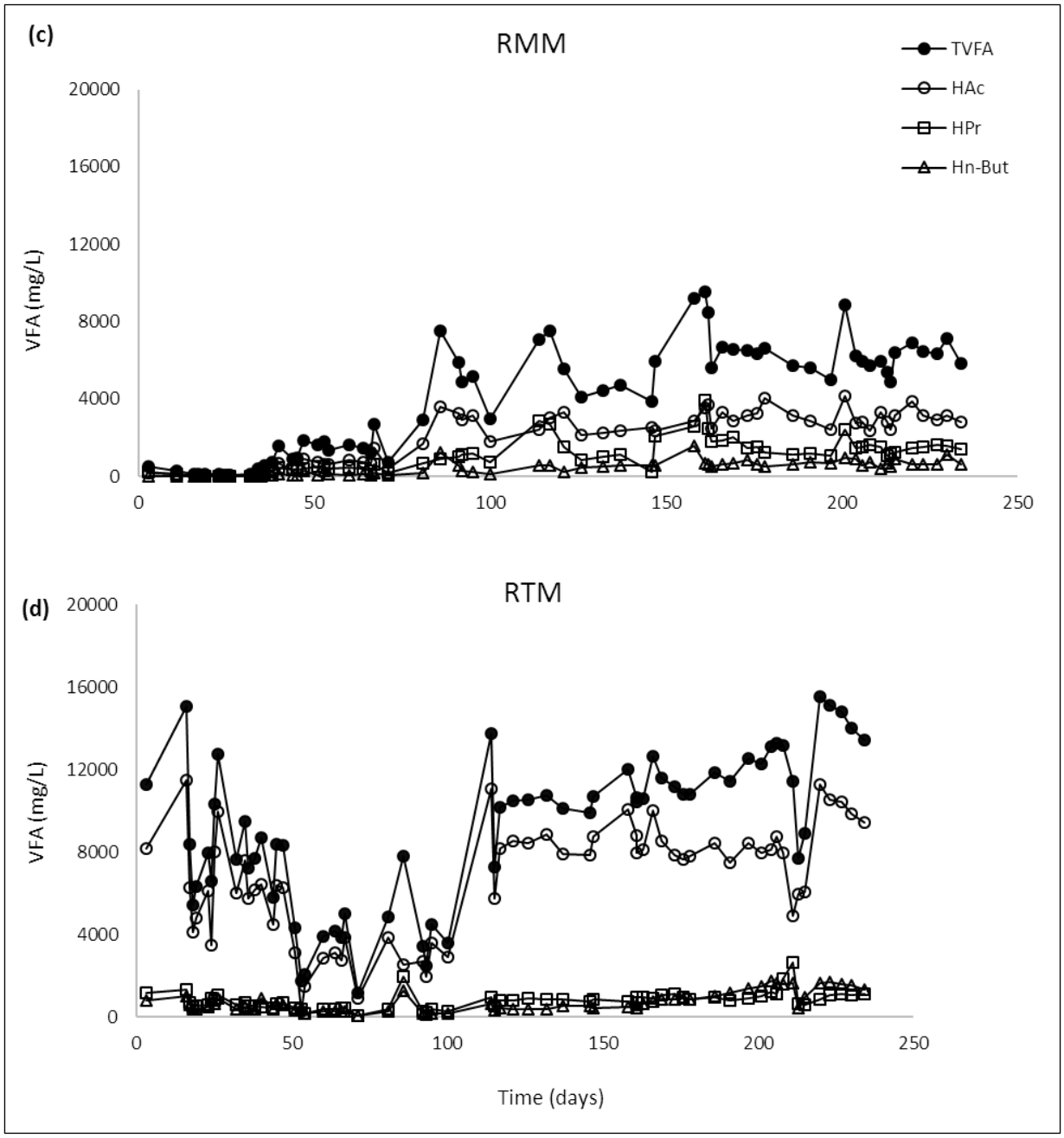

Fig. 2 Volatile fatty acids patterns in the single stage mesophilic and thermophilic reactors

It can be observed from the Fig 1 (c) and (d) that in RTM VFA levels were higher than the obtained in RMM. Nevertheless, in both temperature conditions, the predominant organic acid was the acetate. Thus, the RMM and during the first 70 days, the VFAs does not exceed 4000mg/L, however from that period on, VFAs levels started increasing constantly reaching maximum HAc and HPr concentrations around 3054.61 ± 495.18 mg/L and 1688.11 ± 624.52 mg/L, respectively. For the RTM, the HAc concentrations exceeded 8000 mg/L, indicating activity inhibition or low acitivity of the acetate consuming microorganisms (acetoclastic methanogenesis or syntrophic acetate oxidizing microorganisms) [10,31].

Despite of high VFAs levels, the inhibition was not complete as methane stilled producing even with low productions. It has been reported that inhibition due to VFA accumulation may depend on the substrate characteristics, the inoculum source and other operation conditions [29,32].

Li et al., [33] reported that for AD of raw coffee and ground coffee, the methane production potential decreased by more than 15% under thermophilic condition due to VFAs accumulation, whereas it increased from 35.8% to 48.2% when using a waste activated sludge. Hence, Li et al., [33] observed that acetate concentrations of 10000 mg/L does not affect thermophilic reactors and slow down the methanogenic activity of mesophilic reactors. While concentrations of 20000 mg/L totally inhibited the methanogenic activity at thermophilic conditions. Regarding the propionate inhibition effect, authors reported that 5000 mg/L totally inhibited the methanogenic activity at thermophilic conditions while at the mesophilic ones no significant inhibition was observed. In a previous research of Yu et al., [10] studied the effect of temperature (mesophilic and thermophilic) on the archaeal community in the co-digestion of biowaste and sewage sludge. Authors reported that instead of high biogas obtained at thermophilic conditions, the low temperature. had a positive effect on hydrogenotrophic methanogens.

In another attempt, Koyama et al., [34] studied the effect of alkaline pre-treatment on mesophilic and thermophilic anaerobic digestion of a submerged macrophyte (lignocellulosic-type waste) and reported that during the steady state period, mesophilic conditions achieved a 42% increase in the CH_4 yield using pre-treatment, while thermophilic conditions yielded only an 8% increment. The low methane productions have been related to the volatile fatty acids accumulated in the thermophilic pre-treated reactor.

Riggio et al., [30] compared thermophilic and mesophilic AD of spent cow bedding in leach-bed reactors and they reported the use of thermophilic conditions enhanced only the degradation kinetics of easily-degradable matter during the first days of the digestion, whereas similar methane yields were reached at both temperatures. Therefore, authors reported that the anaerobic digestion in LBRs of spent cow bedding, a substrate rich in slowly-degradable compounds, was not improved in term of methane production considering the overall digestion time by increasing the temperature to thermophilic conditions. Contrastingly, Ghasimi et al., [28] used bench-scale thermophilic and mesophilic anaerobic digestion of fine sieved fraction obtained from the influent of a municipal wastewater treatment plant (mainly composed of cellulose from toilet paper). They reported thermophilic digesters outperformed the mesophilic ones, improving process performance.

The ratio of TVFA to alkalinity has been widely used as indicator of an AD system stability [11]

Fig 1 (e) and (f) shows the profile of TVFA/alkalinity ratio and the pH in the single digestion reactors. It should be noted that due to acidification of the RTM, it was necessary to adjust the pH daily by using an alkali (K_2CO_3).

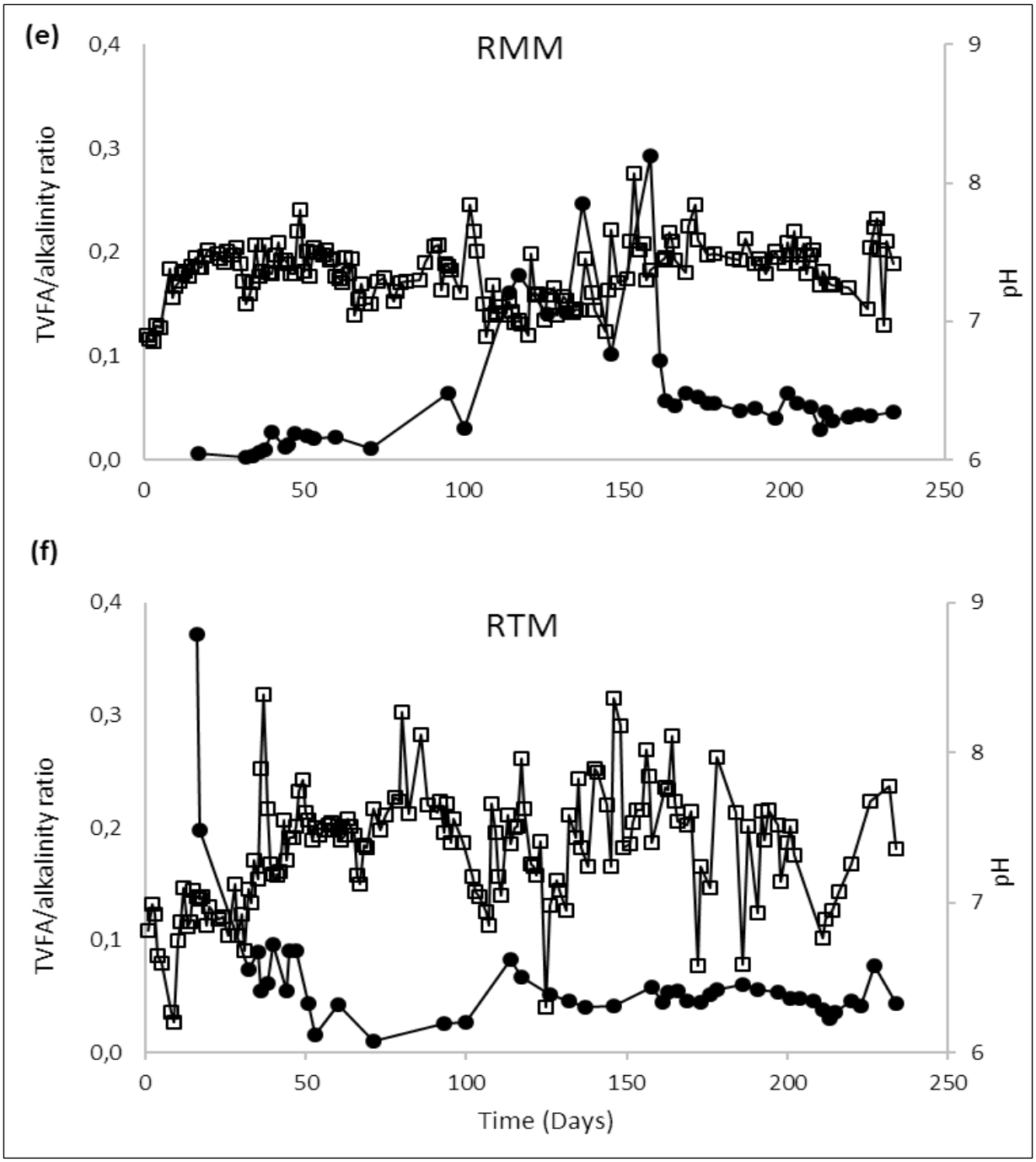

Fig. 3 The TVFA/alkalinity ratio patterns in the single stage mesophilic and thermophilic reactors

Higher fluctuations in the pH values were observed in RTM compared with the RMM (Fig 1 (e) and (f)). It has been reported that mesophilic conditions offer more stability in an anaerobic digestion reactor [29,35]. In both assays the TVFA/alkalinity ratios have been in the required range below 0.4 [11]. However, in the RTM, the TVFA/alkalinity values showed more stability after around 50 days of the assay, likely due to the high alkalinity in the reactor due to the daily alkali addition. In a proper operation of an anaerobic digester, the alkalinity existing inside the digester may neutralize the excess of acidity to maintain an optimum range of the pH. As seen

from Fig 1 (f), there was a severe increase of the ratio TVFA/alkalinity in RMM between days 70 and 150, but without exceeding admissible values [11] , because of the increase in the VFAs values during this period, where adjustment of the pH has been done regularly. In the RTM and instead of the lower TVFA/alkalinity ratio, the existent high alkalinity in the system was no longer sufficient to neutralize VFAs, specifically, the high acetate concentrations prevalent in this reactor. Li et al., [33] reported that for the AD of coffee ground and waste activated sludge, under both mesophilic and thermophilic conditions, the acetate concentrations above 20000mg/L totally inhibited the methanogenic activity in thermophilic reactors while at mesophilic conditions, the methanogenic activity decreased but does not stop. Moreover, they reported that propionate concentrations of 5000 mg/L inhibited thermophilic reactors and led to lower activity in the mesophilic reactor. The same authors reported that instead of the large consideration in the literature of the hydrolysis as the rate limiting step in the AD process, this parameter couldn't be defined generally and that depending on the substrate characteristics and the temperature conditions, the limiting step may vary. In their research, authors reported that acidogenesis was the rate limiting step for mesophilic conditions while the acetogenesis was found to be the rate limiting step for thermophilic reactors, because the conversion of VFAs into acetate has been affected. Contrastingly to the present work and for both RTM and RMM, the methanogenesis was found to be the limiting step, because the conversion of acetate into methane has been altered.

Table 3 shows the organic matter removal efficiencies in the RTM and RMM. The VS removal reached were at 79.2 % and 57.9 %, respectively for the RMM and the RTM.

3.2 Process performance of two-stage anaerobic digestion

3.2.1. Two-phased acidogenic thermophilic-methanogenic mesophilic reactors

Fig 2 shows evolutions of the pH and the biogas production rates (hydrogen (a) or methane (b)) in the acidogenic thermophilic reactor (RTA) and the methanogenic mesophilic (R2TAM), respectively.

As Fig 2 (a) shows, in the start-up of the process, hydrogen productions increased with the pH decrease from 7 to values between 5-6. However, from days 32 to 46, hydrogen productions decreased as a consequence of the pH increase to values above 6.5. Thereupon, and as the pH has been maintained in values ranged of 5.5-6 [36]. The HPR also showed a stability with an average daily productions of 0.45 $LH_2/L_r^*d \pm 0.15$ LH_2/L_r^*d. pH correction has been necessary only when the pH dropped below 5.5 with an alkali (potassium carbonate). This reactor showed a SHP of 87.06 ± 0.34 ml H_2/gVS_{added} (Table 3).

The obtained HPR from the acidogenic reactor has been lower than the obtained from [37]. However, the obtained SHP are higher. Authors studied TPAD of maize silage and reported HPR and SHP of 1.19 ± 0.22 LH_2/L_r^*d and 35.5 ± 16.5 ml $H_2/gVS_{added,}$ respectively. Otherwise, the obtained HPR in the present research was similar to those reported by [38] in a TPAD assays of the organic fraction of municipal wastes (0.43 LH_2/L_r^*d at HRT of 3 days and OLR 16 gVS/Lr*d). However, authors found a significantly lower SHP of 29 ml H_2/gVS_{added}.

It should be noted that the organic matter acidification and conversion into hydrogen, is strictly related to the substrate characteristics. Hence, Lindner et al., [39] studied the suitability of the TPAD assays for different kind of substrates including sugar beet (SB). Authors found that SB had a high sugar content and led to a higher acidification (pH drop) and hence, a high hydrogen production. Similarly, Alkaya and Demirer [40] studied the effect of operational parameters (HRT, OLR, pH) on the enhancement of acidification from sugar beet wastes (wastewater and pulp) and reported that the increase of sugar beet pulp in the mixture with lower HRT operation

conditions led to a high acidification of the digester as a results of the increase of VFA generation from this substrate. Angeriz-Campoy et al., [41] reported a HPR of 0.64 LH_2/L_r^*d from acidogenic thermophilic reactor of OFMSW and lower than 1.07 LH_2/L_r^*d obtained by Romero Aguilar et al., [42].

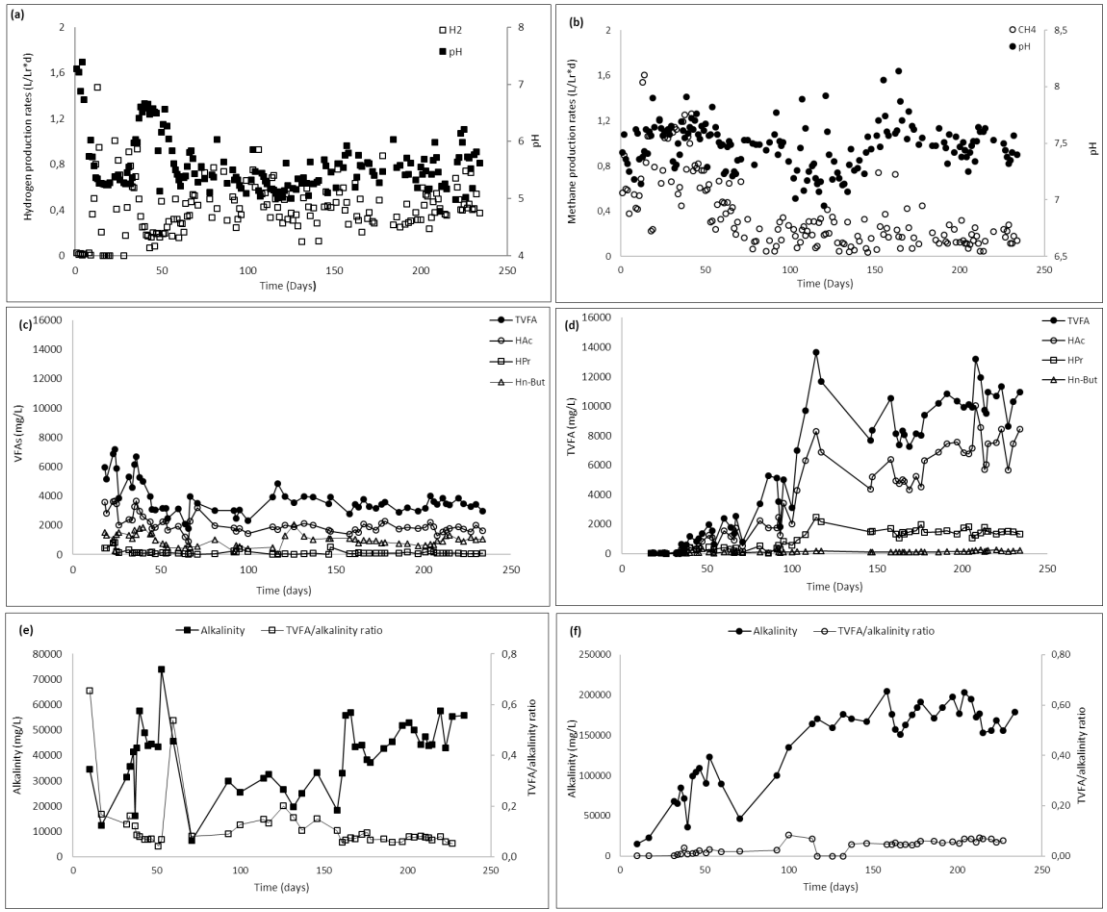

Fig. 4 Biogas production rates (H_2 and CH_4), VFAs, Alkalinity and TVFA/alkalinity patterns in the temperature-phased systems of RTA and R2TM

Fig 2 (c) shows the VFAs pattern in the acidogenic reactor. At the startup of the process, the VFAs level fluctuated with values above 6000 mg/L, however and from the day 67 forward, the TVFA stabilized around 4000 mg/L, which is similar to levels observed by others in an acidogenic digester of vegetable wastes [43]. Based in individual organic acids profiles, their accumulation can be given mainly as acetic followed by n-Butyric acids. Average stable values of 1305.3 ± 116.7 mg/L and 884.3 ± 91.7 mg/L were observed for HAc and HBut, respectively. Accumulation of HAc agrees with results reported by other authors in acidogenic systems of vegetable wastes [43].

The effluent from the acidogenic reactor has been used as a feedstock for the methanogenic reactor in the TPAD system. Characteristics of the acidogenic effluent are shown in Table 3.

In the methanogenic reactor, pH correction has been necessary daily by adding an alkali (potassium carbonate) to the feedstock. Fig 2 (b) shows that pH values were maintained in the optimum range of 7.5-8 to favorite the methanogenic growing [11]. Moreover, as seen in Fig 2 (b), the methane production started with average values of 0.93±0.24 LH_4/L_r^*d. Nevertheless, after about two HRT and stepwise to the feeding with the acidogenic effluent, productions dropped to 0.22 ± 0.14 LH_4/L_r^*d as an average of stable values for a long period time. The methane production decrease was due to accumulation of VFAs in the medium. The SMP obtained in the methanogenic reactor in the TPAD was 97.7 ± 12.3 ml CH_4/gVS_{added} (Table 3), which was two fold lower than the observed in single stage mesophilic reactor (RMM).

Fig 2 (d) depicts the VFAs pattern observed in methanogenic reactor. The VFAs levels have been the double of the observed in the acidogenic reactor. Therefore, it was observed that the VFAs levels increased continuously at the startup of the process before reaching steady levels after about 100 days of operation time. TVFA values above 10,000 mg/L have been observed.

VFAs distribution were composed mainly of 65.8 ± 8.8 % of acetic and 15.8 ± 3.9 % propionic acids. The rest of VFAs content was less than 5-12%.

The high accumulation of organic acids in this digester could explain the low methane production. Especially, acetate accumulation indicated inhibition of the acetate consuming microorganisms. It has been documented that HAc concentration has a crucial impact on the presence and relative abundance of acetotrophic methanogens [10].

Li et al., [33] studied the effect of methanogenic activity with acetate/propionate concentration under thermophilic and mesophilic conditions. Authors reported that in mesophilic conditions, the methanogenic activity decreased by 82.5% when acetic acid concentrations increased from 5000 mg/L to 10,000 mg/L. As reported by the same authors, acetate is the final product of acetogenesis, and all the VFAs needs to be converted to acetate and then they can be converted into methane. These effects indicate that for the methanogenic reactor in the TPAD conditions, the methanogenesis was the rate-limiting step [31,34].

Contrastingly to the present research when acetic acid was found to slow down the methanogenic activity, in a previous single stage Ad of SBB carried out by Aboudi et al., [24], it has been found that propionic acid accumulation produced by the increase in the applied OLR led to inhibit methanogens.

In a batch TPAD study of Xiao et al., [44], authors found that biomass from the second stage of a two-stage anaerobic system could degrade HAc up to 4200mg/L without observable lag-phase. However, at concentrations of 7400 mg/L, an acclimation of biomass was necessary showing a lag-phase before acids degradation.

In another attempt, Montañes Alonso et al., [45] studied single and TPAD of sewage sludge and sugar beet pulp. Authors found a higher MPR and SMP from the mesophilic co-digestion assays in the TPAD at HRTs in the two devices of 10 days/10 days with values of 2.03 LCH_4/L_r^*d and 410 ml CH_4/gVS_{added} respectively for MPR and SMP. It should be noted that in their investigation both reactors in the TPAD were methanogenic. Moreover, co-digestion of sewage sludge with a sugar-rich substrate such as the lixiviated from SBB, led to a high methane production [39].

As reported previously, the TVFA/alkalinity ratio has been used as an indicator of the process stability, and a ratio higher than 0.4 indicated an imminent failure of the methanogenic process [11]. The observed TVFA/alkalinity ratios and alkalinity concentrations in the acidogenic and thermophilic reactors are shown in Fig 2 (e) and (f), respectively. Except for two pics observed in the acidogenic reactor, the TVFA/alkalinity ratio has been in the admissible range in both reactors. Alkalinity in both reactors was higher, however for the methanogenic reactors, alkalinity reached very high stables values around 200 g/L.

Moreover, for the evaluation of the hydrogen production from acidogenic reactors, some authors have proposed the ratio between butyric and acetic acid as indicator of the system performance [46,47] reported that high But/HAc ratio led to a high hydrogen yield. However, other authors indicated that depending on the substrate nature this ratio could be applicable or not [48]. The characteristics of the hydrogenic effluent are presented in Table 3.

The observed Hn-But/HAc ratio in the acidogenic thermophilic reactor arranged of a max-min of 0.07-0.32. The average stable values were 0.18 ± 0.07. Ghimire et al., [46] indicated the higher hydrogen yield can be co-related with a higher Hn-Bu/HAc ratio. However, authors reported that this ratio might not directly co-relate with the hydrogen for all substrates (lower ratio for higher hydrogen production from buffalo manure).

3.2.2. Performance of the two-phased hyper-thermophilic-mesophilic reactors

Biogas productions at the first step hyper-thermophylic and second step mesophilic reactors are shown in Fig 3 (a) and (b), respectively. Table 3 shows the specific biogas production (hydrogen or methane) in the TPAD assay.

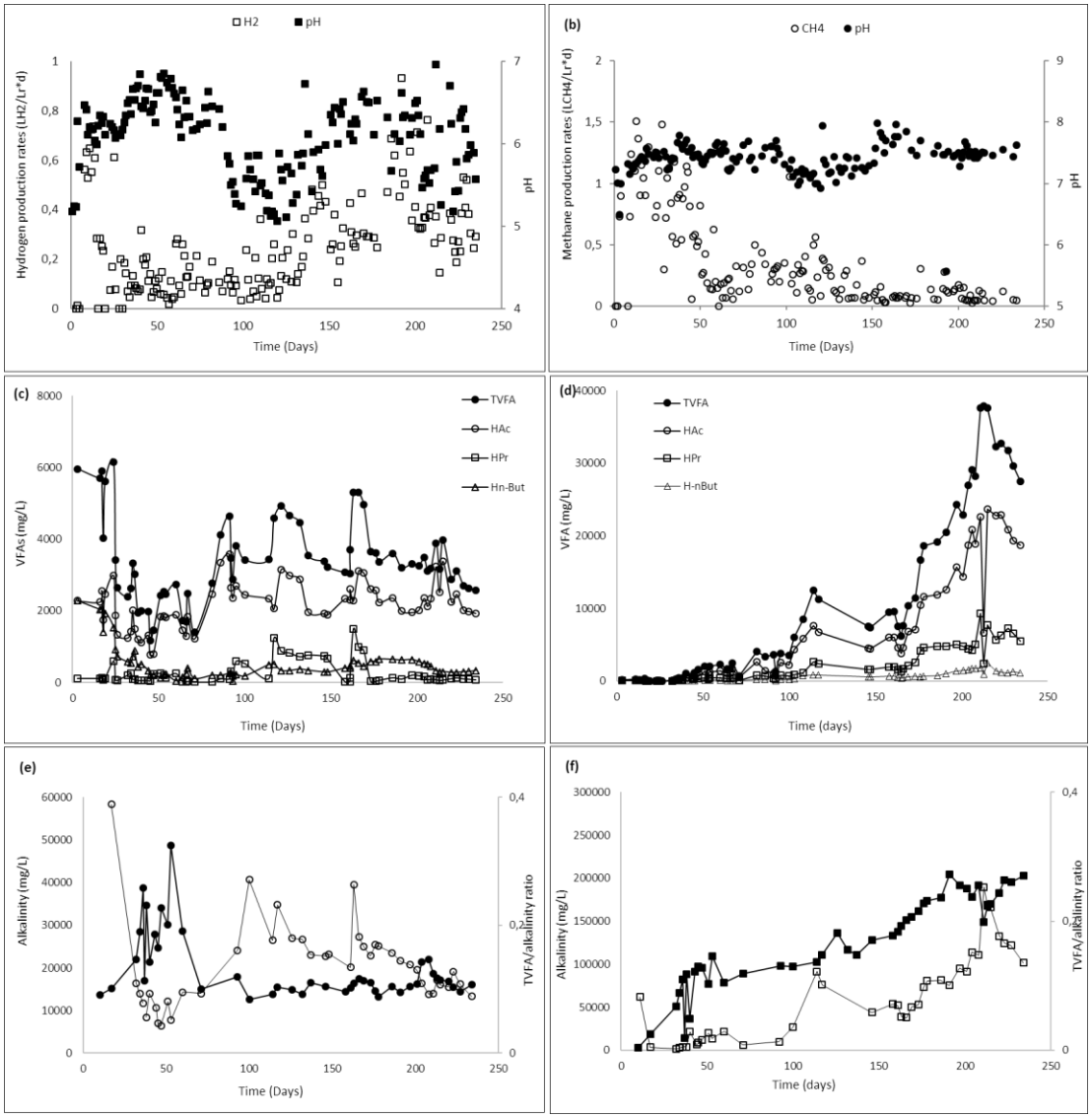

Fig. 5 Biogas production rates (H2 and CH4), VFAs, Alkalinity and TVFA/alkalinity patterns in the temperature-phased systems of RHTA and R2HTM

For the hyper-thermophilic process, the start-up phase shows high fluctuations in the hydrogen productions, as a result of the microorganism's adaptation to the new substrate "SBB". Adaptation period lasted about 30 days. As commented in M&M section, the inoculum used for tis reactor comes from a lab-scale hyper-thermophilic reactor operating with OFMSW, using only the liquid fraction of the effluent. Subsequently, stable lower productions of 0.11 ± 0.04 LH_2/L_r^*d. Nevertheless, and from the day 125 forwards, hydrogen production increased steadily over time, with some higher pics between days 184-207. Stable HPRs of 0.32 ± 0.10 LH_2/L_r^*d have been observed. The increase in the hydrogen productions has been observed once pH values ranged of 5.5-6.5. However, the higher HPR was observed at pH around 6.3. It has been observed that for this reactor values were not stable and had several fluctuations, however, it seemed that the best pH values should be closer to 6. This observation was consistent with results obtained by Lee et al., [12] studying co-digestion of kitchen garbage and excess sludge focusing on the effect of thermophilic and hyper-thermophilic temperature on the process performance. Authors reported an optimal pH of 5.6-6 at 70ºC operation temperature. Other investigations found that pH of 6.8 offer better condition for acidogens in the hyperthermophilic AD of sewage sludge [16]. Kotsopoulos et al., [49] studied dark fermentation of pig slurry at hyper-thermophilic temperature in a SSTR and reported that the pH could reach 7 with no methanogens apparition. Won et al., [50] reported that pH was a critical factor affecting the bacterial species that contributed to H2 production. Khanal et al., [51] studied the effect of pH on hydrogen production and reported that the specific hydrogen production rate was highest for the pH range of 5.5-5.7. Moreover, authors indicated that the optimum pH range, the HAc/HBu ratio was in the range of 3-4 for both sucrose and starch. Algapani et al., [52] compared thermophilic and hyper-thermophilic AD of food wastes for bio-hydrogen production at HRT of 15, 10, 5 and 3 days. Authors reported that the thermophilic temperature was more advantageous than the hyper-thermophilic one. Stable operations were observed at the thermophilic conditions operating at the HRT of 10 days. However, the higher SHP of 70.7 ml H_2/gVS_{added} and hydrogen content in the biogas (58.6 %) were obtained at the HRT of 5 days.

In the methanogenic reactor, it can be observed from the Fig (b) that the methane production rates were higher for the first 50 days of the experiment with average productions of 0.91 ± 0.38 LH_4/L_r^*d. However, MPRs decreased steadily over time indicating system inhibition related to the feedstock characteristics. MPR decreased to an average value of 0.09 ± 0.06 LH_4/L_r^*d despite of stable pH values maintained in the required range for methanogenesis [11]. The methane yield was comparable with the methane yield 192 mlCH4/gVS from potato waste [3], but lower than 500 $mlCH_4/gVS_{added}$ from household solid waste [2].

The biogas production (methane or hydrogen) is strictly related on how the AD phases are coupled. It has been reported that hydrolysis is the rate limiting step in the AD of several organic wastes, mainly due to difficulties in the OM solubilisation [6,33,52]. However, other investigations found that in same case, the high hydrolysis rate could inhibit methanogens which in this case, are not able to convert the hydrolysates, or due to liberation of some inhibitors in the medium [33]. Volatile fatty acids are the main intermediates products of the AD process by the acidogenic pathways. Fig 3 demonstrate the VFAs pattern in the acidogenic hyper-thermophilic and mesophilic reactors in (c) and (d), respectively. As can be observde in the Fig and for the hyper-thermophilic reactor, from the day 95 of the assay, the TVFA stabilised at values around 3529.15 ± 622.67 mg/L. Similarly to the previous thermophilic acidogenic reactor, the main predominant organic acid was acetate which is consistent with others for acidogenic reactors [16,42,41]. Butyrate and propionate were also observed with lower values

than acetate. Butyrate formation is used to be found in acidogenic reactors [42], while propionate was only present at higher concentrations than butyrate between days 117 and 169.

However, Won et al., [50] observed that for hydrogen production from sugar refinery wastewater in a ASBR, the hydrogen productivity was low when operating at pH 5.0 while at a pH of 4.5, a high HPR of 2.18 ± 0.52 LH_2/L_r^*d has been obtained. [53] evaluated the hydrogen and methane production from sugarcane vinasse in an anaerobic fluidized bed reactor. They reported a maximum HPR of 0.57 LH_2/L_r^*d.

Table 3. Final performance and effluent quality of all systems for the steady stable period.

	Single stage		TPAD			
	RMM	RTM	RTA	R2TM	RHTA	R2HTM
pH	7-7-8	7.5-8	5.5-6	7.5-8	5.5-6.5	7.5-8.5
TVFA (g/L)	8.91 ± 1.34	11.69 ± 1.76	3.42 ± 0,34	9.72 ± 1.49	3.63 ± 0,74	28.55 ± 6.32
HPr/Hac	0.31 ± 0.16	0.13 ± 0.08	0.06 ± 0.05	0.24 ± 0.07	0.15 ± 0.17	0.32 ± 0.06
Hbu/HAC	0.13 ± 0.05	0.12 ± 0.06	0.54 ± 0.15	0.02 ± 0.03	0.18 ± 0.07	0.08 ± 0.02
% HAc	61.85 ± 8.33	72.54 ± 8.3	52.27 ± 6.68	67.58 ± 8.81	67.69 ± 9.68	62.65 ± 12.00
%HPr	18.28 ± 6.38	8.69 ± 3.05	3.17 ± 2.34	15.46 ± 3.93	8.82 ± 8.7	19.79 ± 4.72
%Hn-bu	1.83 ± 0.92	1.83 ± 0.61	27.46 ± 5.98	1.62 ± 1.64	11.82 ± 4.11	4.69 ± 1.25
TVFA/alkalinity	0.05 ± 0.01	0.05 ± 0.01	0.07 ± 0.01	0.05 ± 0.02	0.15 ± 0.04	0.13 ± 0.06
Alkalinity (g/L)	178.81 ± 27.73	248.22 ± 46.87	46.88 ± 6.01	174.58 ± 15.12	16.15 ± 2.01	172.54 ± 21.45
SMP ($mLCH_4/gVS_{added}$)	188.3 ± 31.62	90.26 ± 17.09	-	97.77 ± 15.47	-	41.74 ± 12.03
SHP (mLH_2/gVS_{added})	-	-	68.79 ± 0.27	-	48.48 ± 15.81	-
MPR ($LCH_4/L_r{}^*d$)	0.62 ± 0.26	0.30 ± 0.11	-	0.19 ± 0.12	-	-
HPR ($LH_2/L_r{}^*d$)	-	-	0.45 ± 0.15	-	0.32 ± 0.11	0.09 ± 0.06
$\%CH_4$	56.76 ± 10.02	68.19 ± 12.81	-	55.93 ± 9.82	-	59.34 ± 3.6
$\%CO_2$	43.06 ± 10.54	31.81 ± 12	48.49 ± 10.02	44.07 ± 7.68	49.52 ± 13.17	40.66
$\%H_2$	-	-	51.47 ± 10.06	-	50.47 ± 13.06	-
% VS removal	83.09 ± 9.65	65.84 ± 7.54	50.78 ± 12.51	85.12 ± 11.47	44.50 ± 8.54	84.32 ± 7.64
% TS removal	50.78 ± 5.73	51.15 ± 4.23	30.42 ± 8.72	58.51 ± 7.34	28.27 ± 7.23	53.93 ± 5.31

Conclusion

The findings of this study suggest that the two-phased anaerobic system is not suitable for sugar beet by-products (exhausted sugar beet cossettes) anaerobic digestion due to high accumulation of acetate, which inhibited methanogens activity. Single stage mesophilic conditions outperformed both thermophilic single stage and two-phased conditions. It has been deduced that for the studied substrate, temperature is a detrimental factor for the degradation of the organic matter, its conversion into volatile fatty acids, and hence into methane.

Acknowledgments

This research was further supported by the Spanish Ministry of Economy and Competitiveness (CTM2013-43938-R and CTM2016-79071-R), the Spanish Ministry of Science and Innovation (UNCA08-1E-035) and co-funded by the European Regional Development Fund (ERDF). Authors gratefully acknowledge the University of Cadiz (Spain) for the Scholarship UCA-2010-063PU/EPIF-FPI-A/BC and the Agrifood Campus of

References

[1] C. Mao, Y. Feng, X. Wang, G. Ren, Review on research achievements of biogas from anaerobic digestion, Renewable and Sustainable Energy Reviews. 45 (2015) 540–555. doi:10.1016/j.rser.2015.02.032.

[2] Adekunle et al., A Review of Biochemical Process of Anaerobic Digestion, Advances in Bioscience and Biotechnology. 6 (2015) 205–212. doi:http://dx.doi.org/10.4236/abb.2015.63020.

[3] M.H. Gerardi, The Microbiology of Anaerobic Digestrers, 2003.

[4] M.A. Khan, H.H. Ngo, W.S. Guo, Y. Liu, L.D. Nghiem, F.I. Hai, L.J. Deng, J. Wang, Y. Wu, Optimization of process parameters for production of volatile fatty acid, biohydrogen and methane from anaerobic digestion, Bioresource Technology. 219 (2016) 738–748. doi:10.1016/j.biortech.2016.08.073.

[5] W. Kim, S.G. Shin, J. Lim, S. Hwang, Effect of temperature and hydraulic retention time on volatile fatty acid production based on bacterial community structure in anaerobic acidogenesis using swine wastewater, Bioprocess and Biosystems Engineering. 36 (2013) 791–798. doi:10.1007/s00449-013-0905-7.

[6] Y.-Q. Tang, T. Matsui, S. Morimura, X.-L. Wu, K. Kida, Effect of temperature on microbial community of a glucose-degrading methanogenic consortium under hyperthermophilic chemostat cultivation, Journal of Bioscience and Bioengineering. 106 (2008) 180–187. doi:10.1263/jbb.106.180.

[7] M. Lee, T. Hidaka, H. Tsuno, Effect of temperature on performance and microbial diversity in hyperthermophilic digester system fed with kitchen garbage, Bioresource Technology. 99 (2008) 6852–6860. doi:10.1016/j.biortech.2008.01.038.

[8] J.K. Kim, B.R. Oh, Y.N. Chun, S.W. Kim, Effects of temperature and hydraulic retention time on anaerobic digestion of food waste, Journal of Bioscience and Bioengineering. 102 (2006) 328–332. doi:10.1263/jbb.102.328.

[9] C. Gou, Z. Yang, J. Huang, H. Wang, H. Xu, L. Wang, Effects of temperature and organic loading rate on the performance and microbial community of anaerobic co-digestion of waste activated sludge and food waste, 2014. doi:10.1016/j.chemosphere.2014.01.018.

[10] D. Yu, J.M. Kurola, K. Lähde, M. Kymäläinen, A. Sinkkonen, M. Romantschuk, Biogas production and

methanogenic archaeal community in mesophilic and thermophilic anaerobic co-digestion processes, Journal of Environmental Management. 143 (2014) 54–60. doi:10.1016/j.jenvman.2014.04.025.

[11] Y. Chen, J.J. Cheng, K.S. Creamer, Inhibition of anaerobic digestion process: A review, Bioresource Technology. 99 (2008) 4044–4064. doi:10.1016/j.biortech.2007.01.057.

[12] M. Lee, T. Hidaka, W. Hagiwara, H. Tsuno, Comparative performance and microbial diversity of hyperthermophilic and thermophilic co-digestion of kitchen garbage and excess sludge, Bioresource Technology. 100 (2009) 578–585. doi:10.1016/j.biortech.2008.06.063.

[13] J.P. Sheets, X. Ge, Y. Li, Effect of limited air exposure and comparative performance between thermophilic and mesophilic solid-state anaerobic digestion of switchgrass, Bioresource Technology. 180 (2015) 296–303. doi:10.1016/j.biortech.2015.01.011.

[14] L. Appels, J. Baeyens, J. Degrève, R. Dewil, Principles and potential of the anaerobic digestion of waste-activated sludge, Progress in Energy and Combustion Science. 34 (2008) 755–781. doi:10.1016/j.pecs.2008.06.002.

[15] B.E.L. Baêta, D.R.S. Lima, J.G.B. Filho, O.F.H. Adarme, L.V.A. Gurgel, S.F. de Aquino, Evaluation of hydrogen and methane production from sugarcane bagasse hemicellulose hydrolysates by two-stage anaerobic digestion process, Bioresource Technology. 218 (2016) 436–446. doi:10.1016/j.biortech.2016.06.113.

[16] D. Bolzonella, P. Pavan, M. Zanette, F. Cecchi, Two-Phase Anaerobic Digestion of Waste Activated Sludge: Effect of an Extreme Thermophilic Prefermentation, Industrial & Engineering Chemistry Research. 46 (2007) 6650–6655. doi:10.1021/ie061627e.

[17] A. Schievano, A. Tenca, S. Lonati, E. Manzini, F. Adani, Can two-stage instead of one-stage anaerobic digestion really increase energy recovery from biomass?, Applied Energy. 124 (2014) 335–342. doi:10.1016/j.apenergy.2014.03.024.

[18] C. Wang, J. Zuo, X. Chen, W. Xing, L. Xing, P. Li, X. Lu, C. Li, Microbial community structures in an integrated two-phase anaerobic bioreactor fed by fruit vegetable wastes and wheat straw, Journal of Environmental Sciences. 26 (2014) 2484–2492. doi:10.1016/j.jes.2014.06.035.

[19] M. Lee, T. Hidaka, H. Tsuno, Two-phased hyperthermophilic anaerobic co-digestion of waste activated sludge with kitchen garbage, Journal of Bioscience and Bioengineering. 108 (2009) 408–413. doi:10.1016/j.jbiosc.2009.05.011.

[20] A. Schievano, A. Tenca, B. Scaglia, G. Merlino, A. Rizzi, D. Daffonchio, R. Oberti, F. Adani, Two-Stage vs Single-Stage Thermophilic Anaerobic Digestion: Comparison of Energy Production and Biodegradation Efficiencies, Environmental Science & Technology. 46 (2012) 8502–8510. doi:10.1021/es301376n.

[21] J. Bartacek, J. Zabranska, P.N.L. Lens, Developments and constraints in fermentative hydrogen production, Biofuels, Bioproducts and Biorefining. 1 (2007) 201–214. doi:10.1002/bbb.17.

[22] K. Aboudi, C.J. Álvarez-Gallego, L.I. Romero-García, Influence of total solids concentration on the anaerobic co-digestion of sugar beet by-products and livestock manures, Science of The Total Environment. 586 (2017) 438–445. doi:10.1016/j.scitotenv.2017.01.178.

[23] K. Aboudi, C.J. Álvarez-Gallego, L.I. Romero-García, Influence of total solids concentration on the anaerobic co-digestion of sugar beet by-products and livestock manures, Science of the Total

Environment. 586 (2017). doi:10.1016/j.scitotenv.2017.01.178.

[24] K. Aboudi, C.J. Álvarez-Gallego, L.I. Romero-García, Biomethanization of sugar beet byproduct by semi-continuous single digestion and co-digestion with cow manure, Bioresource Technology. 200 (2016) 311–319. doi:10.1016/j.biortech.2015.10.051.

[25] P.C. Hallenbeck, D. Ghosh, Advances in fermentative biohydrogen production: the way forward?, Trends in Biotechnology. 27 (2009) 287–297. doi:10.1016/j.tibtech.2009.02.004.

[26] APHA-AWWA, Standards Methods for the Examination of Water and Wastewater, 20th editi, American Public Health Association Washington DC, USA, 2005.

[27] C.J. Álvarez-Gallego, Testing different procedures for the start up of a dry anaerobic co-digestion process of OFMSW and sewage sludge at thermophilic range, Cádiz Spain, 2005.

[28] D.S.M. Ghasimi, Y. Tao, M. de Kreuk, B. Abbas, M.H. Zandvoort, J.B. van Lier, Digester performance and microbial community changes in thermophilic and mesophilic sequencing batch reactors fed with the fine sieved fraction of municipal sewage, Water Research. 87 (2015) 483–493. doi:10.1016/j.watres.2015.04.027.

[29] C. Da Ros, C. Cavinato, P. Pavan, D. Bolzonella, Mesophilic and thermophilic anaerobic co-digestion of winery wastewater sludge and wine lees: An integrated approach for sustainable wine production, Journal of Environmental Management. (2016). doi:10.1016/j.jenvman.2016.03.029.

[30] S. Riggio, M.A. Hernandéz-Shek, M. Torrijos, G. Vives, G. Esposito, E.D. van Hullebusch, J.P. Steyer, R. Escudié, Comparison of the mesophilic and thermophilic anaerobic digestion of spent cow bedding in leach-bed reactors, 2017. doi:10.1016/j.biortech.2017.02.056.

[31] E. Nordell, B. Nilsson, S. Nilsson Påledal, K. Karisalmi, J. Moestedt, Co-digestion of manure and industrial waste – The effects of trace element addition, Waste Management. 47 (2016) 21–27. doi:10.1016/j.wasman.2015.02.032.

[32] B. Demirel, P. Scherer, The roles of acetotrophic and hydrogenotrophic methanogens during anaerobic conversion of biomass to methane: a review, Reviews in Environmental Science and Bio/Technology. 7 (2008) 173–190. doi:10.1007/s11157-008-9131-1.

[33] Q. Li, W. Qiao, X. Wang, K. Takayanagi, M. Shofie, Y.-Y. Li, Kinetic characterization of thermophilic and mesophilic anaerobic digestion for coffee grounds and waste activated sludge, Waste Management. 36 (2015) 77–85. doi:10.1016/j.wasman.2014.11.016.

[34] M. Koyama, K. Watanabe, N. Kurosawa, K. Ishikawa, S. Ban, T. Toda, Effect of alkaline pretreatment on mesophilic and thermophilic anaerobic digestion of a submerged macrophyte: inhibition and recovery against dissolved lignin during semi-continuous operation, Bioresource Technology. (2017). doi:10.1016/j.biortech.2017.04.046.

[35] J. Fernández-Rodríguez, M. Pérez, L.I. Romero, Comparison of mesophilic and thermophilic dry anaerobic digestion of OFMSW: Kinetic analysis, Chemical Engineering Journal. 232 (2013) 59–64. doi:10.1016/j.cej.2013.07.066.

[36] F.M. Espinoza-Escalante, C. Pelayo-Ortíz, J. Navarro-Corona, Y. González-García, A. Bories, H. Gutiérrez-Pulido, Anaerobic digestion of the vinasses from the fermentation of Agave tequilana Weber to tequila: The effect of pH, temperature and hydraulic retention time on the production of hydrogen and methane, Biomass and Bioenergy. 33 (2009) 14–20. doi:10.1016/j.biombioe.2008.04.006.

[37] P.C. Benito Martin, M. Schlienz, M. Greger, Production of bio-hydrogen and methane during semi-continuous digestion of maize silage in a two-stage system, International Journal of Hydrogen Energy. 42 (2017) 5768–5779. doi:10.1016/j.ijhydene.2017.01.020.

[38] S. Zahedi, R. Solera, F. Micolucci, C. Cavinato, D. Bolzonella, Changes in microbial community during hydrogen and methane production in two-stage thermophilic anaerobic co-digestion process from biowaste, Waste Management. 49 (2016) 40–46. doi:10.1016/j.wasman.2016.01.016.

[39] J. Lindner, S. Zielonka, H. Oechsner, A. Lemmer, Is the continuous two-stage anaerobic digestion process well suited for all substrates?, Bioresource Technology. 200 (2016) 470–476. doi:10.1016/j.biortech.2015.10.052.

[40] E. Alkaya, G.N. Demirer, Anaerobic acidification of sugar-beet processing wastes: Effect of operational parameters, Biomass and Bioenergy. 35 (2011) 32–39. doi:10.1016/j.biombioe.2010.08.002.

[41] R. Angeriz-Campoy, C.J. Álvarez-Gallego, L.I. Romero-García, Thermophilic anaerobic co-digestion of organic fraction of municipal solid waste (OFMSW) with food waste (FW): Enhancement of bio-hydrogen production, Bioresource Technology. 194 (2015) 291–296. doi:10.1016/j.biortech.2015.07.011.

[42] M.A. Romero Aguilar, L.A. Fdez-Güelfo, C.J. Álvarez-Gallego, L.I. Romero García, Effect of HRT on hydrogen production and organic matter solubilization in acidogenic anaerobic digestion of OFMSW, Chemical Engineering Journal. 219 (2013) 443–449. doi:10.1016/j.cej.2012.12.090.

[43] Z. Zuo, S. Wu, W. Zhang, R. Dong, Performance of two-stage vegetable waste anaerobic digestion depending on varying recirculation rates, Bioresource Technology. 162 (2014) 266–272. doi:10.1016/j.biortech.2014.03.156.

[44] K. Xiao, C. Guo, Y. Zhou, Y. Maspolim, W.-J. Ng, Acetic acid effects on methanogens in the second stage of a two-stage anaerobic system, Chemosphere. 144 (2016) 1498–1504. doi:10.1016/j.chemosphere.2015.10.035.

[45] R. Montañés Alonso, R. Solera del Río, M. Pérez García, Thermophilic and mesophilic temperature phase anaerobic co-digestion (TPAcD) compared with single-stage co-digestion of sewage sludge and sugar beet pulp lixiviation, Biomass and Bioenergy. 93 (2016) 107–115. doi:10.1016/j.biombioe.2016.05.028.

[46] A. Ghimire, L. Frunzo, L. Pontoni, G. d'Antonio, P.N.L. Lens, G. Esposito, F. Pirozzi, Dark fermentation of complex waste biomass for biohydrogen production by pretreated thermophilic anaerobic digestate, Journal of Environmental Management. 152 (2015) 43–48. doi:10.1016/j.jenvman.2014.12.049.

[47] F.R. Hawkes, I. Hussy, G. Kyazze, R. Dinsdale, D.L. Hawkes, Continuous dark fermentative hydrogen production by mesophilic microflora: Principles and progress, International Journal of Hydrogen Energy. 32 (2007) 172–184. doi:10.1016/j.ijhydene.2006.08.014.

[48] S.-H. Kim, S.-K. Han, H.-S. Shin, Effect of substrate concentration on hydrogen production and 16S rDNA-based analysis of the microbial community in a continuous fermenter, Process Biochemistry. 41 (2006) 199–207. doi:10.1016/j.procbio.2005.06.013.

[49] T.A. Kotsopoulos, I.A. Fotidis, N. Tsolakis, G.G. Martzopoulos, Biohydrogen production from pig slurry in a CSTR reactor system with mixed cultures under hyper-thermophilic temperature (70 °C),

Biomass and Bioenergy. 33 (2009) 1168–1174. doi:10.1016/j.biombioe.2009.05.001.

[50] S.G. Won, S.A. Baldwin, A.K. Lau, M. Rezadehbashi, Optimal operational conditions for biohydrogen production from sugar refinery wastewater in an ASBR, International Journal of Hydrogen Energy. 38 (2013) 13895–13906. doi:10.1016/j.ijhydene.2013.08.071.

[51] S.K. Khanal, W.-H. Chen, L. Li, S. Sung, Biological hydrogen production: effects of pH and intermediate products, International Journal of Hydrogen Energy. 29 (2004) 1123–1131. doi:10.1016/j.ijhydene.2003.11.002.

[52] D.E. Algapani, W. Qiao, M. Su, F. di Pumpo, S.M. Wandera, F. Adani, R. Dong, Bio-hydrolysis and bio-hydrogen production from food waste by thermophilic and hyperthermophilic anaerobic process, Bioresource Technology. 216 (2016) 768–777. doi:10.1016/j.biortech.2016.06.016.

[53] C.M. dos Reis, M.F. Carosia, I.K. Sakamoto, M.B. Amâncio Varesche, E.L. Silva, Evaluation of hydrogen and methane production from sugarcane vinasse in an anaerobic fluidized bed reactor, International Journal of Hydrogen Energy. 40 (2015) 8498–8509. doi:10.1016/j.ijhydene.2015.04.136.

[54] G. Luo, L. Xie, Z. Zou, W. Wang, Q. Zhou, H. Shim, Anaerobic treatment of cassava stillage for hydrogen and methane production in continuously stirred tank reactor (CSTR) under high organic loading rate (OLR), 2010. doi:10.1016/j.ijhydene.2010.08.033.

[55] R. Chandra, H. Takeuchi, T. Hasegawa, Methane production from lignocellulosic agricultural crop wastes: A review in context to second generation of biofuel production, Renewable and Sustainable Energy Reviews. 16 (2012) 1462–1476. doi:10.1016/j.rser.2011.11.035.

[56] Hutnan et al., two-Step Pilot-Scale Anaerobic Treatment of Sugar Beet Pulp, Polish Journal of Environmental Studies. 10 (2001) 237–243.