Enhancement of Methane Production in Thermophilic Anaerobic Co-Digestion of Exhausted Sugar Beet Pulp and Pig Manure

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Abstract: In this paper, the viability of thermophilic anaerobic co-digestion of exhausted sugar beet pulp (ESBP) and pig manure (PM) was evaluated. The effect of the proportion of ESBP on biogas production was investigated by using a series of lab-scale batch assays, in duplicates. The following five ESBP:PM mixture ratios were studied: 0:100, 10:90, 25:75, 50:50, and 100:0. The highest cumulative methane production (212.4 mL CH₄/g VS added) was reached for the mixture 25:75. The experimental results showed that the increase in the proportion of ESBP in the mixture led to the distortion of the process, due to acidification by the volatile fatty acids generated. Acetic acid was the predominant acid in all the cases, representing more than 78% of the total acidity. Moreover, the results obtained by operating at thermophilic temperatures have been compared with those obtained in a previous study conducted at mesophilic temperatures. The results have shown that in the individual digestion of ESBP, the activity of acetoclastic methanogens was affected in both temperatures, but especially in thermophilic conditions. Thus, the methane produced in the individual thermophilic digestion of ESBP came almost entirely from the activity of hydrogen-utilizing methanogenic archaea.

Keywords: exhausted sugar beet pulp; pig manure; anaerobic co-digestion; thermophilic; lignocellulosic waste

1. Introduction

The main component of agro-industrial waste is lignocellulose, which is constituted by three fractions—lignin, hemicellulose and cellulose [1]. Lignocellulosic biomass can be converted into different products, including biofuels and fine chemicals with a high added-value, and can also be used as a cheap substrate source in microbial fermentation and enzyme production [2].

An interesting by-product generated in the process of sugar extraction from sugar beet (Beta vulgaris), is the exhausted sugar beet pulp (ESBP), which has the following composition—24%–32% of hemicellulose, 22%–30% of cellulose, 3%–4% of lignin, and 24%–32% of pectin [3]. Therefore, ESBP is a suitable substrate for biogas production, through the anaerobic digestion process (AD) [4–6]. The use of biogas produced from ESBP could generate economic and environmental benefits for energy-intensive consumers, such as sugar beet industries and, hence, contribute to the circular economy.

AD is a mature, highly efficient, and economically viable technology for transforming organic wastes into methane [7–9]. The process occurs under anaerobic conditions, requiring considerably less energy with respect to the aerobic treatment processes [10].

Agro-industrial by-products could release, depending on their nature and origin, toxic or inhibitory compounds for microorganisms, such as ammonia, sulphides, heavy metals, long chain fatty acids (LCFAs), and other organic compounds. The intensification of the microorganisms activity in the system, especially the increase of their hydrolytic ability, could be counterproductive if the breakdown of the lignocellulosic material resulted in the release of intermediate inhibitory compounds. The accumulation of these compounds would lead to the destabilization of the AD process [11]. Therefore, in order to improve the efficiency and stability of the AD process, a deep knowledge of the critical process parameters, affecting the different microbial groups is required [12,13].

According to the literature, operations in thermophilic temperature ranges (50–60 °C) lead to a higher biogas production than in mesophilic range (30–40 °C), but compromises the stability of the process [14,15].

However, the decomposition of agro-industrial by-products could present limitations due to the presence of non-biodegradable lignocellulose and the deficit in the nitrogen source, with respect to the high content in organic matter [1,16].

Aboudi et al. [11] have reported that thermophilic or hyperthermophilic acidogenic anaerobic digestion of ESBP led to the accumulation of LCFAs, such as palmitate and lignocerate. The use of the effluents from these reactors for the feeding of two subsequent mesophilic reactors led to the inhibition of methanogenic archaea population. LCFAs are considered inhibitory compounds for the AD process, especially for methanogenic microorganisms [17].

With respect to the deficit of nitrogen in ESBP, several authors [18,19] have shown that the anaerobic co-digestion (AcoD) of ESBP with animal manure, solves this problem. Thus, animal manure supplies the necessary nutrients, but it also provides alkalinity and specific microorganisms, originating from the digestive tract of animals, which are capable of degrading vegetal fibers [20,21]. Therefore, animal manure improves, both, the biodegradability of the lignocellulosic material and the stability of the AD process. Aboudi et al. [22], have studied the AcoD of ESBP with pig manure (PM) in continuous stirred tank reactor (CSTR) reactors operating in the mesophilic range of temperature. Other authors [23–25] have studied the AcoD of different agricultural wastes with animal manure and have shown that co-digestion improves the anaerobic process and waste digestibility.

The main objective of this study was to determine the optimal mixture ratio of ESBP and PM for the thermophilic anaerobic co-digestion of both wastes. In addition, the effect of temperature on the process performance was studied by performing a comparison with the previous results reported in the literature, for mesophilic AcoD, using similar wastes.

2. Materials and Methods

2.1. Substrate and Inoculum

The ESBP samples came from a high-size sugar beet factory (AB Sugar™) located in Jerez de la Frontera ( Cádiz, Spain). The ESBP samples have a very high solid content (TS range 80%–90%) that facilitated their conservation. The samples of PM were collected in a local farm placed in San José del Valle ( Cádiz, Spain). The farm did not have any device for the separation of urine and feces. The samples were frozen at −20 °C, unfrozen, and stored at 4 °C, when they were close to being used.

The mixtures of ESBP and PM were prepared, considering the proportions of each waste, on a wet weight basis, and a total solid content of 8% was maintained. ESBP samples were previously rehydrated for 24 h, in deionized water [11]. ESBP is highly hygroscopic and, hence, the total solids percentage must be limited to 8%, to avoid rheological problems into the reactors. Indeed, previous studies determined that the increase in solids content impeded an adequate mixing in the reactor, due to the high water holding capacity of ESBP [26–30]. The following mixtures of ESBP:PM (weight basis) were studied: 0:100, 10:90, 25:75, 50:50, and 100:0.

The inoculum used in the experiments came from a lab-scale semi-continuous reactor for the AcoD of ESBP and PM. The reactor has been working in the thermophilic temperature range (55 °C)
and was fed with an ESBP:PM mixture of (10:90), also with a total solids content of 8%. When the inoculum samples were taken, the reactor was operating in stable conditions with a hydraulic retention time of 30 days, a methane yield around 105–110 mL CH₄/g VS added, and a productivity of 0.22–0.24 L CH₄/L reactor·day. The use of an adapted inoculum to carry out the AD assays guaranteed an efficient start-up of the process [30]. The inoculum to substrate ratio was at 1:1 (volume basis).

The physicochemical characteristics of the substrates and the inoculum are shown in Table 1.

### Table 1. Physicochemical characteristics of the substrates and inoculum.

<table>
<thead>
<tr>
<th>Component</th>
<th>Units</th>
<th>ESBP</th>
<th>PM</th>
<th>Inoculum</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>-</td>
<td>6.22 ± 0.03</td>
<td>8.26 ± 0.10</td>
<td>7.96 ± 0.08</td>
</tr>
<tr>
<td>TS</td>
<td>g/kg</td>
<td>857.7 ± 0.04</td>
<td>276.3 ± 0.27</td>
<td>55.67 ± 0.15</td>
</tr>
<tr>
<td>VS</td>
<td>g/kg</td>
<td>758.6 ± 0.06</td>
<td>156.8 ± 0.30</td>
<td>28.43 ± 0.11</td>
</tr>
<tr>
<td>sCOD</td>
<td>g/kg</td>
<td>21.0 ± 0.42</td>
<td>7.0 ± 0.28</td>
<td>3.4 ± 0.39</td>
</tr>
<tr>
<td>TVFA</td>
<td>g/kg</td>
<td>1.99 ± 0.31</td>
<td>1.2 ± 0.02</td>
<td>0.72 ± 0.10</td>
</tr>
<tr>
<td>Alkalinity</td>
<td>g/kg</td>
<td>2.19 ± 0.10</td>
<td>33.1 ± 0.50</td>
<td>11.8 ± 0.77</td>
</tr>
<tr>
<td>C/N ratio</td>
<td>-</td>
<td>37.4 ± 0.22</td>
<td>3.03 ± 0.27</td>
<td>-</td>
</tr>
<tr>
<td>Pectins</td>
<td>%</td>
<td>55.54</td>
<td>-</td>
<td>69.31</td>
</tr>
<tr>
<td>Hemicellulose</td>
<td>%</td>
<td>22.52</td>
<td>-</td>
<td>11.29</td>
</tr>
<tr>
<td>Cellulose</td>
<td>%</td>
<td>21.14</td>
<td>-</td>
<td>11.90</td>
</tr>
<tr>
<td>Lignin</td>
<td>%</td>
<td>3.50</td>
<td>-</td>
<td>5.61</td>
</tr>
</tbody>
</table>

All results were calculated in a wet basis. ESBP: Exhausted Sugar Beet Pulp, PM: Pig Manure, TS: Total Solids, VS: Volatile Solids, sCOD: Soluble Chemical Oxygen Demand, TVFA: Total Volatile Fatty Acid, -: No unit/Not analyzed.

### 2.2. Experimental Design

A series of 10 stainless steel reactors with a working volume of 1.7 L were operated in batch mode. Each reactor had independent devices for heating and stirring. The biogas production was continuously measured using a MilliGas counter (Ritter®) and a series of specific infrared on-line sensors for CH₄ and H₂ determinations (Calibrated and certified by Bluesens®). Figure 1 shows an illustration of the equipment used for the biogas measurement and the characterization.

![Figure 1. Equipment used for the biogas measurement and characterization. From left to right—infrared on-line sensors, the MilliGas counter, and the data interface module.](image)

Subsequently, the reactors were hermetically sealed and purged with N₂ to remove the residual O₂. The temperature was continuously measured by an inner sensor and was maintained at 55 ºC. The mixing was performed by paddles (20 rpm).

### 2.3. Analytical Methods

All analytical determinations were performed according to the Standard Methods [31]. The following reference methods were used—Total Solids (TS) by 2540B, Volatile Solids (VS) by 2540E, total Chemical Oxygen Demand (tCOD), and soluble Chemical Oxygen Demand (sCOD) by 5220C,
Dissolved Organic Carbon (DOC) by 5310B, using a carbon/nitrogen analyzer (Analytic-Jena multi N/C 3100 with chemiluminescence detector, Endress+Hauser Company®, Waldheim, Germany), alkalinity by 2320B and pH by 4500H⁺. In addition, the concentration of the main individual volatile fatty acids (VFAs), from C2 to C7, were measured by using a gas chromatograph GC-2010 (Shimadzu Corporation®, Kyoto, Japan) with flame ionization detector and a Nukol® capillary with a diameter of 0.25 mm and 30 m of length column (Merck KGaA®, Darmstadt, Germany). The hydrogen was used as carrier gas with a flow of 50 mL/min and 75.5 kPa; synthetic air (400 mL/min and 50 kPa) and hydrogen (40 mL/min and 60 kPa) were used for the flame ignition, and nitrogen (30 mL/min and 75 kPa) was used as the make-up gas. All the gases were provided by Abello-Linde® (Barcelona, Spain).

The pH was measured daily and directly into the reactor. The remaining parameters were analyzed three times per week. Prior to the analysis of sCOD, DOC, and VFA, the samples were centrifuged in a Consul-21 Ortoalresa® equipment (Madrid, Spain) at 3220 x g during 15 min and filtered with a 0.47 µm glass microfiber filter (ref. GF52047, Hahnemühle®, Dassel, Germany). For the analysis of VFAs, an additional filtration was carried out through a 0.22 µm PTFE filter manufactured by Rephile Bioscience® (Shanghai, China) [32].

To determine the lignocellulosic fractions of the samples (cellulose, hemicellulose, and lignin), the FIBERTEC™ 8000 equipment (FOSS®, Hilleroed, Denmark) was used by applying the Van Soest method [33].

2.4. Indirect Parameters

Fdez-Güelfo et al. [8] have proposed a series of indirect parameters that can be used to analyze the performance of the different stages of the anaerobic digestion process and to determine the rate-limiting stage for specific operating conditions.

These parameters include the Dissolved Acid Carbon (DAC) and the Acidogenic Substrate as Carbon (ASC). The DAC is the carbon contained in the volatile fatty acids (C2 to C7) present in the medium. An increase in DAC is related to a failure in the methanogenic stage. The ASC corresponds to the difference (expressed in carbon units) between the Dissolved Organic Carbon (analytical value of DOC) and the DAC (calculated value). The ASC represents the solubilized organic carbon that has not been transformed into acids, and an increase in this parameter could be interpreted as a distortion of the acidogenic stage.

Angeriz-Campoy et al. [34] have used the indirect parameter ASC and have corroborated that the failure in the AcoD of organic fraction of municipal solid waste and sewage sludge, was due to a distortion in the hydrolytic phase. Aboudi et al. [35] have used the indirect parameters to analyze the AcoD of exhausted sugar beet pulp and cow manure, in the mesophilic range of temperature as well.

3. Results and Discussion

3.1. Waste Characteristics

As can be seen in the Table 1, the ESBP is a lignocellulosic material with a very high organic matter content (high values of VS y COD). However, the alkalinity of ESBP is relatively low with respect to its organic content. Therefore, a mixture with PM could be favorable, since PM has a 12 times higher alkalinity. In addition, the C/N ratio indicates that ESBP is deficient in nitrogen and, hence, co-digestion with PM might adjust the nutrient content.

3.2. Process Stability

The pH is the result of the acid–base equilibrium in the process and, therefore, is related to the production of VFAs and alkalinity. The pH is considered a key parameter in the stability of the anaerobic digestion process, since a low pH value implies that the organic acids are mainly in their undissociated form, which is the most inhibitory for the anaerobic microbiota [19]. In fact, if the pKa of the acetic acid (pKa = 4.76 at 25 °C) is considered, approximately 15% of the acid would be
present in its undissociated form at pH = 5.5, while at pH = 4.76 the undissociated form could reach a 50%. The low alkalinity of the lignocellulosic waste leads to an eventual acidification of the system, because VFAs accumulation significantly diminishes the buffer capacity, affecting the growth rate of the microorganisms capable of transforming the organic matter into biogas [36].

The buffer capacity of the system could be increased by the addition of strong bases or carbonate salts, even though the CO₂ percentage in biogas could be affected [37]. Figure 2 shows the pH and the acidity/alkalinity ratio evolution in the experiments.

![Figure 2. pH evolution (a) and acidity/alkalinity ratio evolution (b) in all ESBP:PM ratios.](image)

In this work, during the first days of operation, the pH diminished below the required range, especially in the reactors with high content in ESBP, as can be seen in Figure 2a. The daily correction of pH with potassium carbonate, permitted all reactors to operate in the appropriate pH range, within a few days [38]. However, for reactors with the mixture ratios 0:100, 10:90, and 25:75, the pH was stabilized rapidly and the required additions of the alkaline reagent were very low. Specifically, the additions of potassium carbonate were 3, 5, and 8 g for the tests 0:100, 10:90, and 25:75, respectively, whereas for reactors 50:50 and 100:0 were necessary 12 and 46 g, respectively. To the end of the process, the pH in all reactors were in the required range.

The acidity/alkalinity ratio has been used as an indicator of acidification problems in anaerobic reactors. In the literature, three different ranges have been identified for this parameter—values below 0.4 indicate an optimal process performance; values between 0.4 and 0.8 are evidence of the beginning of process distortion and, finally, values higher than 0.8 indicate that a total or a partial inhibition of the methanogenic activity occurred [39–41].
Figure 2b shows that for the mixture 0:100, the ratio was in the optimum range, throughout the whole assay, while for the mixture 10:90, this ratio was over 0.4 for the first days, and then rapidly reached the optimum range. However, for the mixtures 25:75 and 50:50, the acidity/alkalinity ratio initially exceeded 0.8 and a longer period was required (which increased with an in increase in the ESBP content), to reach the optimum level. For the reactor containing only ESBP (100:0), the ratio remained above 0.8 for most of the test, and only reached values between 0.4 and 0.8 at the end of the assay.

Therefore, according to the acidity/alkalinity ratio, the increase in the percentage of animal manure (PM) in the mixture had a beneficial effect on the stability of the AcoD of ESBP and PM.

Figure 3a shows the evolution of the total volatile fatty acidity (TVFA) in all tests. The initial acidification was due to the rapid release of VFAs and it seemed to be a characteristic of the ESBP, as a substrate in the AD process [4]. Indeed, as the content of ESBP in the mixtures increased, the release of VFAs was more abrupt, reaching higher maximum concentrations of TVFA. Moreover, the time needed to reduce the TVFA concentration from the maximum value was longer for reactors with a high fraction of ESBP in the mixture, showing a decoupling between the acidogenic and acetoclastic-methanogenic activities. In the case of the mixture 100:0, the higher values of VFAs seemed to have inhibited the activity of the acetoclastic-methanogenic archaea population, since the VFAs had not degraded over the 40 days of operation.

Figure 3b shows the main individual VFAs observed in the different tests. Acetic (HAc), propionic (HPr), and butyric (HBu) acids together represented more than 80%–90% of the TVFA (when the maximum of TVFA was reached). Acetic acid was the predominant in all the cases, representing more than 78% of TVFA. It could be observed that an increase in the concentrations of the three main organic acids occurred when the content in ESBP was increased in the mixtures. In the test 100:0, the maximum concentration of acetic acid was 19.5 g/L, whereas the concentrations of propionic and butyric acids were 1.4 and 2.5 g/L, respectively. Probably, these high concentrations of VFAs had inhibited the acetoclastic-methanogenic stage [11]. Thus, despite the slight decrease in the acetic acid concentration at the end of the test, the methanogenic activity was clearly affected and, hence, the observed methane production must have come mainly from the activity of the hydrogen-utilizing methanogenic archaea. As can be seen later, in Section 3.5, there was a high fraction of organic matter as VFAs, which was not transformed into methane at the end of the tests.

The relationship between the concentrations of propionic and acetic acids (HPr/HAc ratio) was another very useful parameter to study the stability of the AD process. According to the literature, when the HPr/HAc ratio exceeds 1.4, the destabilization of the process occurs [42]. However, other studies have determined that the threshold value of this relationship depends on the characteristics of the substrates used in co-digestion [35,43]. Figure 3c shows the evolution of the HPr/HAc ratio in all the mixtures. In this study, despite certain initial high values, the HPr/HAc ratio was usually less than 2. For the mixture 50:50, values above 1.4 were recorded for 7 days, but a decrease was observed from day 20 of the test. However, for the mixture 100:0, the HPr/HAc ratio remained much lower with respect to the mixtures 25:75 and 50:50 throughout the assay. This behavior was due to the fact that the initial production of acetic acid in the mixture 100:0 was very high. Thus, although the highest concentration of propionic acid was obtained in this test, the HPr/HAc ratio remained at low level.

It can be pointed out that the microbiota coming from the inoculum was very effective to remove propionic acid. Therefore, the transitory accumulation of propionic acid could be interpreted as a decoupling between the acidogenic and acetogenic stages for a specific period.

In the literature, different threshold values of HPr have been reported as causing inhibition. Barredo and Evison [44] have indicated that concentrations of propionic acid higher than 1500–2220 mg/L could lead to inhibition. However, in the mesophilic AcoD of ESBP and PM, concentrations of HPr in the range 1099–5500 mg/L were observed and the system was not inhibited [35].
that the SMP was improved in all co-digestion mixtures, with respect to the individual digestion of ESBP. Figure 4 shows the specific methane production (SMP) obtained in all the digesters. It can be seen that the microbiota coming from the inoculum was very effective to remove propionic acid. Therefore, the transitory accumulation of propionic acid could be much lower with respect to the mixtures 25:75 and 50:50 throughout the assay. This behavior was observed for all the mixtures. In this study, despite certain initial high values, the HPr/HAc ratio was usually less than 2. For the mixture 50:50, values above 1.4 were recorded for 7 days, but a decrease was observed in the ratio after this time, and the ratio was less than 1.4 after 10 days. Although the highest concentration of propionic acid was obtained in this test, the HPr/HAc ratio did not exceed 1.4. This indicates that the process was stable, and the system was not inhibited.

In this work, the maximum concentrations of propionic acid detected were: 125.42 mg/L, 277.02 mg/L, 447.75 mg/L, 972.88 mg/L, and 1401.26 mg/L for the mixtures 0:100, 10:90, 25:75, 50:50, and 100:0, respectively. Therefore, concentrations of propionic acid higher than 1500–2220 mg/L could lead to inhibition. However, in the mesophilic AcoD of ESBP and PM, concentrations of propionic acid lower than 2. For the mixture 50:50, values above 1.4 were recorded for 7 days, but a decrease was observed in the ratio after this time, and the ratio was less than 1.4 after 10 days. Although the highest concentration of propionic acid was obtained in this test, the HPr/HAc ratio did not exceed 1.4. This indicates that the process was stable, and the system was not inhibited.

3.3. Methane Production

The reactors were maintained in operation until no significant methane production was detected. Figure 4 shows the specific methane production (SMP) obtained in all the digesters. It can be seen that the SMP was improved in all co-digestion mixtures, with respect to the individual digestion of ESBP. The following data were obtained for the different tests: 19.2; 108.1; 212.4; 141.4, and 47.9 mL CH₄/gVS_added for the mixtures 0:100; 10:90; 25:75; 50:50, and 100:0, respectively.
were improved. In addition, pig manure added microorganisms from its digestive tract which could facilitate the anaerobic digestion of PM and ESBP. The synergy observed was due to the presence of typical animal manure toxics (ammonia nitrogen, drugs, and antibiotics). The inhibition by these parameters in all reactors.

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3.3. Methane Production

In terms of methane production, the best mixture was 25:75. In fact, the reactor with the mixture 25:75 had rapidly reached the maximum SMP of 212.4 mL CH4/g VSadded with a very short lag phase (2 days). Aboudi et al. [40] found an optimal ratio of 32:68 with an SMP of 494 mL CH4/g VSadd at the mesophilic range. Ohuchi et al. [6] obtained an SMP of 377–422 mL CH4/g VSadd for anaerobic co-digestion of cow manure with ensiled sugar beet tops at the proportion of 40%. The maximum SMP found for the mixture 25:75 in this work was approximately 11 times higher than for the mixture 0:100 (PM alone) and 5 times higher than 100:0 (ESBP alone), highlighting the synergy of the co-digestion of both substrates.

Therefore, it could be admitted that the co-digestion of ESBP and PM in the thermophilic range showed a synergistic effect in the methane production and the rate of the organic matter degradation, with respect to the individual anaerobic digestion of PM and ESBP. The synergy observed was due to the fact that the mixture of both wastes generated more appropriate environmental conditions for the development of the process; that is, both the nutrient balance (C/N ratio) and the available alkalinity were improved. In addition, pig manure added microorganisms from its digestive tract which could enhance the hydrolysis of the lignocellulosic material present in the ESBP. These results were in line with what has been reported by several authors studying AD of agro-industrial wastes [45,46].

Nevertheless, it must be noted that the SMP obtained for the PM used in this study was clearly lower than that obtained in a previous study, conducted under mesophilic conditions [46], where a value of 468 mL CH4/g VSadded was reached. Moreover, this effect seemed to extend to the assay with the ratio 10:90, due to its high PM proportion. This aspect could be due to the different characteristics of the manures used in both tests. Another possible explanation for this difference could be related to the presence of typical animal manure toxics (ammonia nitrogen, drugs, and antibiotics). The inhibition by ammonia nitrogen was especially relevant in the thermophilic anaerobic digestion [47] and several authors reported that animal manures were a reservoir of antibiotic-resistant genes, outlining that for PM, around 149 antibiotic-resistant genes were found [48–50]. However, to support this hypothesis, additional specific analytical determinations should be performed.

3.4. Acidogenic Substrate as Carbon (ASC)

In order to obtain a more detailed interpretation of when the process failed (see Section 2.4), the indirect parameters ASC and DAC were calculated since they allowed a better understanding of the origin of the distortions [8]. The study of the ASC parameter, which represented the hydrolyzed and solubilized organic matter that had not been degraded to VFAs, permitted to interpret the appearance of the inhibitory effects in acidogenesis stage [34]. A transitory accumulation of ASC denoted the existence of some type of limitations in acidogenesis because the hydrolysis stage was normally the rate-limiting step of the anaerobic degradation of solid wastes [32]. Figure 5 shows the evolution of these parameters in all reactors.

Figure 4. Specific accumulated methane production for the different mixtures tested.
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It could be observed that the ASC was not accumulated in any test. In fact, ASC reached rapidly the final level for this parameter, indicating that this value represented the concentration of the organic carbon that could not be transformed into VFAs. This behavior was observed for all tests, including the mixture 100:0 (ESBP alone). Moreover, the increase of organic matter added to the medium, when the ESBP proportion in the mixture increased, led to an increase in the maximum TVFA obtained. Therefore, it could be deduced that the inhibition of the acidogenic stage did not occur and that the hydrolytic and acidogenic stages were well-coupled in all reactors.

However, the acidogenic and methanogenic stages were not coupled in the determined periods in each test. In these periods, the acidogenesis rate was higher than that of methanogenesis and, hence, an accumulation of VFA was observed. The decoupling periods were longer in reactors with high proportions of ESBP in the mixtures. Indeed, the decoupling period covered the entire test for the mixture 100:0 (ESBP alone), due to

### 3.5. Influence of the Temperature Range

Temperature is a key variable in all biological processes. Although it is generally admitted that thermophilic anaerobic digestion improves the kinetics and the methane yield of the process, in comparison to the mesophilic range, there are some possible drawbacks in the function of the substrate and the operational conditions. Therefore, when the hydrolytic and acidogenic stages are predominant in a thermophilic system, a significant accumulation of VFAs occurs due to the inhibition of acetoclastic methanogenic microorganisms, which are the most sensitive microorganisms to temperature [51].

As was previously pointed out in Section 3.3, a significant improvement in methane production was obtained by applying the co-digestion option with respect to the anaerobic digestion of the individual substrates. However, the calculated SMPs were markedly lower than those obtained in the mesophilic process [46].

Figure 6 depicts the DOC evolution in this study, together with data from a previous mesophilic study of Aboudi et al. [46]. Maximum values of DOC have been taken into account for this comparison, since they represent the situation of maximum unbalance between solubilization and consumption of organic matter in batch processes. At the thermophilic temperature conditions, the evolution of DOC was very similar to that previously commented on TVFA and the maximum of DOC achieved in each test was directly related to the ESBP content. In fact, and for the mixture

**Figure 5.** Evolution of dissolved organic carbon (DOC), dissolved acid carbon (DAC), and acidogenic substrate as carbon (ASC) in each reactor. (a) Reactor 0:100; (b) Reactor 10:90; (c) Reactor 25:75; (d) Reactor 50:50, and (e) Reactor 100:0.
It could be observed that the ASC was not accumulated in any test. In fact, ASC reached rapidly the final level for this parameter, indicating that this value represented the concentration of the organic carbon that could not be transformed into VFAs. This behavior was observed for all tests, including the mixture 100:0 (ESBP alone). Moreover, the increase of organic matter added to the medium, when the ESBP proportion in the mixture increased, led to an increase in the maximum TVFA obtained. Therefore, it could be deduced that the inhibition of the acidogenic stage did not occur and that the hydrolytic and acidogenic stages were well-coupled in all reactors. However, the acidogenic and methanogenic stages were not coupled in the determined periods in each test. In these periods, the acidogenesis rate was higher than that of methanogenesis and, hence, an accumulation of VFA was observed. The decoupling periods were longer in reactors with high proportions of ESBP in the mixtures. Indeed, the decoupling period covered the entire test for the mixture 100:0 (ESBP alone), due to the inhibition of the acetoclastic metanogenic population, as a response of the excessively high level of TVFA.

3.5. Influence of the Temperature Range

Temperature is a key variable in all biological processes. Although it is generally admitted that thermophilic anaerobic digestion improves the kinetics and the methane yield of the process, in comparison to the mesophilic range, there are some possible drawbacks in the function of the substrate and the operational conditions. Therefore, when the hydrolytic and acidogenic stages are predominant in a thermophilic system, a significant accumulation of VFAs occurs due to the inhibition of acetoclastic methanogenic microorganisms, which are the most sensitive microorganisms to temperature [51].

As was previously pointed out in Section 3.3, a significant improvement in methane production was obtained by applying the co-digestion option with respect to the anaerobic digestion of the individual substrates. However, the calculated SMPs were markedly lower than those obtained in the mesophilic process [46].

Figure 6 depicts the DOC evolution in this study, together with data from a previous mesophilic study of Aboudi et al. [46]. Maximum values of DOC have been taken into account for this comparison, since they represent the situation of maximum unbalance between solubilization and consumption of organic matter in batch processes. At the thermophilic temperature conditions, the evolution of DOC was very similar to that previously commented on TVFA and the maximum of DOC achieved in each test was directly related to the ESBP content. In fact, and for the mixture 100:0, the removal of the dissolved organic matter (as DOC) was very low, since an important fraction remained in the medium as VFAs, mainly as acetic acid.

![Figure 6](image_url). Maximum values of DOC in thermophilic assays (this study) and mesophilic assays [46].

Aboudi et al. [46] have found the same trend in the increase of the maximum values of DOC when the ESBP proportion in the mixture increased. However, as can be seen in Figure 6, these maximum
values were generally lower with respect to those observed in this study. The reason is that hydrolysis rate was normally sharper in the thermophilic process than in the mesophilic one [14,15].

Likewise, the PM degradation was not complete in the reactors with a high proportion of PM, as has been discussed previously. Considering all these issues, the comparison between the mesophilic and thermophilic assays needed a new indicator, allowing the estimation of the theoretical methane that could be obtained from the non-degraded organic matter. This variable would be the percentage of the non-produced methane from ASC or VFA in the system, with respect to the total theoretical methane (non-produced and produced) that could be generated if all the organic matter was converted.

VFAs and ASC concentrations refer to the organic material which has not been converted into biogas. For this, the final levels of VFAs and the ASC were considered for the theoretical calculations. Only concentrations of the predominant VFAs (HAc, HPr, and HBu) were used. Moreover, the ASC was considered to be completely converted to VFAs in the same proportion observed at the end of the tests.

The following equations were considered for the calculations:

\[
\text{CH}_3\text{COO}^- + \text{H}_2\text{O} \rightarrow \text{CH}_4 + \text{HCO}_3^- \tag{1}
\]

\[
\text{CH}_3\text{CH}_2\text{COO}^- + 3\text{H}_2\text{O} \rightarrow \text{CH}_3\text{COO}^- + \text{HCO}_3^- + \text{H}^+ + 3\text{H}_2 \tag{2}
\]

\[
\text{CH}_3\text{CH}_2\text{CH}_2\text{COO}^- + 2\text{H}_2\text{O} \rightarrow 2\text{CH}_3\text{COO}^- + \text{H}^+ + 2\text{H}_2 \tag{3}
\]

\[
\text{CO}_2 + 2\text{H}_2 \rightarrow \text{CH}_4 + 2\text{H}_2\text{O} \tag{4}
\]

In an efficient AD system, the combination between the above-mentioned equations enables the calculation of the carbon proportions converted into methane for each substrate. These carbon proportions are 1/2, 7/12, and 5/8 for acetic, propionic, and butyric acids, respectively. Table 2 shows the obtained results of this estimation.

<table>
<thead>
<tr>
<th>Assay Reference</th>
<th>CH4 (HAc) (L/Lreactor)</th>
<th>CH4 (HPr) (L/Lreactor)</th>
<th>CH4 (HBu) (L/Lreactor)</th>
<th>CH4 (ASC) (L/Lreactor)</th>
<th>CH4 (VFAs+ASC) (L/Lreactor)</th>
<th>CH4 (Produced) (L)</th>
<th>CH4 (Non-Produced) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0:100T</td>
<td>0.052</td>
<td>0.010</td>
<td>0.077</td>
<td>0.106</td>
<td>0.42</td>
<td>0.86</td>
<td>32.7</td>
</tr>
<tr>
<td>10:90T</td>
<td>0.100</td>
<td>0.006</td>
<td>0.042</td>
<td>0.100</td>
<td>0.42</td>
<td>4.03</td>
<td>9.40</td>
</tr>
<tr>
<td>25:75T</td>
<td>0.039</td>
<td>0.011</td>
<td>0.012</td>
<td>0.100</td>
<td>0.27</td>
<td>9.16</td>
<td>2.90</td>
</tr>
<tr>
<td>50:50T</td>
<td>0.064</td>
<td>0.039</td>
<td>0.051</td>
<td>0.103</td>
<td>0.39</td>
<td>8.43</td>
<td>4.40</td>
</tr>
<tr>
<td>100:0T</td>
<td>4.056</td>
<td>0.499</td>
<td>1.121</td>
<td>0.098</td>
<td>9.82</td>
<td>2.92</td>
<td>77.1</td>
</tr>
<tr>
<td>0:100M</td>
<td>0.004</td>
<td>0.002</td>
<td>0.002</td>
<td>0.102</td>
<td>0.22</td>
<td>7.63</td>
<td>2.8</td>
</tr>
<tr>
<td>32:68M</td>
<td>0.003</td>
<td>0.000</td>
<td>0.002</td>
<td>0.100</td>
<td>0.21</td>
<td>8.48</td>
<td>2.4</td>
</tr>
<tr>
<td>48:52M</td>
<td>0.011</td>
<td>0.001</td>
<td>0.004</td>
<td>0.099</td>
<td>0.23</td>
<td>9.34</td>
<td>2.4</td>
</tr>
<tr>
<td>72:28M</td>
<td>0.041</td>
<td>0.025</td>
<td>0.003</td>
<td>0.099</td>
<td>0.34</td>
<td>10.19</td>
<td>3.2</td>
</tr>
<tr>
<td>100:0M</td>
<td>0.266</td>
<td>0.200</td>
<td>0.022</td>
<td>0.107</td>
<td>5.19</td>
<td>11.04</td>
<td>32.0</td>
</tr>
</tbody>
</table>

T—thermophilic; M—mesophilic. It can be observed that the methane production associated with the theoretical ASC conversion was very low and was similar in all the assays. This finding enforces the fact that the hydrolysis and acidogenesis stages have not been limited in any operational condition.

As can be observed, for all co-digestion assays, the organic matter was almost completely converted into methane, and the percentage of the non-produced methane ranged from 2.4%–4.4%. However, a different behavior was observed for the two thermophilic reactors with high PM content, in which a noticeable fraction of the methane, associated with the non-converted ASC and VFAs (9.4% and 32.7% in 10:90T and 0:100T, respectively), was observed. In terms of the estimated methane from ASC and VFAs, both data were similar (0.42 L) and the final difference was attributed to the additional organic matter provided by the ESBP in the reactor 10:90T. In addition, the differences with respect to the mesophilic tests could be attributed to the PM characteristics, as previously commented.
The results from the reactors containing only the ESBP as the substrate have demonstrated that the methanogenesis stage was clearly affected, in both 100:0T and 100:0M reactors.

In the reactor 100:0T, the fast release of VFAs had induced a maximum level of acetic acid of 25 g/L around day 13 (Figure 3), which had prevented a normal development of the methanogenesis, leaving a final concentration of acetic acid equivalent to 4 L CH₄/Lreactor. The estimation of the methane fraction from the non-converted ASC and VFAs was 77.1%. This percentage was similar to that proposed by Smith and Mah [52], who estimated that 73% of the total methane was produced by acetoclastic methanogenesis, while the remaining 27% was produced by the hydrogen-utilizing methanogens.

However, the estimation of the methane fraction from the non-converted ASC and VFAs in the reactor 100:0M was 32.0%. Thus, the acetic acid was degraded in a higher proportion than in the thermophilic reactor, while the propionic acid had slightly increased with respect to the other mesophilic co-digestion tests. The different behavior of methanogenesis in both cases could be likely related to a possible accumulation of intermediate inhibitory compounds in the thermophilic reactor, such as long chain fatty acids (LCFAs) [17,53,54].

In a recent work, Aboudi et al. [11] had studied the anaerobic digestion of ESBP in a temperature phased CSTR reactors (hyperthermophilic at 65 °C–mesophilic at 35 °C and thermophilic at 55 °C–mesophilic at 35 °C). Authors reported an LCFAs accumulation in the hyperthermophilic and thermophilic reactors. Hence, palmitic acid concentrations of 140 and 210 mg/L and lignoceric acid concentrations of 80 and 125 mg/L were detected in thermophilic and hyperthermophilic reactors, respectively. The concentration of these acids was significantly reduced in the subsequent mesophilic reactors. Therefore, the inhibition of methanogenic acetoclastic archaea in the anaerobic digestion of ESBP, could be linked to the operations at high temperatures.

4. Conclusions

The thermophilic co-digestion of the exhausted sugar beet pulp with pig manure allowed for the achievement of an increase in the methane yield, with respect to the anaerobic digestion of ESBP, individually. The addition of PM had a beneficial effect, as it provided additional alkalinity and nitrogen and, consequently, increased the stability of the process. Moreover, the ESBP provided carbon and had contributed to a better C/N balance in the process. Therefore, the mixture of both wastes had a synergistic effect on the process. Thus, the ratio 25:75 (ESBP:PM) was the best proportion tested with a specific methane production of 212 mL/VSadded.

Despite the thermophilic range being normally considered favorable for increasing the process rate and product yield, the comparison of the results obtained with previous mesophilic studies from the literature indicated that the temperature had a detrimental effect on the process. Thus, in this study, the population of methanogenic acetoclastic archaea seems to have been affected and the methane production would have been mostly generated by the pathway of the hydrogen-utilizing microorganisms.

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Conflicts of Interest: The authors declare no conflict of interest.

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