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ANAEROBIC DIGESTION OF AGRICULTURAL WASTES: STUDIES OF TEMPERATURE, CO-DIGESTION AND POTENTIAL INHIBITORS

BEATRIZ DE DIEGO DÍAZ

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Memoria presentada por Dª Beatriz de Diego Díaz para aspirar al grado de Doctor por la Universidad de Navarra

El presente trabajo ha sido realizado bajo nuestra dirección en el Departamento de Química y autorizamos su presentación ante el Tribunal que lo ha de juzgar.

Pamplona, 2 de julio de 2018

Dr. Francisco Javier Peñas Esteban | Dra. Juana Fernández Rodríguez

A mis padres y a mi hermana

Nada te turbe,
Nada te espante,
Todo se pasa,
Dios no se muda.
La paciencia
Todo lo alcanza.

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Abstract

The high global generation of residue that derives from the activity of the agricultural sector makes of its efficient treatment an issue of capital importance. The treatment of wastes through biological pathways, such as anaerobic digestion (AD), is increasingly being encouraged. However, a successful performance of AD is yet to be fully achieved.

AD is a microbiological process that takes place in the absence of oxygen leading to organic substrate consumption and biogas production. The waste valorization is thus twofold: energetic, as biogas combustion can be used for electricity or heat production; and agricultural, due to the feasible production of compost from the waste after AD.

The agricultural wastes herein employed were chosen attending to their worldwide relevance and their role in the economic activity of Navarre (Spain). These are: artichoke, asparagus, carrot, bean, pea, green bean, cabbage, sloe and malt. Additionally, manure was assessed as result of the international stay in the Technical University of Denmark.

As temperature has shown to be a determinant condition in the process, this project has covered different operation temperatures, namely mesophilic (35 °C), intermediate (42 °C) and thermophilic (55 °C) ranges. Also, anaerobic co-digestion (AcoD) of several types of agricultural wastes has been studied throughout the whole study.

Firstly, as preliminary assessments, the use of a nutrient solution in AcoD was evaluated. Results clearly showed that the use of it was beneficial for the performance of the reactor and, therefore, was implemented in the following studies.

The next step was the AD of lignocellulosic substrates, artichoke and asparagus, whose composition makes of them little accessible for the microbial community. Single-stage (at the three aforementioned temperatures) and two-phased anaerobic digestion (TPAD; thermophilic and mesophilic phases) were compared.

The following stage of the project has been to approach the microbiology underlying the anaerobic process. To this aim, bacterial identification of AD reactors treating thermally pretreated and non-pretreated substrates (sloe and malt) was performed. Further microbiological studies based on 16S rRNA were carried out on continuous anaerobic reactors treating manure and subjected to biogas upgrading through H_2 addition. These allowed confirming that biodiversity within reactors is enriched as the process progresses.

Finally, this dissertation has also addressed potential inhibitors arising from the substrate. Two commonly used pesticides were added in anaerobic reactors. Scale-up has also been carried out with pesticides and further studies are recommended to be pursued.

Overall results showed that mesophilic range provides the most stable operation but thermophilic range yields more efficacious removals and shorter processes. Also, AcoD leads to higher improvements than TPAD on specific substrates. Lastly, the presence of pesticides has positive effects on general performance.

Resumen

La alta generación de residuos a nivel mundial que se deriva de la actividad del sector agrícola hace de su tratamiento eficiente una cuestión de capital importancia. El tratamiento de desechos a través de vías biológicas, como la digestión anaerobia (DA), se fomenta cada vez más. Sin embargo, el proceso óptimo de DA está todavía por alcanzar.

La DA es un proceso microbiológico que tiene lugar en ausencia de oxígeno e implica el consumo de un sustrato orgánico y la producción de biogás. La valorización del residuo es, por tanto, doble: energética, ya que la combustión de biogás puede utilizarse para producir electricidad o calor; y agrícola, debido a la posible producción de compost a partir del residuo tras su tratamiento anaerobio.

Los residuos agrícolas aquí empleados se eligieron atendiendo a su relevancia mundial y a su papel en la actividad económica de Navarra (España). Estos son: alcachofa, espárrago, zanahoria, haba, guisante, judía verde, col de bruselas, endrina y malta. Además, se trabajó con estiércol a raíz de la estancia internacional en la Universidad Técnica de Dinamarca.

La temperatura ha demostrado ser una condición determinante en el proceso y es por ello que este proyecto ha estudiado diferentes temperaturas de operación, a saber, mesófila (35 °C), intermedia (42 °C) y termófila (55 °C). Además, se investigó la codigestión de varios tipos de desechos agrícolas a lo largo de todo el estudio.

En primer lugar, como análisis preliminar, se evaluó el uso de una solución de nutrientes en codigestión. Los resultados claramente mostraron que el uso de la misma fue beneficioso para el rendimiento del reactor y, por lo tanto, fue implementada en los siguientes estudios.

El siguiente paso fue el tratamiento de sustratos lignocelulósicos, alcachofa y espárrago, cuya composición los hace poco accesibles para la comunidad microbiana. Se comparó la DA en una sola etapa (a las tres temperaturas mencionadas anteriormente) y en dos fases (DAFT, fases termofílica y mesofílica).

La siguiente etapa del proyecto abordó la microbiología subyacente al proceso anaerobio. Con este objetivo, se realizó la identificación bacteriana en reactores anaerobios que trataban sustratos pretratados y no pretratados térmicamente (endrina y malta). También se llevaron a cabo otros estudios microbiológicos basados en ARNr 16S en reactores anaerobios continuos que trataban estiércol y que acoplaban un proceso de "biogas upgrading" mediante la adición de H_2. Esto permitió confirmar que la biodiversidad dentro de los reactores se enriquece a medida que avanza el proceso.

Finalmente, este proyecto también abordó los potenciales inhibidores que surgen del sustrato. Se agregaron en reactores anaerobios dos pesticidas de uso común. El escalado del proceso también se llevó a cabo con pesticidas y se recomienda una investigación más amplia al respecto.

Los resultados generales mostraron que el rango mesofílico proporciona un proceso más estable, mientras que el rango termófilico acarrea eliminaciones más eficaces y procesos más cortos. Además, la codigestión conduce a mayores mejoras que la DAFT en sustratos específicos. Por último, la presencia de pesticidas tiene efectos positivos en los rendimientos generales.

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I. State of the art of anaerobic digestion of agricultural wastes

1. Current scenario of agricultural waste treatment

Agricultural wastes, in some cases referred to as agri-food wastes, can be difficult to degrade through biological pathways. This issue relies on two facts: a very high worldwide generation of this kind of waste, which derives from a big industry with high productions, and the huge heterogeneity of its composition, which leads to arduous generalization of its treatment. The two most extended treatment approaches that are used nowadays on general waste are landfilling and incineration. However, their implementation on agricultural residue is not permitted, and biological treatments are being highly encouraged (Appels et al., 2008; European Commission, 2017; BOE, 2009 –still in force-). Moreover, incineration and landfilling present many drawbacks such as pollution of water streams of all types by leachates, greenhouse gas emissions (GHGE), toxic gas emissions, high costs of waste handling due to the rise in environmental awareness by governments and administrations, etc. (Ren et al., 2018). Energy and gas recovery through the aforementioned treatments is lost in big proportions, as in the best case scenario less than 60 % of the gas is collected. Furthermore, in growing countries like China, that has a great amount of waste generation, only the 20 % is recovered. This derives in increasing methane emissions of anthropogenic origin as stated by the United States Environmental Protection Agency (USEPA), which estimated that up to 16 % of global emissions in (1999-2015) were methane (Intergovernmental Panel on Climate Change, 2014; USEPA, 2017). Additionally and highly related with waste life cycle, in 2014 the European Union was the third responsible (9 %) of global CO_2 emissions from fossil fuel combustion and some industrial processes (China was the first with 30 %, followed by the USA with 15 %) (Boden et al., 2016; USEPA, 2017).

Within the varied composition and structure of agricultural waste, the presence of lignocellulose makes of it a recalcitrant substrate with low vulnerability to be broken down by anaerobic digestion (AD). This approach that will be further described later on has been encouraged for this type of waste by public organisms and an expanding scientific knowledge.

2. Anaerobic Digestion

Agricultural waste treatment is not only interesting from an environmental point of view. One of the main characteristics is its high organic content (Uçkun Kiran and Liu, 2015) that can be further taken advantage from for several purposes such as energy recovery through AD. Vast literature, industrial development and scientific interest are clear signs of AD having great potential for agricultural waste treatment. Agricultural waste is, to a certain extent, vulnerable to biodegradation, has high moisture content and the aforementioned organic matter. This makes of this residue a perfect candidate as AD substrate, also supported by the wide implementation of biogas plants for agricultural treatment as summarized in Figure 1 (e.g. at the end of 2015 Europe had 17358 biogas plants in total, accounting Germany with 63 % of all of them. Spain reached 139; European Biogas Association, 2016). Additionally, it is abundant in availability (Vasco-Correa et al., 2018).

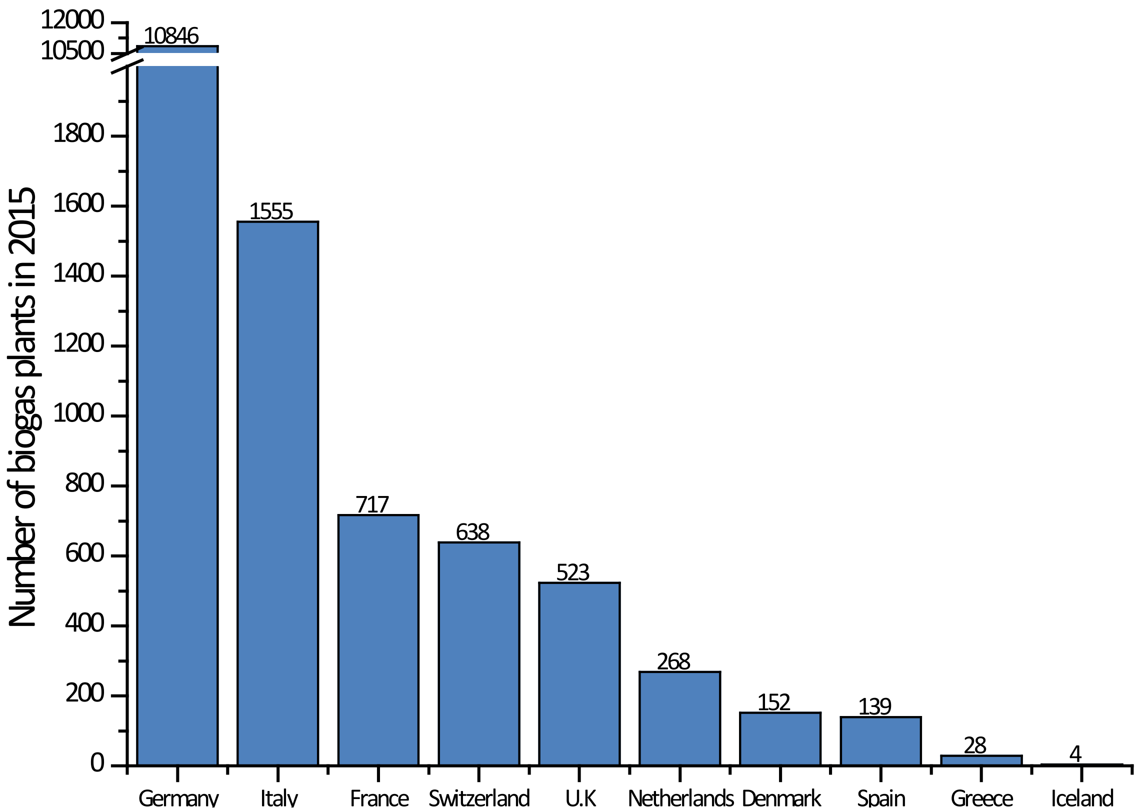

Figure 1. Number of biogas plants in some European countries at the end of year 2015. Adapted from the European Biogas Association (2016).

Other more specific studies (Oldfield et al., 2016) have suggested that waste prevention provides the best benefits regarding environmental impact. However, this is followed by AD that, in comparison to composting and incineration, yielded lower global warming potential, lower eutrophication and acidification potentials, and a better carbon return on investment. However, agricultural wastes also present characteristics that pose obstacles to their biological degradation such as the diverse content in total solids (TS), that can range from a 10 to 80 % depending on the crop, and high lignocellulosic content (cellulose, 35–50 %; hemicellulose, 20-35 %; lignin, 10-25 %), where lignin is the bottleneck for agricultural residue break-down, followed by cellulose crystallinity. Therefore, pretreatments are generally required for the successful performance of AD on this type of substrate, especially to improve their biogas yield, which is slightly lower (0.2-0.5 L/g-volatile solids) than for other types of waste (0.4-0.8 L/g-volatile solids) (Chen et al., 2014; Liu et al., 2008; Monlau et al., 2012; Vasco-Correa et al., 2018; Weiland, 2010).

2.1 Principles of Anaerobic Digestion

AD is a complex process carried out by a consortium of microorganisms, namely bacteria and archaea. Both operate synergycally in order to degrade organic matter in a sequential and parallel utilization as macromolecules are broken-down throughout four stages: hydrolysis, acidogenesis, acetogenesis and methanogenesis (Figure 2), which are described in detail below. In this biochemical process, microorganisms play a key role, being different communities involved according to the stage of the degrading process. These are so varied that, for instance, up to 50 different bacteria can take part in the hydrolytic and acidogenic steps. Furthermore, methanogenic archaea include, as reported up to date, 65 different species from 3 different orders, 7 families and 19 genus (Wang et al., 2018).

In this process, products from each phase are utilized as substrates for the following stage, leading to the formation of biogas. Biogas is mainly formed out of CH_4 (50-75 %) and CO_2 (25-50 %) and other elements in trace concentrations (H_2S, N_2, H_2, etc.). Nevertheless, it should be noted that specific strategies can lead to biogas upgrading (up to 99 % of CH_4) through hydrogen utilization, for instance (Angelidaki et al., 2018). Due to its composition, similar to natural gas, biogas can be used as fuel and produce heat or electric energy through its combustion. Moreover, it can be canalized for its direct use in an adapted boiler or injected after its purification (e.g decarbonising, dehydration, desulphurization, elimination of organo-halogen and heavy metals) in already operating natural gas infrastructures either for its transport or distribution. Biogas has been reported to provide 6.0-6.5 kWh/m^3 (Deublein and Steinhauser, 2008). Additionally, CH_4 has a 21-fold higher warming potential than CO_2, hence the reduction of GHGE by its use is also a positive aspect of biogas utilization.

Attending to the added value that biogas production entails, some of the positive aspects of agricultural AD are the potential additional income from energy generation, agronomic valorization, obtaining of natural fertilizers, and energetic and economic independence in a global scenario in which fossil fuel prices are constantly rising, among others. Regarding environmental aspects, biogas is a renewable source of energy, as it is able of replacing a part of the aforementioned fossil energy, and the agricultural waste management is therefore sustainable (Castellano-Hinojosa et al., 2018).

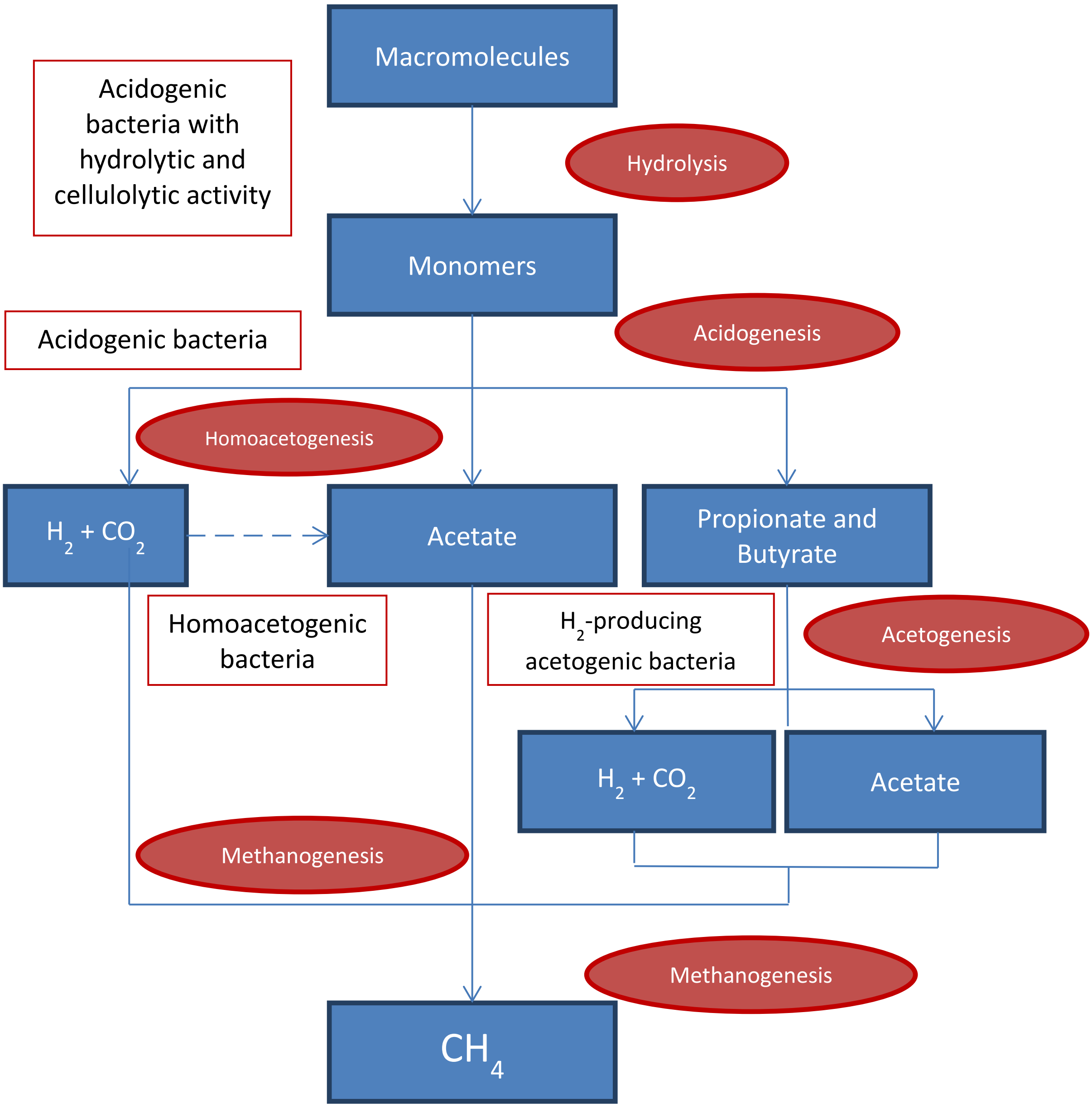

Figure 2. Stages of anaerobic digestion with involved microorganisms (adapted from Barlaz, 1998).

2.1.1 Hydrolysis

Hydrolysis is carried out by exoenzymes that are secreted in order to break-down organic macromolecules into smaller monomers that can surpass the microbial cell wall. Therefore, proteins, carbohydrates and lipids are degraded into aminoacids, smaller sugars and long-chain fatty acids and polyols, respectively. The vast community that can be found in this step includes aerobic and facultative anaerobic species from the genus *Clostridium, Bacteroides, Bifidobacterium, Butyrivibrio, Proteobacteria, Pseudomonas, Bacillus, Streptococcus* and *Eubacterium* (Wang et al., 2018).

The hydrolytic step is widely considered a rate-limiting step for many reasons. The most worthmentioning is the high generation of intermediate products. This can directly lead to inhibition processes, especially due to reactor acidification. Additionally, other toxic compounds such as complex heterocyclic by-products or excessive volatile fatty acids (VFA) can hinder the whole process (Yuan and Zhu, 2016; Wang et al., 2018).

Another aspect that can have an influence in the course of this step is the composition of the substrate. Waste from agricultural origin, is a recalcitrant substrate to a certain extent due to the presence of lignocellulose in its composition. Lignocellulose is known to have a compact crystalline structure that is difficult to be attacked by the microbial machinery and difficults the stabilization of the substrate (Kleinert and Barth, 2008). Lignin is *a priori* the most arduous component to be degraded due to its compact phenolic structure, which not only hinders its own break-down, but that of hemicellulose and cellulose as well. Furthermore, the presence of sulfur and oxygen can be translated into an obstacle for fuel production, among other products (International Lignin Institute, 2018). Also, the by-products from lignocellulose break-down include additional toxicants, such as aromatic intermediates or aldehydes, that can further pose a problem to the process (Malherbe and Cloete, 2002).

This first step of AD can be also conditioned by particle size and level of crushing. Surface contact is determinant in hydrolysis. For this and other reasons that will be further described, the pretreatment of substrate has been widely studied in the literature.

2.1.2 Acidogenesis

This step is characterized by further break-down of hydrolysis products. Monomeric substrates are degraded into alcohols, short-chain fatty acids as well as VFA, namely lactic, pyruvic, acetic and formic acids, among others. In this stage the main phyla that take part are *Bacteroidetes, Chloroflexi, Firmicutes* and *Proteobacteria* (Wang et al., 2018).

Additionally, NH_3, H_2, CO_2 and other subproducts from previous reactions can be also present. These play an important role regarding the preferred reactions that will take place during the acidogenic phase. For instance, high H_2 generation directs the process towards propionate and butyrate production. However, this should be highly controlled since excessive H_2 production can lead to inhibition. On the other hand, when H_2 pressure is low, the system shifts towards the metabolic pathway of acetate or CO_2 and CH_4 generation (Bassani et al., 2015; Mosey, 1983; Sparling et al., 1997).

Hydrogen production throughout acidogenesis can be highly conditioned by operating conditions as it will be described in following sections. For instance, higher temperatures (like in the thermophilic range) speed up the process as the thermodynamics are more favorable to organic matter degradation. Therefore, H_2 generation is generally higher under these conditions, leading the process towards propionate and butyrate.

Additionally, due to the high H_2 generation rates that some substrates and systems can yield, H_2 production has been encouraged through incomplete degradation of the waste as to use H_2 as the ultimate product. However, as aforementioned, inhibition at these temperatures can also contribute to system arrest.

2.1.3 Acetogenesis

Acetogenic phase is characterized by the formation of acetic acid and H_2 from substrates like lactic acid and pyruvic acid.

This phase is highly interesting as two different pathways can be followed for further organic matter degradation. Acetogens are obligate anaerobes that use a reductive acetyl-CoA or Wood-Ljungdahl (W-L) pathway for energy conservation and CO_2 for the generation of acetyl-CoA and cell carbon. Therefore, acetogens can be classified into homoacetogens or CO_2-reducing acetogens. The former produces acetate as the only fermentation product.

The W-L pathway is carried out by a wide variety of microorganisms including species from many phylogenetic classes (methanogenic archaea and acetogenic bacteria) and both oxidative and reductive reductions can be utilized. The latter is used for energy conservation and carbon assimilation, using acetyl-CoA or generating it and cell carbon from CO_2, respectively. The W-L basically uses CO_2 for its reduction into CO that then is converted into acetyl-CoA.

The W-L pathway takes place through two branches, namely Eastern and Western (Figure 3). In the former, CO_2 is reduced by 6 electrons into a methyl group. In the meantime, the latter takes place reducing another CO_2 into CO. Also, the condensation of the methyl group bound to the CO and the coenzyme A takes place to generate acetyl-CoA. This can be transformed to cell carbon or acetyl phosphate that would transfer its phosphoryl group to ADP to produce ATP and acetate. This acetate is key in the growth of acetogenic bacteria (Ragsdale and Pierce, 2008).

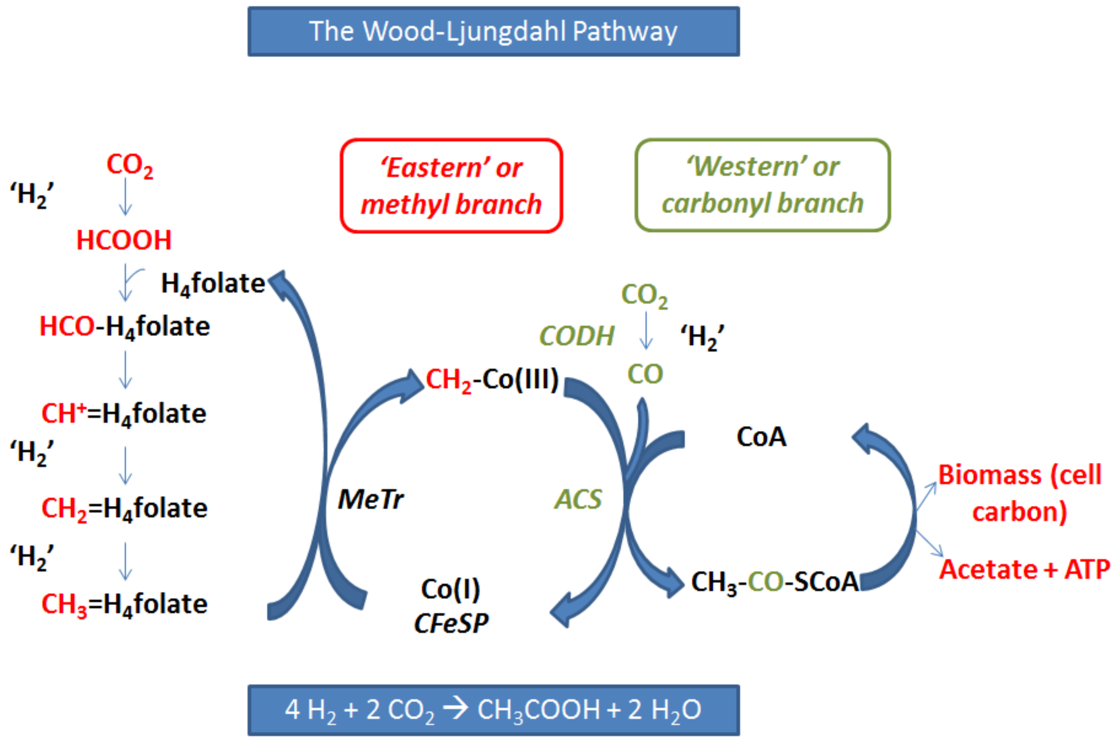

Figure 3. The Wood-Ljungdahl pathway. 'H_2' refers to the need of two electrons and two protons. CFeSP: corrinoid iron–sulfur protein; MeTr: methyl-H_4folate:CFeSP methyltransferase; ACS: acetyl coenzyme A synthase CODH: CO dehydrogenase; SCoA: succinyl-CoA. Adapted from Ragsdale and Pierce (2008).

It is worth-noting that gens for the Eastern branch are ubiquitous and spread along the whole genome. On the other hand, Western branch genes are exclusive of the W-L-utilizing microorganisms. Also, these are located in *acs* gene cluster as shown in Figure 4 for *Moorella thermoacetica* (Roberts et al., 1989; Ragsdale and Pierce, 2008).

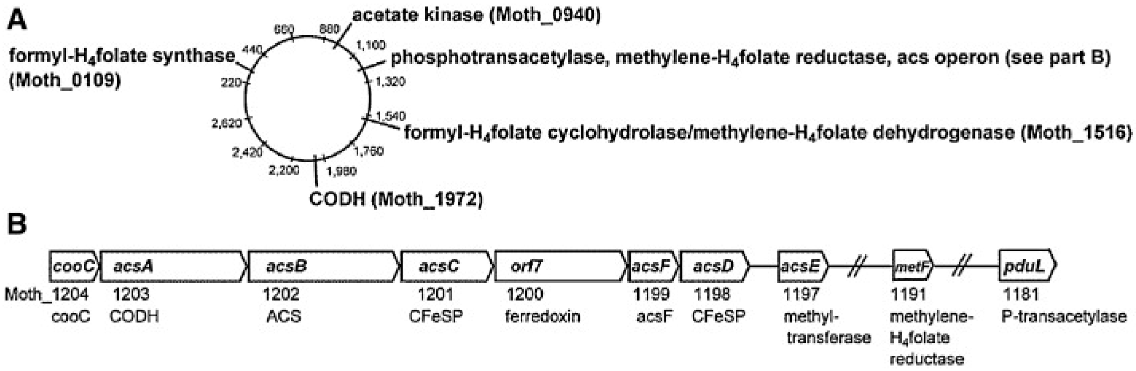

Figure 4. Western branch encoding genes in *acs* cluster from *Moorella thermoacetica* in the chromosome (A) W-L genes on the circular chromosome (B). Adapted from Ragsdale and Pierce (2008) and Roberts et al. (1989).

Growth conditions and environment are determinant of the pathway that acetogens and methanogens carry out. In this way, when both H_2 and CO_2 are abundant, reductive W-L pathway takes over for CO_2 fixation. On the other hand, energy conservation takes place through the conversion of both substrates into CH_4.

2.1.4 Methanogenesis

This is the last phase of the AD process in which methane is produced after a series of break-downs of organic matter into smaller molecules (Figure 5). However, the two most common are the production of CH_4 through CO_2 reduction and from organic molecules break-down. The former uses carbon from CO_2 or acetate as the ultimate electron acceptor while the latter uses simple organic substrates (such as acetic acid, formate, methanol, methylamine, dimethyl sulfide or methanethiol). The use of these organic molecules makes of this pathway a typical fermentation.

Generally, the most occurring pathway is the first one. This is the generation of CH_4 from acetate, carried out by aceticlastic methanogens, and oxidation of acetate to CO_2

and H_2 by syntrophic acetate oxidizing bacteria (SAO). This route accounts for more than 70 % of CH_4 generation while the rest is mainly formed directly from CO_2 and H_2. This last reaction is implemented by hydrogenotrophic methanogens (Bassani et al., 2015).

It is carried out by methanogenic archaea that convert all the energy in the substrate into CH_4. Methanogens are more than 65 species present to a wide variety of genus, up to 19, distributed along 3 different orders. Among them, *Methanobacterium, Methanococcus, Methanobrevibacter, Methanomicrobium, Methanosarcina* and *Methanosaeta* are the most worth-mentioning as they account as the principal CH_4 producers (Wang et al., 2018).

This step can be considered the most crucial, as these microorganisms require a very specific growth environment. In this sense, they are highly sensitive to changes and the fulfilling of the process can be arrested if methanogens find limitations. These archaea make use of H_2 and CO_2, acetate, methanol and mono-, di- and tri-amines.

Depending on the pathway that methanogens utilize, they can be classified as acetoclastic or as H_2-utilizing archaea.

Considering their important role in utilizing the products of the previous steps, methanogens are also important in avoiding inhibition from acidification.

$$\mathbf{4\,H_2 + CO_2 \longrightarrow CH_4 + 2\,H_2O}$$

$$\mathbf{4\,HCOO^- + 2\,H^+ \longrightarrow CH_4 + CO_2 + 2\,HCO_3^-}$$

$$HCOO^- + 3\,H_2 + H^+ \longrightarrow CH_4 + 2\,H_2O$$

$$4\,CO + 2\,H_2O \longrightarrow CH_4 + 3\,CO_2$$

$$4\,CH_3COH \longrightarrow 3\,CH_4 + CO_2 + 2\,H_2O$$

$$CH_3COO^- \longrightarrow CH_4 + HCO_3^-$$

$$CH_3COH + H_2 \longrightarrow CH_4 + H_2O$$

$$4\,CH_3NH_2 + 2\,H_2O + 4\,H^+ \longrightarrow 3\,CH_4 + CO_2 + 4\,NH_4^+$$

$$2\,(CH_3)_2NH + 2\,H_2O + 2\,H^+ \longrightarrow 3\,CH_4 + CO_2 + 2\,NH_4^+$$

$$4\,(CH_3)_3N + 6\,H_2O + 4\,H^+ \longrightarrow 9\,CH_4 + 3\,CO_2 + 4\,NH_4^+$$

$$2\,CH_3CH_2\text{-}N(CH_3)_2 + 2\,H_2O \longrightarrow 3\,CH_4 + CO_2 + 2\,CH_3CH_2NH_2$$

Figure 5. Different possible pathways for CH_4 production. Reactions in bold are those that most commonly take place.

The complexity of the whole process leads to the establishment of some syntrophic interactions, as some of the steps are thermodynamically unfavorable due to the small limits in which products and substrate concentrations favor the course of the process. Additionally, the course of the process is highly dependent on the type of substrate that is fed to the reactor.

2.2 Parameters of interest

These four stages that comprise AD require of a thorough control. A successful operation of waste stabilization can be quantified and monitored attending to different parameters. Organic matter indicators, biogas generation, biogas quality and composition, and production yields, are some of the parameters that can be mentioned.

Organic matter is to be reduced as the process progresses. This is generally quantified as total organic carbon (TOC), chemical oxygen demand (COD), biochemical oxygen demand (BOD), total solids (TS) and volatile solids (VS). Overall biogas production is more important in batch experiments, while for continuous or semi-continuous operation, production per organic matter added or reduced is more interesting, as it describes better the performance of the process. Theoretical productivity attending to stoichiometry ($CH_4 + 2\ O_2 \rightarrow CO_2 + 2\ H_2O$) indicates that 0.35 L of CH_4 can be produced per g-COD but this value is in practice not reached. Theoretical productivity in reference to VS, on the other hand, is more difficult to determine as it depends on the specific composition of the substrate (e.g. carbohydrates, lipids, proteins, etc.). Biogas quality is as well of capital importance, for its content in CH_4 is advised to be around 60-90 %, depending on the substrate and digestion strategy. Also, when the H_2S content in the substrate is high, it has to be taken into account when designing downstream processes. This ($H_2S \rightarrow H_2SO_4$) deteriorates pipelines and infrastructure and, therefore, in many cases it is recommendable that biogas is purified in order to avoid damage.

Alkalinity and pH are also key parameters to be thoroughly controlled. Optimal pH for AD operation ranges from 6.5 to 8. However, each stage of biomethanization can release different compounds in the media that alter these conditions. For instance, the risk of drastically lowering the pH during hydrolysis and acidogenesis is higher than in the following steps as VFA can be accumulated when the operation is not being efficacious enough, with the subsequent biogas formation loss. This is also related with the organic load. As a great amount of available substrate can trigger bacterial growth for its rapid degradation, more intermediate compounds such as VFA are accumulated because of the slower growth rate of acetogenic bacteria and methanogenic archaea.

Therefore, pH has to be carefully monitored as to avoid process failure due to inhibition. Also, pH has an effect on the other variables such as CO_2 solubility or chemical composition through equilibrium shifts. As it will be later on described, pH impacts ammonium/ammonia concentration, which can inhibit AD to a certain extent.

However, changes in pH are closely related to alkalinity, this is, the capacity of the system of facing pH changes and act as a buffer (Schnürer, 2016). To this matter, total alkalinity and partial alkalinity should be distinguished. The former refers to all the buffering systems present in the reactor summed up, while the later only describes the carbonate and ammonium/ammonia system. Additionally, intermediate alkalinity is the difference between both of them and basically accounts for the buffering activity of VFA. It has been shown that a stable operation is that in which the ratio between the intermediate and the partial alkalinity is 0.3. When this value gets closer to 0.8 the probability of instability increases, and above it, instability can be declared (McGenity et al., 2017; Lahav and Morgan, 2004; Schnürer, 2016).

2.3 Variables affecting the process

Microbial performance and diversity are dependent on incoming material but also on operational conditions. Therefore, it is necessary to identify all the variables that have an influence in the process.

2.3.1 Mixing

Biogas production and process success is very much dependent on stirring. This ensures contact of the microbial community with the substrate, as well as minimization of temperature gradient, foaming avoidance and homogeneity. Since stratification of the digester is generally seen as negative, stirring is key in avoiding heavier substrates to sink and lighter ones to float.

Overall, stirring can be classified as hydraulic, pneumatic or mechanical, being the latter the most commonly used. Hydraulic stirring recirculates sludge with the help of pumps and, finally, pneumatic makes use of part of the produced biogas that is injected through the bottom of the reactor and rises up managing to generate horizontal and vertical mixing. Intensity and continuous or intermittent mixing are parameters that affect digester performance (e.g. gas from within the digestate is more easily released) and economic viability. Energy demand of mixing in a full-scale plant can vary from 14-54 % of the total demand of operation and it can be importantly reduced, for instance, by working intermittently. However, each specific substrate and operative conditions have unique mixing requirements, especially considering that too much stirring can be followed by microbial shear stress and less syntrophic interactions between microorganisms that would form flocks if it was not for the disruption that excessive mixing provokes (Kowalczyk et al., 2013; Lindmark et al., 2014).

2.3.2 Substrate

It is widely known that the substrate has an effect in the performance of the process. Its nature affects kinetics, biodegradability, biogas yield and total CH_4 production. In this way, it is not the same stabilizing the organic fraction of a waste that is recalcitrant to biological conversion due to its crystalline structure, or of a waste that is already highly degraded. Many aspects play a role in the influence that the substrate has. Some of them are: inoculum/substrate ratio (Fabbri et al., 2014), micro- and macronutrients balance or C/N ratio, which should be around 15-25. All of them are necessary to meet microbial nutritional requirements. To this concern, some substrates may be advised to be used separately and others in combination with others to fulfill this balance, which is known as anaerobic co-digestion (AcoD). Also, when AcoD is proposed, inhibitors from one of the substrates can be diluted. AcoD is an example of optimization strategy that has shown to have very positive effects on anaerobic treatment as described by the literature, where the rise in interest is remarkable. Mata-Álvarez et al. (2014) reported how up to 50 % of AD publications in the period of 2012-2013 discussed AcoD, and up to 23 % of all of them used agricultural waste as co-substrate. Despite the high dependence on specific characteristics of the substrates, AcoD can result in optimized performance through higher yields, nutritional balance and variability, process stability, toxicity reduction, upgraded microbial community, etc. (Astals et al., 2014; Mehariya et al., 2018).

Also, additives such as buffers, antifoamers or trace elements can be added to ensure a proper operation of the AD process.

Substrate composition, hence microbial activity, is as well important to ensure the achievement of a useful digestate without contaminants and high nutrients content so that it can be applied on agricultural fields (Schnürer, 2016).

Proteins, carbohydrates and lipids differ in their theoretical CH_4 potential. Slaughterhouse wastes, agricultural and some food wastes have high contents of protein, leading to high CH_4 yields but also high ammonium release, which contributes with alkalinity but can also cause process inhibition, to which methanogenic archaea are especially sensitive (Westerholm et al., 2016). However, other factors influence ammonia concentration such as reactor configuration, operative conditions, etc. In addition to ammonia, sulphide is a commonly released compound from substrates which are rich in protein. High amounts of sulphide not only affects the bacterial community, but also its complexation with metals can lead to lower access to the organic matter to be degraded or elements that are necessary for an appropriate microbial activity (Shakeri Yekta et al., 2014). From an industrial and economic point of view, sulphide also contaminates the biogas formed, affecting on its way pipes and engines by corrosion due to its conversion to sulfuric acid. Thus, sulphide removal from biogas is usually carried out downstream by its reaction with ferrous scrap.

For substrates with low nitrogen content, the risk that is posed is nitrogen limitation and quick acidification due to a faster process that releases a greater amount of intermediate products. This is the case of starch, straw and crop wastes or wastes from the paper industry. However, some of these wastes, depending on its nature, can in the contrary face very slow break-downs due to their high lignocellulosic content.

Lipidic substrates (fish or slaughterhouse wastes), on the other hand, trigger the liberation of long-chain fatty acids. Consequently, acidification of the process, combined with foaming and toxicity, can result in process failure. Furthermore, their break-down can hinder the complete biomethanization as their degradation rate is very low in comparison with their oxidation. The imbalance between those rates can as well lead to inhibition (Schnürer, 2016).

Finally, substrates like agricultural waste lack trace elements that are crucial for the effective activity of the microbial population, especially methanogens. Among them, iron, cobalt, nickel, molybdenum, selenium, zinc and tungsten are worth-mentioning. Therefore, stability of the process is highly dependent on the presence of them and appropriate CH_4 yields can be compromised if these elements are missing (Demirel and Scherer, 2011; Sawatdeenarunat et al., 2015).

The specific flaws derived from substrate composition can be overcome through upstream processes such as pretreatments.

- **Pretreatments in anaerobic digestion**

Agricultural wastes have been described to present specific burdens to biological degradation. Therefore, the use of pretreatment strategies that give higher access to the microbial community for organic break-down is a widely studied field for AD enhancement. Attending to the substrate characteristics, many pretreatments have been proposed by the literature (chemical, mechanical, thermal, biological, the combination of two approaches, etc.). In regard to agricultural wastes, their lignocellulosic composition makes of them a substrate whose pretreatment is preferred to be physico-chemical at laboratory and pilot-scale. However, at industrial level biological and mechanical are the most implemented.

- Thermal pretreatment

Thermal pretreatment allows solubilization or early break-down of the organic matter, especially when this is crystalline and its availability is low. Nonetheless, depending on the specific pretreatment, industrial implementation may not be cost-effective. Steam explosion is one of the main strategies and, despite not being generally applied at full-scale, the enhancement of the process has been proven. It involves high-pressure saturated steam on the substrate for a short time (minutes) and then a rapid reduction of it to achieve an acute decompression. This can result in easier hydrolysis but the release of substances such as melanoidins, furans or phenols can hinder the enzymatic activity of the anaerobic consortia, especially methanogens (Carrere et al., 2016; Ferreira et al., 2013; Liu et al., 2012; Monlau et al., 2012). Direct heating of the substrate through high (150-220 °C) or low (60-120 °C) temperature pretreatments have also been reported. The former has been described to result in recalcitrant compounds formation, which hinder the AD process, and the latter requires longer times of exposure (Ferreira et al., 2013; Mata-Álvarez et al., 2014).

- Chemical pretreatment

Thermal pretreatment is often combined with a chemical one, either acid or alkaline. The former generally uses H_2SO_4, H_3PO_4, HNO_3 and HCl and it manages to disrupt the lignocellulosic structure to such level that cellulose turns highly available for enzymatic degradation. Alkaline pretreatments, mainly NaOH, $Ca(OH)_2$ or ammonia, on the contrary, attack lignin through its depolymerization and exposes hemicellulose, being cellulose the least altered (Carrere et al., 2016; Chen et al., 2014).

- Biological pretreatment

Biological approaches are varied. Among them, the most characteristic are enzymatic, degradation by fungi, composting and ensiling. Enzymatic is directed to the reduction of feedstock viscosity as well as the increase of biogas yield. However, some drawbacks can be mentioned such as the requirement of a sterilization step. Fungal pretreatment is also related to the enzymatic one, as many of the enzymes used come from this type of organisms. It manages to increase the methane potential through lignin disruption, but attending to organic matter removal this approach is not the most efficacious. Lastly, composting and ensiling are simple upstream strategies that manage to reduce operational costs through auto-heating of the substrate and lactic fermentation, respectively (Carrere et al., 2016).

- Mechanical pretreatment

Size reduction has been proven to have an effect on substrate degradation, as supported by lab-scale assays and full-scale plants. This can be achieved through shredding, grinding and milling, and there is a great amount of technologies available, whose success relies on the specific type of substrate. For instance, wet substrates with more than 15-20 % of moisture are preferably pretreated by colloid mills and extruders.

The strategy is costly when refering to industrial plants, but digestion rates have shown to be improved (Carrere et al., 2016; Kratky and Jirout 2011).

2.3.3 Organic loading rate

Organic loading rate (OLR) can be defined as the amount of organic matter (expressed as BOD, COD, VS, etc.) that is added to a given volume of media per unit of time (Sivanandan, 2009). This can be applied in continuous or semicontinuous mode (e.g. once a day, several times a week, etc.). OLR should not be too high as this can result in inefficient process, especially if the retention time is low as the substrate would not be fully degraded and the microbial population can be washed away. Additionally, this could lead to inhibition from product accumulation or even acidification and process failure. OLR affects kinetics, total biogas production, microbial diversity and stability, and intermediate compounds formation. However, it has not shown to impact final biogas yield, which is more dependent on temperature and type of substrate rather than OLR (Schnürer, 2016).

2.3.4 Temperature

Temperature is one of the variables that most influences the AD process. Also, its wide interest (scientific, technological and economical) makes of temperature a key operative condition for a successful reactor performance, as it can be seen from the literature.

Temperature has influence on the energy balance of the process, microbial growth rates, reaction rates and thus potential inhibition, community structure, chemical equilibrium shifts (e.g. ammonium/ammonia), microbial diversity, degradation pathways, etc. (Schnürer, 2016). Therefore, thorough studies that include different substrates, inoculums, reactor types, etc., have been carried out in the past years in order to elucidate the advantages and disadvantages of each temperature range.

- **Temperature ranges**

Temperature as an operative condition can be classified in four different ranges:

- psychrophilic (< 20 °C)
- mesophilic (20-45 °C)
- thermophilic (45-60 °C)
- hyperthermophilic (> 60 °C)

As shown in Figure 6, each type of bacteria can grow in a wide spectrum of temperatures. However, each of them has an optimal temperature of growth at which the growth rate is the highest. This is close to 15 °C, 35 °C and 55 °C for psychrophiles, mesophiles and thermophiles, respectively. In the case of hyperthermophiles, the optimal growth temperature is more variable, 80-95 °C. Despite being bacteria able to grow in psychrophilic conditions, no methanogenic archaea has been described to be able of growing under these conditions. Therefore, this operative range is not taken in consideration at an industrial level. Also, solids retention times (SRTs) would be unaffordable as up to 100 days would be required (Zeeman and Lettinga, 1999). This graph is dependent on two variables: the rise in cellular activity due to higher temperatures, and inactivation or bacterial death due to protein denaturation.

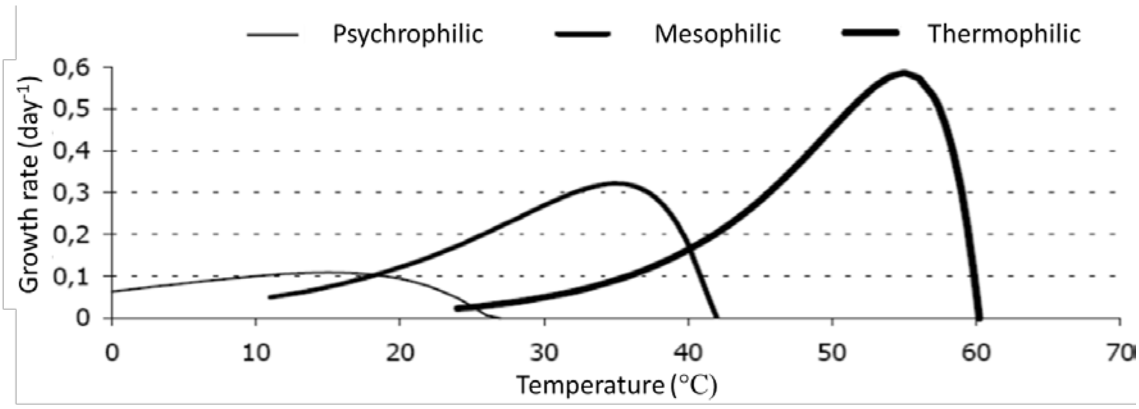

Figure 6. Bacterial growth rates at different temperatures. Adapted from Romero et al. (2002).

As aforementioned, the preferred industrial conditions are mesophilic and thermophilic, having each of them advantages and disadvantages of their own and being the former the most widely implemented. For instance, the mesophilic range prevents inhibitors and toxicants accumulation, it is less sensitive to inhibitors like ammonia, it provides a more stable environment of the process, industrial operative costs are lower, and it can handle small changes in operative conditions to a certain point in comparison to thermophilic processes. On the other hand, thermophilic AD generally manages to remove a bigger amount of VS and COD, inactivate pathogens that can hinder the process and yield better efficiencies. However, not only temperature has to be taken into account. The type of inoculum, the type of temperature changes that the reactor is exposed to, the time of different conditions at which the reactor is exposed to, etc., can also determine the growth rate and overall reactor performance.

The fact that different temperatures lead to different AD performances relies on break-down and substrate transformation reactions being more or less favorable. This means that higher temperatures require less energy for the aforementioned reactions, which directly leads to a faster process. ΔG^0 is an extensive magnitude that describes thermodynamic potential, this is, it yields information regarding equilibrium and spontaneity of a chemical reaction at specific conditions of temperature and pressure. As shown in Table 1 for a given reaction, ΔG^0 is lower at thermophilic range in comparison to the mesophilic range.

Table 1. Some break-down reactions of organic matter in the AD process with their corresponding ΔG^0 under mesophilic and thermophilic conditions.

Reaction	**ΔG^0 (kJ/mol)**	
	35 °C	**55 °C**
$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^-$	31.0	-34.0
$CH_3COO^- + 4\ H_2O \rightarrow 2\ HCO_3^- + H^+ + 4\ H_2$	104.2	89.8
$HCO_3^- + 4\ H_20 \rightarrow CH_4 + 3\ H_2O$	-135.6	122.5
$C_2H_5COO^- + 3\ H_2O \rightarrow HCO_3^- + CH_3COO^- + H^+ + 3\ H_2$	76.1	62.3
$C_3H_7COO^- + 2\ H_20 \rightarrow CH_3COO^- + H^+ + 2\ H_2$	48.1	37.9

Additionally, the thermophilic range provides a less viscous environment, which results in lower stirring requirements, thus less energy input needed. However, the literature has also identified that the microbial population can be less diverse at higher temperatures than at mesophilic conditions. This is highly related with the above-mentioned fact that this range takes lower changes in operative conditions and less efficiency in the degradation of some compounds such as phenols (Schnürer, 2016).

The optimal growth temperatures of each range have been widely studied, but intermediate temperatures are gaining more and more importance, as they manage to yield suitable performance. As described by the graph, microbial growth is meant to be the best at specific conditions. Nonetheless, the close synergic relationships that are established within an anaerobic reactor, and the influence of other aspects (e.g. substrate), may provide a scenario in which the most favorable performance is achieved at intermediate temperatures (such as 42 °C), between the optimal of mesophilic and thermophilic ranges (Hansen et al., 1998; Sakar et al., 2009).

- **Temperature-phased anaerobic digestion (TPAD)**

Classic AD takes place at one temperature in a single reactor in which the four phases of waste stabilization take place. However, a temperature-phased AD (TPAD) in which methanogenesis is carried out separately from hydrolysis, acidogenesis and acetogenesis offers an optimized process with many advantages over single-phase AD (SPAD). As outlined in the previous section, each temperature range has advantages and disadvantages; hence the combination of two conditions can lead to a successful agricultural waste treatment, reducing, for instance, operation times.

TPAD was first described by Pohland and Ghosh in 1971. It started to make its way when it was realized that hydrolytic, acidogenic and acetogenic bacteria have growth rates that are considerably faster than those of methanogenic archaea. Also, the operative conditions highly affect the growth of microorganisms. For instance, high SRTs and neutral pH may hinder VFA generation from acidogenic bacteria. However, short SRTs may lead to methanogens being washed out from the reactor due to their slow growth rate. Therefore, phase separation can avoid the so-called “sour digestion” (Ghosh, 1987; Maspolim et al., 2014).

In TPAD, the steps are split up in two reactors in which, generally, the first operates in the thermophilic range and the second one under mesophilic conditions. Also an initial hyperthermophilic stage followed by a thermophilic one can be implemented. The core benefit that is achieved via TPAD is the stability. Ventura et al. (2014) described the first step as a metabolic buffer that avoids pH shock or accumulation of highly concentrated intermediate products that can act as inhibitors or toxic compounds. This allows higher OLR that otherwise would inhibit the process during the first steps of AD. Additionally, shorter hydraulic retention times (HRT) are attained (Solera et al., 2002; Ventura et al., 2014).

Further advantages include higher solids and pathogen removals, improved biogas productions and foaming prevention. Additionally, the fact that SRTs can be shortened, leads to appropriate performances in smaller reactors, being the investment, potential losses and the handling considerably lower (Ghosh et al., 1995; Maspolim et al., 2014).

Among the drawbacks of TPAD when compared to SPAD we find a longer start-up time, higher initial investment due to the need of an additional reactor and low granulation that normally contributes to higher contact area (Ventura et al., 2014).

The optimization that is achieved through TPAD when compared to SPAD has generated great interest as shown by the literature. Many studies have addressed TPAD under different scopes: improvements on methane yields (Fernández-Rodríguez et al., 2016; Fernández-Rodríguez et al., 2015; Lim et al., 2013; Nathao et al., 2013; Shen et al., 2013), microbial characterization of TPAD (Shin et al., 2010; Lim et al., 2013), pretreatment of substrates in TPAD (Stabnikova et al., 2008; Shahriari et al., 2013), and overall performance (Lim et al., 2013; Lin et al., 2012; Lin et al., 2013), among others.

2.4 Anaerobic digestion technology and configurations

Section 2.3 went through all variables affecting the process, meaning that the correct performance of AD is dependent on various factors whose conditions determine success or failure of operation. Also a separate section exposing the technology and different configurations is highly related to the fate of the process, as they are variables of operation.

AD at industrial level is carried out using different types of reactors, which are elected according to different criteria such as TS content or OLRs. The first classification discriminates wet (< 10 % of TS) and dry (15-35 % of TS) processes, while OLRs can be divided in low and high rate processes, depending on each specific case.

Wet AD is usually carried out at higher loading rates, shorter retention times and in smaller reactors than dry AD. Additionally, the reactor type aims granulation or flocculation by self-attachment or on specific inert surfaces. This keeps microorganisms from being washed away and increases resistance to shock-loads and toxic compounds due to the close aggregation. The types of wastes that are generally treated in these configurations are from municipal, agro-food industry, slaughter, dairy industry, manure and pulp and paper industry origin. The main purpose is thus energy, nutrient and water recovery with low costs and high effluent quality (Lim and Kim, 2014; Schnürer, 2016).

Granule formation has proven to be more dependent on wastewater properties than any other factor (e.g. reactor type). Retention times, pH, temperature, microbiological factors, etc. are also very determinant. Granulation of the microbial community happens on microbial conglomerates or on inert surfaces, and develops by granule growth through adhesion by extracellular polymers, insertion in cavities and holes, presence of cations, etc. (Lim and Kim, 2014; Schnürer, 2016).

As the requirements of this strategy are very specific, the reactors that fulfill them are upflow anaerobic sludge blanket (UASB) reactors (Figure 7a) or variations of it. UASB manages to generate granules of sludge (0.5-5 mm in diameter or even higher) that are suspended inside the tank and that get in contact with the inlet which is pumped from the bottom and circulated upwards. However, UASBs can still improve the biomass-substrate contact. Therefore, optimizations of this common technology has led to the development of expanded granular sludge blanket (EGSB) reactor, which uses internal recycling, and static granular sludge reactor (SGBR), which suppresses flow recirculation and operates in downflow mode. These can work at higher organic loads and flow speeds, and even manages to perform stable methanogenesis at very low temperatures (Bhatia and Yang, 2017; Lettinga et al., 2001; Schnürer, 2016; Verstraete et al., 1996).

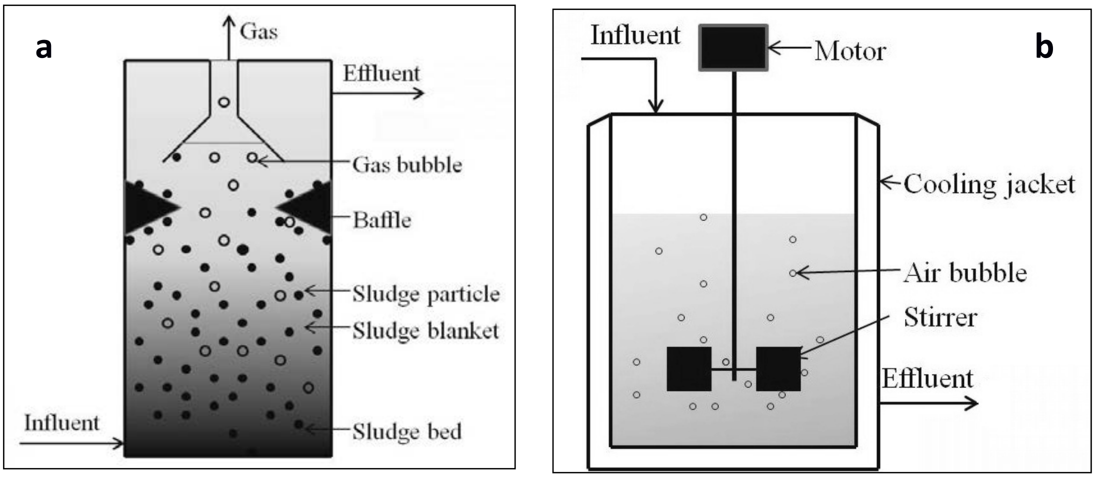

Figure 7. a) UASB reactor b) CSTR reactor (Bhatia and Yang, 2017).

In contrast, lower loading rates usually handle dry AD, where continuous stirred-tank reactors (CSTR) (Figure 7b) are the most widely spread. Also, less implemented plug flow (PF) reactors are key for the treatment of very dry substrates. Among the waste streams handled in this configuration the most worth-mentioning are sludge, slaughterhouse, food, manure and other industrial wastes such as crops. These are generally fed continuously or semi-continuously and operative parameters such as retention times (15-40 days) or loading rates (2-6 g-VS/L·day) are overall determined by substrate availability (i.e. how often can the reactor be fed).

Among the advantages of dry AD, the most worth noting are lower reactor volumes, thus, lower material and heating costs. Another remarkable advantage and added value that dry AD yields is the valuable digestate that is generated. This has comparatively lower water content than wet AD, which means that nutrients are more concentrated and the digestate can potentially be used as fertilizer. A common approach to properly handle dry wastes is the use of diluting water or other substrates for its AcoD. The purpose of it is guaranteeing microbial-substrate contact, and bettering stirring and its homogeneity.

A third form of process classification, which has been mentioned along the whole section, is the loading periods during operation. This allows separating continuous, semicontinuous and batch regimes attending to continuous, intermittent or single loading of the reactors. This mainly has an effect on the times that the microbial population is retained in the digesters. Almost every industrial plant operates at continuous or semicontinuous; however, batch is not a discarded option, especially for treating small volumes of waste. Each of them presents advantages and drawbacks.

Batch operation is characteristic because of the separation of AD in two phases: one in which acidification takes place much faster than methanogenesis, and another one at which acids are converted into biogas (Bouallagui et al., 2005). Attending to organic removal and productions, batch happens to be less efficient than continuous processes attending to organic removal and productions. On the other hand, the former is considered a much more simple process that is easier to control and design. Moreover, this regime of operation provides robustness against contaminants. Batch processes also entail lower economic investments, which gives rise to an especial interest from developing countries (Bouallagui et al., 2005; De Baere, 2000).

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II. Objectives

Based on the state of the art, the main objective of the doctoral dissertation is the optimization of anaerobic digestion of agricultural wastes, focusing on the recalcitrant substrates and the potential chemical inhibitors, under different temperature strategies and batch regime.

The specific objectives of the project are the following:

1. **To study the influence of nutritional supplementation on anaerobic digestion of agricultural wastes.**

Despite the great amount of literature related to co-digestion, some nutritional limitations may arise. In order to fulfill this objective, a complex mineral solution was employed to assess whether it enhanced the process on agricultural waste (co-digestion of green pea, cabbage, green bean, carrot, artichoke and bean). This has been studied in 1-L reactors at three different temperatures of operation: mesophilic (35 °C), intermediate (42 °C), and thermophilic range (55 °C). Previous studies had shown how trace elements have an effect on the microbial metabolism as they play a key role in their enzymatic activity (C/N balance, cofactors, etc.). Therefore, this assessment can provide valuable information as to how to proceed in the following stages of the project.

2. **To optimize anaerobic digestion on agricultural wastes with high content in lignocellulose.**

Lignocellulosic biomass has been reported to present lower susceptibility to biological degradation due to its crystalline structure. Artichoke and asparagus wastes were used as representative substrates due to their local importance in Navarre and their high consumption worldwide. Additionally, kinetic modeling was performed as to give a broader view to each process and easier comparison between configurations.

To this concern, three main approaches have been carried out in 1-L reactors:

- The effect of digestion temperature (35 °C, 42 °C, and 55 °C) for each waste separately.
- Based on the previous studies, the effect of temperature-phased anaerobic digestion (TPAD) with two phases: one first stage at thermophilic range (for 5 or 7 days) followed by a mesophilic one to complete the degradation of each substrate.
- The assessment of co-digestion of artichoke and asparagus wastes at three different temperatures (35 °C, 42 °C, and 55 °C) and two different ratios (1:2 and 2:1), in order to determine which of them enhances or hampers the process.

3. To study the performance and microbial populations of anaerobic digestion of agricultural wastes.

Microbiological studies on complex populations can contribute to the elucidation of the relationships (synergies, competition, syntrophy, etc.) established within the anaerobic consortia and also provide information regarding the operation of the digesters. In order to assess the evolution of the microbiological consortium in the systems, several studies were approached:

- Sloe and malt were used as organic substrates in 1-L reactors in order to evaluate the effect of thermal pretreatment (110 °C for 2 hours) on their respective anaerobic treatments at 35 °C and 55 °C and, more specifically, on the bacterial population throughout time. Commercial kits of anaerobic microbial identification (API20A) were used.

- 16S rRNA amplicon analyses were also performed on biogas-upgraded reactors treating cattle manure for two years. This will lead to a deeper insight of microbial communities' stabilization and specialization in steady-state anaerobic reactors (Technical University of Denmark – DTU).

4. **To study the effect of pesticides on the anaerobic digestion of local agricultural wastes.**

Agricultural wastes can contain chemical pesticides that are used during harvesting and can potentially affect microbiological processes such as AD. In this project, the study of the effect of two widely used pesticides (mancozeb -a fungicide- and tefluthrin -an herbicide-) has been assessed in 1-L reactors. For this purpose, the co-digestion of several wastes (green pea, cabbage, green bean, carrot, artichoke and bean) has been used to assess the influence of each pesticide alone and the combined effect of both in the anaerobic reactor. This has been carried out at the three aforementioned operation temperatures (35 °C, 42 °C and 55 °C).

Additionally, a scale-up of this topic was carried out in batch reactors (from 1-L to 5-L reactors), using four different agricultural wastes, namely sloe, malt, asparagus and artichoke. This study was conducted at mesophilic range, for being the widespread range at industrial scale, and the obtained results can be key to pursue future research.

This dissertation follows the approach represented below (Figure 1):

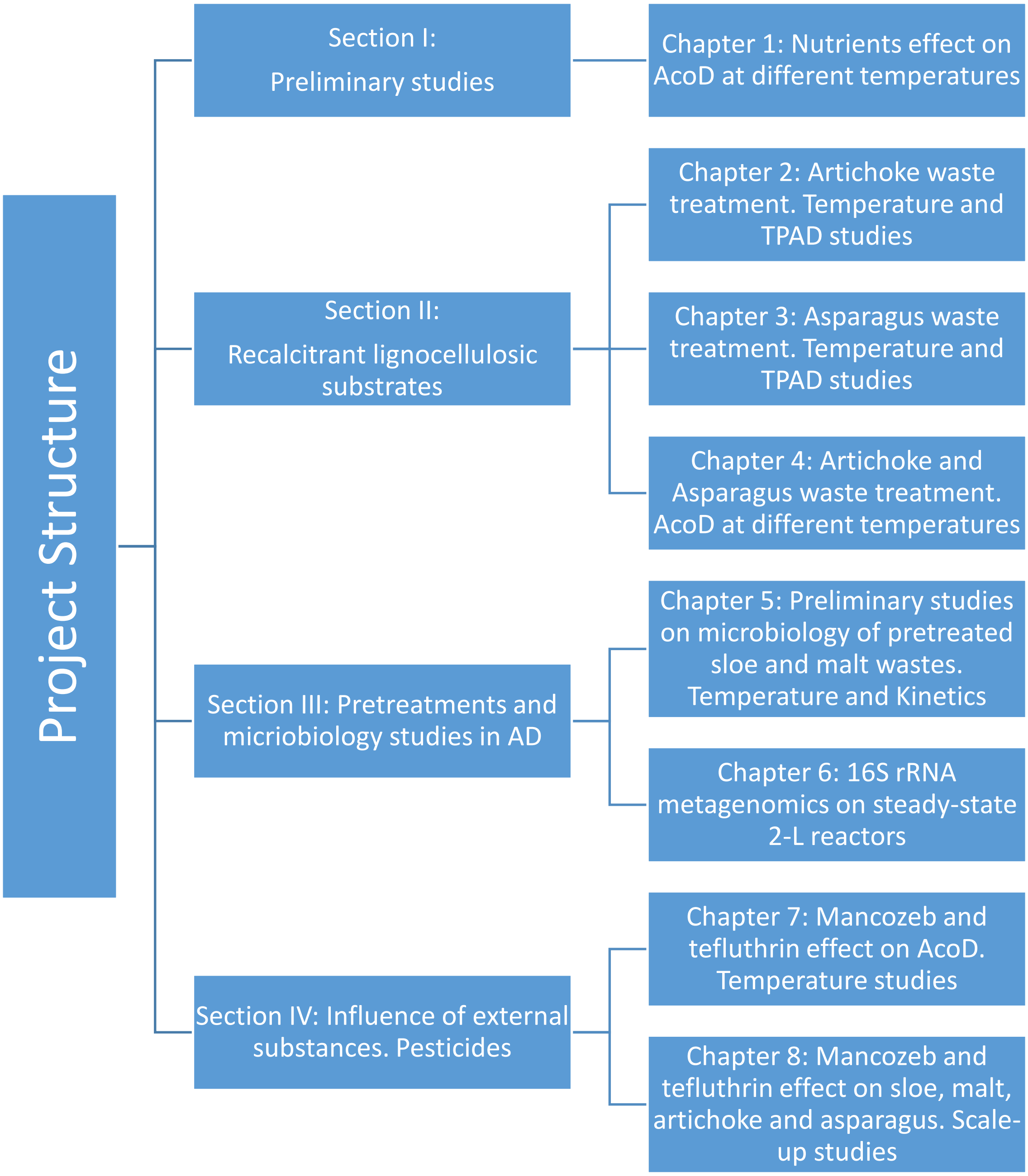

Figure 1. Project structure scheme.

III. Results and Discussion

Section I: Preliminary studies

Chapter 1

Impact of supplementary nutrients on codigestion of agricultural waste: Study of temperatures

Published in Food and Bioproducts Processing

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Section II: Recalcitrant lignocellulosic substrates

Chapter 2

Kinetic analysis of single-stage and temperature-phased anaerobic digestion on lignocellulosic waste: artichoke

Biochemical Engineering Journal. With Editor

Kinetic analysis of single-stage and temperature-phased anaerobic digestion on lignocellulosic waste: artichoke

Beatriz de Diego-Díaz[a], Francisco J. Peñas[a], Juana Fernández- Rodríguez[a]

[a] University of Navarra, Department of Chemistry, School of Sciences, Irunlarrea 1, 31009 Pamplona, Spain.

Abstract

Lignocellulosic wastes are organic substrates that generally present high resistance to be degraded through biological treatments, for instance, anaerobic digestion (AD). However, they are potentially a good source of organic molecules that can become biogas. Additionally, sustainability plans encourage their biodegradation. Therefore, AD optimization was assessed in this study approaching the temperature of operation and using artichoke as a representative of lignocellulosic waste.

The studies were performed at mesophilic (35 °C), intermediate (42 °C) and thermophilic (55 °C) ranges. Results showed that thermophilic and mesophilic conditions yielded similar biogas production and productivities (6.0 and 6.1 L/reactor, this is 0.04 kWh; and 360.1 and 336.0 mL-biogas/g-VS; 236.2 and 284.3 mL-CH_4/g-VS, respectively).

On the other hand, intermediate temperature condition did not have a significant impact on the AD.

Additionally, temperature-phased anaerobic digestion (TPAD) – thermophilic:mesophilic - was studied in two configurations: 5 days thermophilic-range (TPAD5) and 7 days thermophilic-range (TPAD7). Both somehow improved single-stage

AD, being TPAD7 more efficacious (7.8 L/reactor, 48.8 kW/mL, 441.6 mL-biogas/g-VS, 375.3 mL-CH_4/g-VS).

The kinetic modelling with the experimental data, considering VS and biogas, fitted accurately (R^2 > 0.9697). The non-biodegradable substrate (S_∞) was lower in TPAD systems (around 15.4 mg/L), comparing with single-stages (average 17.4 mg/L). Additionally maximum specific growth rates (μ_M) were higher for TPAD, specifically TPAD7 (0.0630 d^{-1}) as opposed to single-stages, being the minimum in mesophilic (0.0308 d^{-1}) and maximum in thermophilic (0.0615 d^{-1}).

Based on the obtained results, TPAD configurations have shown better performance dealing with lignocellulosic waste in front of single-stage temperatures, being the TPAD7 the best condition studied. The optimization of AD using lignocellulosic waste would recover energy from an abundant source of residual organic matter.

Keywords: temperature-phased anaerobic digestion (TPAD); single-stage temperature; lignocellulosic waste; kinetic modelling; energetic valorization.

1. Introduction

Anaerobic digestion (AD) is a natural-occurring process in which waste is degraded and stabilized. This can be controlled at industrial level for organic waste minimization and generation of an added value product: biogas. However, not every substrate is equally susceptible to biological break-down. Some lignocellulosic compounds such as those derived from the agricultural industry, forestry or plant biomass in general, are more recalcitrant and, therefore, show more difficulties to be degraded.

Lignocellulose is the most abundant renewable material on earth, being a good source of organic molecules that can be transformed into useful products such as chemicals, materials and biofuels, being lignin the most difficult to be decomposed through biological treatments due to its chemically stable structure (phenolic and non-phenolic polymer). This entails a difficult degradation of the rest of the lignocellulosic structure. Additionally, the presence of sulfur and oxygen results in an obstacle to produce fuels or liquid additives from lignocellulose. These are the main reasons for waste lignin being mainly used nowadays for combustion (Ge et al., 2016; International Lignin Institute, 2018; Kleinert and Barth, 2008).

The crystalline structure of lignocellulosic matter, which has a very strong bond force, prevents microorganisms from accessing it, which directly relates to retarded mass transfer; but also some other problems arise from its composition (Shi et al., 2013). Among others, we find that inhibitor accumulation is a bottleneck when digesting this type of substrates, as the difficulty to break-down organic matter leads to intermediate products awaiting to be further degraded (Yang et al., 2015). However, lignocellulose is potentially and theoretically a good source of organic substrate for biogas generation, which yields 6.0-6.5 kWh/m^3 (Deublein and Steinhauser, 2008). Moreover, Spanish legislation such as the National Plan for Waste Research 2008-2015 (BOE, 2009), still in force, encourages biological approaches (AD and/or composting) for organic waste treatment, as they are more sustainable comparing, for example, with combustion. Therefore, the optimization of AD on lignocellulosic substrates is of capital importance and it can be pursued through various strategies such as co-digestion or temperature phases.

Temperature is a key condition that has been widely studied in the literature as a parameter to be considered when optimizing industrial biomethanization of wastes. Temperature conditions have been classified into psychrophilic (< 20 °C), mesophilic (20-45 °C), thermophilic (45-60 °C) and hyperthermophilic (> 60 °C) range. For mesophilic and thermophilic ranges, the most common ranges, 35 °C and 55 °C have been determined to be the optimal temperatures. However, also intermediate temperatures such as 41-45 °C are also implemented (Sakar et al., 2009).

Previous studies have shown that thermophilic ranges lead to higher velocities in the degradation of substrates, shortening operation times. High temperatures lead to higher microbiological growth and conversion rates that are translated into more efficacious degradation of organic matter (De Vrieze et al., 2016). On the other hand, mesophilic ranges provide more stability to the process, as rapid accumulation of potentially inhibitory products is avoided.

Attending to operative costs at industrial scale, the literature shows how the payback time of the net investment of biogas plants is 3-5 years, considering only the selling of electric energy from AD. For instance, a plant operating with a thermophilic reactor

(55 °C) improved revenue from a milder temperature (47 °C), reaching generations of 8,789 MWh/year. This would entail 52,000 €/year of energy investment (total costs of 719,000 €/year) while obtaining benefits of 1,933,473 €/year (Cavinato, et al., 2010).

In the 1990s, a new temperature configuration with the aim of joining the advantages of both temperature ranges was proposed. Han and Dague (1997) worked with domestic primary sludge that combined two phases of temperatures (temperature-phased anaerobic digestion, TPAD): first stage in thermophilic (55 °C) and second stage in mesophilic (35 °C). Since then, TPAD has been widely studied as a good strategy for treating organic waste by AD comparing with the single stage temperature (Aslanzadeh et al., 2014; Chu et al., 2008; Fernández-Rodríguez et al., 2016 and 2015; Kim et al., 2011). Generally, the configuration used entails an initial thermophilic stage followed by a mesophilic one. This leads to the speeding up of the bottleneck of the process in early stages of AD in batch and semicontinuous configurations.

As a representative lignocellulosic waste, in this study, biomethanization of artichoke is addressed. Artichoke is one of the most characteristic vegetables in the Mediterranean agriculture. Italy and Spain are the world leading producers with up to 747,700 tonnes (total world production is estimated in 1.79 million tonnes) (FAO, 2014). Artichoke is mainly directed to market consumption and industrial purposes, both of which generate a considerable amount of waste; around 50 % of the total input. Leaves, bracts of heads and 30 % of the edible part of the vegetable are discarded and can be used for waste valorization through animal feed (García et al., 2005) or AD (Fabbri et al., 2014; Ros et al., 2013). In fact, through AD treatment up to 826.03 GWh/year could be recovered from this kind of waste (Deublein and Steinhauser, 2008; Fisgativa, et al., 2010).

Biomethanization is a widely applied technology; therefore, the approaches to its improvement for recalcitrant substrates are manifold. In this study, the main objective is the enhancement of AD on artichoke through the following strategies: temperature studies in the mesophilic range (M35), an intermediate temperature (I42) and thermophilic range (T55). Additionally two configurations of TPAD (TPAD7 and TPAD5) were carried out for biomethanization of the lignocellulosic waste: artichoke; to compare the performance of the overall processes.

2. Materials and Methods

2.1 Experimental System

Continuous stirred-tank reactors (CSTR) were used for batch assays according with Holliger et al. (2016) (Figure 1). The main advantage of using CSTRs is a more complete and uniform mixing, as well as homogeneity within the digester (e.g. temperature and density) (Bhatia and Yang, 2017). Each reactor consisted in a sealed bottle (1.0 L of total volume and 0.6 L of working volume) with a port for biogas collection in 5L-fluorinated ethylene propylene bags (SKC). Magnetic stirring was used for each reactor to maintain homogeneity and these were kept in a thermostatic bath filled with oil to avoid evaporation of the heating fluid. Digesters were set up according to total solids (TS) (≃ 6 %), working in the wet range (4-10 % of TS).

Each reactor contained 0.2 L of inoculum (1/3 of the working volume of the reactor), which did not significantly add to the total organic matter content of the digesters, and 0.05 L of a nutrient solution based on that used by Sevillano et al. (2011).

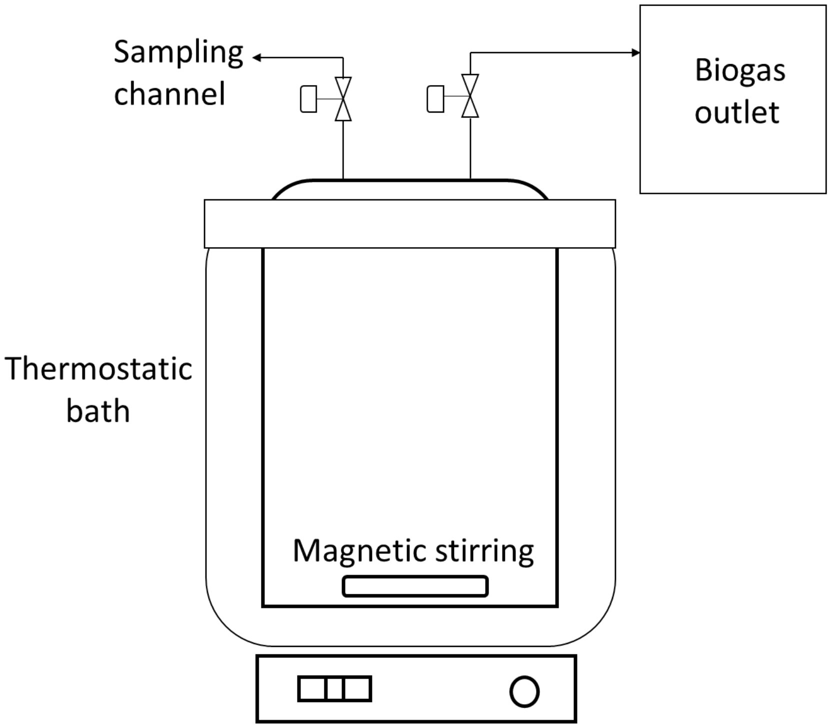

Figure 1. Continuous stirred-tank reactor (CSTR) diagram used in this study.

Several temperature configurations were carried out: mesophilic range (M35), an intermediate temperature (I42) and thermophilic range (T55). Also, temperature-phased anaerobic degestion (TPAD-T55:M35) was assayed for AD optimization. The first phase at 55 °C (T) and the second at 35 °C (M). Two TPAD configurations were elected according to preliminary studies: one with a first phase of 5 days (TPAD5) and another one of 7 days (TPAD7). Every configuration was kept operating until the total arrest of biogas production.

2.2 Analytical Methods

Analytical methods were carried out according to standardized methods (APHA, 2012). The parameters monitored were total and soluble chemical oxygen demand (tCOD and sCOD, respectively), pH, alkalinity, TS, volatile solids (VS). For sCOD, samples were previously centrifuged and the supernatant was filtered with 0.45 µm and 0.20 µm filters (Starstedt). Both tCOD and sCOD were analyzed in a spectrophotometer (Agilent

Technologies 8453). Alkalinity was determined in a 1:50 dilution in deionized water with a final point of pH 4.3.

Biogas production was determined by bubbling it through a gasometer and CH_4 content was analyzed with a gasometer containing an alkaline solution (Holliger et al., 2016). Additionally, a biogas analyzer (Geotech Biogas5000) was used to quantify the composition.

2.3 Substrates and initial characterization

Artichoke used in this study was locally harvested. The inoculum was collected from the anaerobic reactor of a municipal wastewater treatment plant and adapted to each of the temperatures of study through previous incubation. The characterization for artichoke and sludge is shown in Table 1 and digesters at starting point in Table 2.

Table 1. Characterization of substrate and inoculum used in this study.

Waste	tCOD ($g O_2/L$)*	sCOD ($g O_2/L$)*	TS (%)	VS (%)***	pH	Alkalinity ($mgCaCO_3/L$)
Inoculum	7.50	5.50	1.10 ± 0.05	1.00 ± 0.06	6.60	900
Artichoke	72.59**	24.68**	13.87 ± 0.37	12.17 ± 0.33	5.86**	1305

*Standard error of 0.2 gO_2/L (equipment error)
** Measured with 1:10 dilution of the sample
*** Percentage from the total fraction

2.4 Statistical analysis

All statistical analyses were carried out with Microsoft Excel software that uses the following equations:

Correlation coefficient (ρ), which determines the statistical relationship between two variables, according with:

$$Correl\ (\mathrm{X,Y}) = \frac{\sum(x-\bar{x})(y-\bar{y})}{\sqrt{\sum(x-\overline{x})^2 \sum(y-\overline{y})^2}}$$

Being x: each value of variable 1; $\bar{x}$=average value of variable 1; y= each value of variable 2; $\overline{y}$= average value of variable 2.

Asymmetry coefficient (AC), which allows establishing the symmetry degree in a probability distribution of a random variable, according with:

$$\frac{n}{1(n-1)(n-2)}\sum\frac{(x-\bar{x})^3}{s}$$

Being n= sample size; x: each value of the variable; $\bar{x}$=average value of the variable; s: standard deviation.

2.5 Kinetic modelling

Kinetic modelling for this study was already successfully used in de Diego-Díaz et al. (2018), based in substrate consumption (S) and biogas production (B), where:

$$-\mathrm{d}S/\mathrm{d}t = \mu_M\,(S_M - S)\,(S - S_\infty)\,/\,(S_0 - S_\infty)$$

$$-\mathrm{d}B/\mathrm{d}S = \alpha$$

μ_M: maximum specific growth rate of biomass; S_M: maximum theoretical substrate concentration of initial biomass, S_∞: non-biodegradable substrate concentration; S_0: initial substrate concentration; α: biogas yield coefficient.

3. Results and Discussion

The characterization of digesters at starting point was carried out and is shown in Table 2.

Table 2. Characterization of reactors at starting point.

Waste	tCOD ($g\ O_2/L$)*	sCOD ($g\ O_2/L$)*	TS (%)	VS (%)**	pH	Alkalinity ($mg\ CaCO_3/L$)
M35	74.06	29.33	5.87	5.36	7.06	1100
I42	59.90	19.39	5.92	5.24	6.93	1090
T55	59.47	29.78	6.27	5.50	6.36	745
TPAD7	50.27	15.58	6.06	5.45	6.93	1105
TPAD5	51.60	19.95	5.41	4.92	7.14	1250

*Standard error of 0.2 gO_2/L (equipment error)
** Percentage from the total fraction

3.1 Study of single-stage temperature: M35, I42 and T55

All the studied configurations have shown a good performance, providing higher productions of biogas than those stated in previous studies (Fabbri et al., 2014). However, in this study some differences can be noted among the three conditions assessed (Table 3). Total biogas production was the best of all three temperatures at M35 (6.1 L); however, this temperature was not successful at removing solids, as only 34.12 % of TS were eliminated. On the other hand, biogas production per TS consumed was the best of all three operative conditions with up to 507.9 mL-biogas/g-TS.

Additionally, the other productivities were only slightly surpassed by T55 with 360.1 mL-biogas/g-VS and 236.2 mL- CH_4/g-VS.

The substrate that the system is no longer able to degrade is also considered important. Both M35 and I42 showed a very similar amount of final VS, 2.33 % and 2.40 %, respectively (Figure 2). However, T55 showed a final amount of VS of 2.73 %, meaning that this operative condition does not manage to reduce organic matter as effectively as others do. Moreover, it is M35 that manages to eliminate more relative VS with up to 56.53 % of removal. Considering that T55 is supposedly the condition at which organic matter would be removed more efficaciously due to thermodynamics, this can be explained through intermediate products formation. The initial high efficacy in breaking down polymers is translated in slight inhibition of the system as supported by a lower pH in this stage. Therefore, it can be concluded that under milder conditions, the reactor could still eliminate the remaining substrate.

Being artichoke so difficult to biodegrade, solids removal was the main objective of the study. The best range to this aim was thermophilic, as it got to remove 45.45 % of TS (1.3-fold the eliminations at 35 °C). Additionally, total biogas production was not far from that of mesophilic range, with 6.0 L. The quality of this biogas was, on the other hand, better for mesophilic range as the total amount of CH_4 generated was 5.2 L, the highest amount in proportion to the biogas produced and the highest in absolute terms. This absolute proportion, 85.2 %, overtakes that from the reported by Ros et al. (2013) with a 65.0 %. Additionally, CH_4 production from VS_{added} has previously been reported to be 160 mL-CH_4/g-VS_{added} under mesophilic conditions for a recalcitrant substrate (food waste and straw), which is practically the same for M35 in this study

(161.7 mL-CH_4/g-VS_{added}) (Kim et al., 2003). Moreover, biogas and CH_4 yields were the best of all conditions but it should be noted that T55 is not far from these values.

The intermediate temperature evaluated, 42 °C, provided positive results only for solids removal (43.93 % of TS and VS 54.20 %), but still thermophilic range slightly surpassed TS removal with up to 45.45 % but relegated VS elimination (1.1-fold higher for I42). On the other hand, biogas and CH_4 generation (4.8 L and 2.2 L, this is 0.03 and 0.01 kWh, respectively) and their ratio to solids reduction are far away from what the other two temperatures studied managed to yield, probably due to the non-optimal temperature that could affect methanogenic activity.

Process optimization can take place through TPAD, as an initial thermophilic phase and a following mesophilic one can lead to a more stable process in which organic matter hydrolysis is accelerated. The stabilization of biogas production and organic matter consumption appeared to take place during the first 7 days of the process as seen in Figure 2. Therefore, TPAD were performed accordingly, choosing 5 and 7 days for the thermophilic phase.

From these results, it can be observed that thermophilic and mesophilic ranges are the most suitable for biomethanization of artichoke regarding removal of organic matter and productivities. Intermediate range does not show remarkable performance in this case.

Table 3. General results for artichoke AD. Temperature studies.

	M35	I42	T55
TS removal (%)	34.12	43.93	45.45
VS removal (%)	56.53	54.20	50.36
COD consumed (%)	13.82	27.41	20.69
Biogas (L)	6.1	4.8	6.0
CH_4 (L)	5.2	2.2	3.9
Biogas/TS removed (mL/g)	507.9	310.5	350.0
Biogas/VS removed (mL/g)	336.0	284.4	360.1
CH_4/TS removed (mL/g)	429.8	141.0	229.5
CH_4/VS removed (mL/g)	284.3	129.1	236.2
Time (≃days)	11	7	17

3.2 Temperature-phased anaerobic digestion of artichoke: TPAD5 and TPAD7

Temperature-phased reactors were set-up with an initial stage in the thermophilic range of 5 and 7 days (TPAD5-T and TPAD7-T, respectively) and a second step in the mesophilic range (TPAD5-M and TPAD7-M, respectively). These studies took the similar time overall (21 and 19 days, respectively), being 14 days in mesophilic until the biogas production was stable and the organic matter was no longer degradable by the system (Table 4 and Figure 3). For the phase at 55 °C for each configuration, results are somehow similar bearing in mind that each of them were kept for different times. For TPAD5-T, it can be seen how solids removal is more successful as it manages to reduce 43.62 % of VS, while TPAD7-T reaches a similar result with 42.77 %. Throughout the following phase at 35 °C, both TPAD5-M and TPAD7-M also eliminated almost the same VS, meaning that 55 °C and its duration are key to achieve high organic reductions as it is at this temperature when the most solids are removed.

Related to biogas production, the major amount is generated at 55 °C for both configurations, which is logical due to the hydrolytic phase in which many H_2 is released from the break-down of macromolecules. In TPAD7-T, the production of biogas was higher, which could have to do with the fact that 55 °C is kept for two days longer. However, the expected biogas production for TPAD5-T was higher than that obtained, which also proves that a longer thermophilic phase is positive for the system, probably due to the conditioning of the system to the presence of intermediate products. The difference of production for both systems in early stages (Figure 3b) is attributed to the heterogeneity of the inoculum, and the complexity of working with solid substrates and the overall process. Biogas production at 35 °C, on the other hand, can be considered to show less difference, as TPAD7-M produced 1.0 L and TPAD5-M almost reached 0.7 L.

A different scenario can be seen attending to productivities, which show how TPAD7 is more efficient. TPAD7-T reaches 482.0 mL-biogas/g-VS, while TPAD5-T barely produced 305.4 mL-biogas/g-VS. However, 35 °C for both configurations yielded productions per VS that can be contemplated as the same, ranging 283.1-252.1 for mL-biogas/g-VS and 188.4-172.5 for mL-CH_4/g-VS, for TPAD7-M and TPAD5-M, respectively.

Additionally, each TPAD experiment was analyzed as a black box, considering the starting and the ending point. Comparing both configurations, TPAD5 and TPAD7, the second one provided general better results. Biogas and CH_4 productions and yields relating TS consumption were the best when the thermophilic phase took 7 days rather than 5. Total biogas production reached 7.8 L (0.05 kWh) with TPAD7. TPAD5, on the other hand, managed to produce 4.7 L-biogas (0.03 kWh), the lowest production including those for the single-stage temperature. However, CH_4 yield was 248.9 mL-CH_4/g-VS, which still is a very positive result. Interestingly, the improvement of the biogas:CH_4 ratio of single-stage (T55) through TPAD was 27.7 % and 30.2 % for TPAD5 and TPAD7, respectively. This is in accordance with the literature, which has shown that this enhancement generally is of the 26-50 % (Ge et al., 2011).

Regarding VS elimination, TPAD7 was also insignificantly more efficacious, as this configuration managed to consume 53.69 % of the total VS added, while TPAD5 reached 53.38 %. To this concern, improvement of VS removal for T55 was around 6.2-6.8 % approximately, slightly lower than that reported by the literature, 7-11 % (Ge et al., 2011). However, other authors such as Wu et al. (2015) have shown how TPAD has no effect on VS removal when compared to mesophilic single-stage, which is consistent with our results. For biogas productivity, TPAD7 reached a higher yield than TPAD5 for VS removal, with 441.6 mL-biogas/g-VS as opposed to 295.6 mL-biogas/g-VS, 1.5 times less than TPAD7. Also, TPAD7 (418.1 mL-biogas/g-TS) showed a biogas production per g-TS consumed that was better than TPAD5 (332.9 mL-biogas/g-TS).

Artichoke stabilization through TPAD yielded better results when compared to single-stage configuration regarding some of the parameters studied. VS removal only improved when compared with thermophilic digestion, as TPAD7 managed to eliminate 53.6 % while 35 °C condition only reached 50.99 %. TS elimination was also improved through TPAD7, which was 1.5 times higher than at M35. Total biogas and productivity per VS consumed were only improved through TPAD7 (441.6 mL/g-VS; 1.3-fold and 1.2-fold that of M35 and T55, respectively), while TPAD5 showed lower results than both single-stage temperatures (295.6 mL/g-VS; 1.1-fold and 1.2-fold lower than that of M35 and T55, respectively). Additionally, total biogas production reached 7.8 L with TPAD7, a considerable optimization of both temperatures (6.1 L at M35 and 6.00 L at T55 - 0.04 kWh).

Regarding parameters related to CH_4, total production was improved for mesophilic and thermophilic conditions via TPAD7 with up to 6.6 L. Additionally, the efficiency of CH_4 generation through solids removal, especially VS, was optimized with the temperature-phased digestion system: TPAD5 (248.9 mL-CH_4/g-VS) slightly improved T55. TPAD7 (375.3 mL-CH_4/g-VS), on the other hand, surpassed both T55 and M35. The latter was enhanced in 12 %, which is in accordance with Xu et al. (2018) that showed how CH_4 yields are improved by 10-20 % with TPAD.

Based on the above-discussed results, it seems that the TPAD7 configuration yielded the best performance compared to the other configurations.

Table 4. Results for artichoke AD. TPAD studies.

	TPAD7		TPAD5		TPAD7	TPAD5
	TPAD7-T	**TPAD7-M**	**TPAD5-T**	**TPAD5-M**	**Black box**	
TS removal (%)	25.95	33.81	37.78	8.53	50.99	43.08
VS removal (%)	42.77	19.08	43.62	17.30	53.69	53.38
COD consumed (%)	9.85	9.71	1.96	52.14	18.60	53.08
Biogas (L)	6.7	1.0	3.9	0.7	7.8	4.7
CH_4 (L)	5.9	0.7	3.4	0.5	6.6	3.9
Biogas/TS removed (mL/g)	714.2	111.1	320.5	421.1	418.1	332.9
Biogas/VS removed (mL/g)	482.0	283.2	305.4	252.1	441.6	295.6
CH_4/TS removed (mL/g)	626.8	73.9	279.2	288.1	355.3	280.3
CH_4/VS removed (mL/g)	423.0	188.4	266.0	172.5	375.3	248.9
Time (≃days)	7	14	5	14	21	19

3.3 Statistical analysis

Statistical analysis was carried out to establish correlation between processes of different conditions regarding solids and biogas production. Correlation coefficient (ρ) showed a high relation (> 0.720) between TS and VS for all the processes except for M35 (Table 5). Asymmetry coefficient (AC) for both TS and VS is positive for all the processes as well, except for M35 and T55 (for the latter the value is not far from 0). For M35, the low value can be explained by considering that a negative AC could be translated into a slower hydrolytic phase as there are more values that are further to the average TS and VS at the left tail. Therefore, M35 is the condition at which hydrolytic phase is slower than the other four processes that have a positive AC.

Also ρ for biogas production was calculated between processes. Data show that I42 and T55 are highly correlated with $\rho_{x.y}$=0.968, meaning that the production in both conditions can be considered equal. On the other hand, M35 and T55 show a low correlation of 0.685. It is worth-mentioning that the correlation between M35 and T55 is higher than the one of M35 and I42, while the temperature difference is bigger between M35 and T55 than for M35 and I42. This can be due to the optimal temperatures at which biomethanization can take place. Therefore, thermophilic and mesophilic ranges are more highly related than an intermediate temperature with the mesophilic range, in spite of being only 7 °C away instead of 13 °C.

Table 5. Statistical analysis for TS, VS and biogas at different conditions. ρ=correlation coefficient. AC=asymmetry coefficient.

	M35	**I42**	**T55**	**TPAD7**	**TPAD5**
$\rho_{TS.VS}$	0.450	0.991	0.723	0.901	0.949
AC_{TS}	-0.791	1.898	-0.143	1.036	2.289
AC_{VS}	1.668	2.012	1.316	2.272	2.149
	M35/I42	**M35/T55**	**I42/T55**	**TPAD7/TPAD5**	-
$\rho_{x.y\ (biogas)}$	0.587	0.685	0.968	0.806	-

3.4 Kinetic modelling

The fitting results for the kinetic model are shown in Table 6. These refer to VS elimination and biogas production. Also, both experimental and predicted data are reflected in Figures 2 and 3. It can be seen how these are in close agreement with each other thanks to the used model. Moreover, regression coefficients quantitatively indicate that calculated results and the experimental ones fit appropriately, as combined R^2 for the substrate consumption and biogas generation reaches 0.9660.

Table 6. Fitting coefficients for kinetic modelling of organic matter consumption and biogas generation in the biomethanization of artichoke under different temperature configurations.

System	S_∞ (mg/L)	S_M (mg/L)	μ_M (d^{-1})	α (L/g-VS)	R^2 (for VS)	R^2 (for biogas)
M35	17.09	182.00	0.0308	0.3635	0.9442	0.9725
I42	16.67	182.00	0.0587	0.3083	0.9768	0.9832
T55	18.28	148.91	0.0615	0.3837	0.9411	0.9795
TPAD7	15.58	139.00	0.0630	0.4280	0.9447	0.9697
TPAD5	15.20	138.50	0.0619	0.3047	0.9712	0.9749

For maximum specific growth rate (μ_M) it is worth-mentioning that it increases with temperature for single-stage studies, reaching 0.0615 d^{-1} for T55. This can be translated into a faster growth under thermodynamically favorable conditions. Therefore, and considering a lower experimental efficiency in VS removal, it can be again concluded that the process was somehow inhibited by intermediate products accumulation. Attending to TPAD, both configurations surpass the μ_M of single-stage digesters; and, what is more, TPAD7 showed the highest value, managing to double the maximum specific growth rate of M35. Additionally, the theoretical elimination of VS ($1–S_\infty/S_0$) by TPAD7 was the highest of all (52.3 %), 1.12-fold that of M35 and T55. Moreover, being the predicted non-biodegradable substrate (S_∞) almost the lowest (15.59 mg/L; only slightly lower for TPAD5 and with 1.7 times less biogas production) for TPAD7 it can be concluded that this configuration is potentially the most efficacious. Additionally, it is worth-noting that S_∞ for both TPAD studies are considerably lower than T55 while μ_M remains comparable. This can lead to the conclusion that with better-expected eliminations, the operative costs derived from heating of the reactor can be lowered through TPAD at industrial scale.

Biogas yield (α) was around the same order for all the conditions, ranging from 0.3047 – 1.9 Wh/g-VS (TPAD5) to 0.4280 L/g-VS – 2.7 Wh/g-VS (TPAD7). However, it is again worth noting that TPAD7 yielded the best-predicted productivity, being 30 % higher than the lowest one.

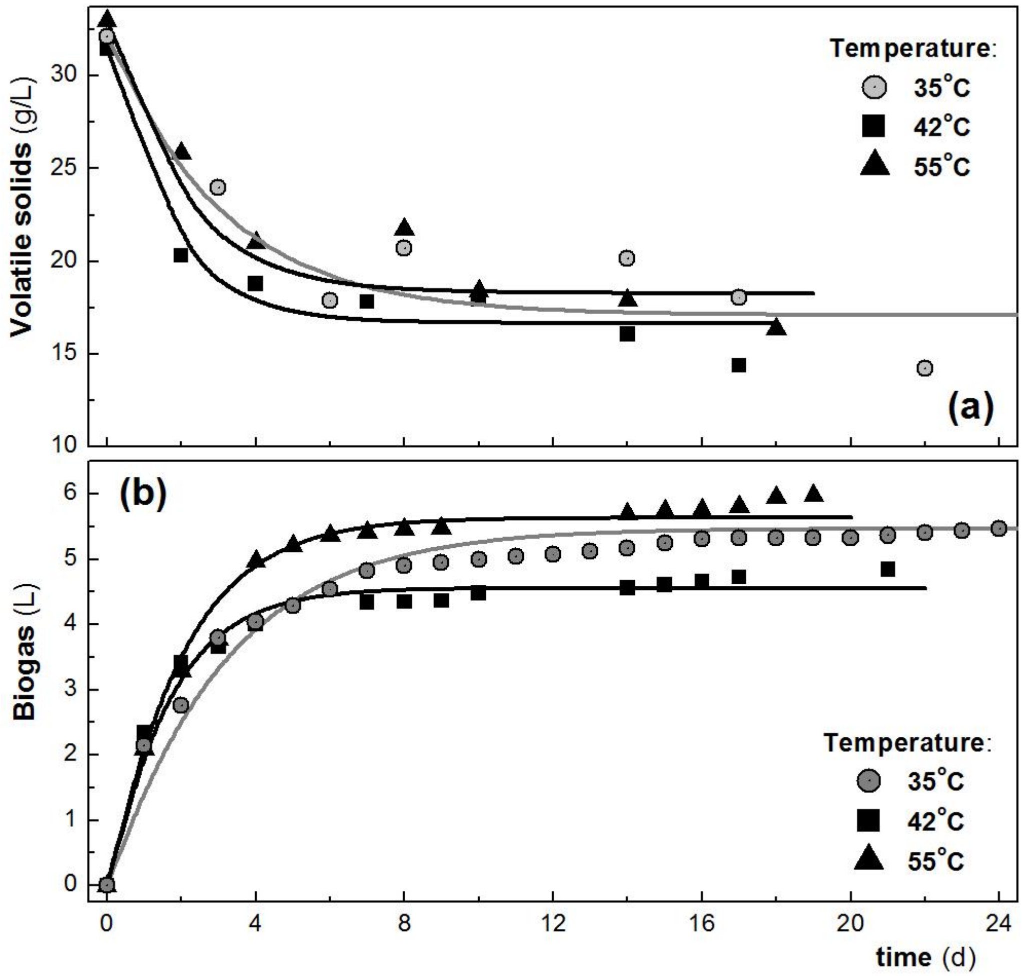

Figure 2. Kinetic modelling of single-stage temperature. a) VS; b) Biogas production; • 35 °C; ■ 42 °C; ▲ 55 °C.

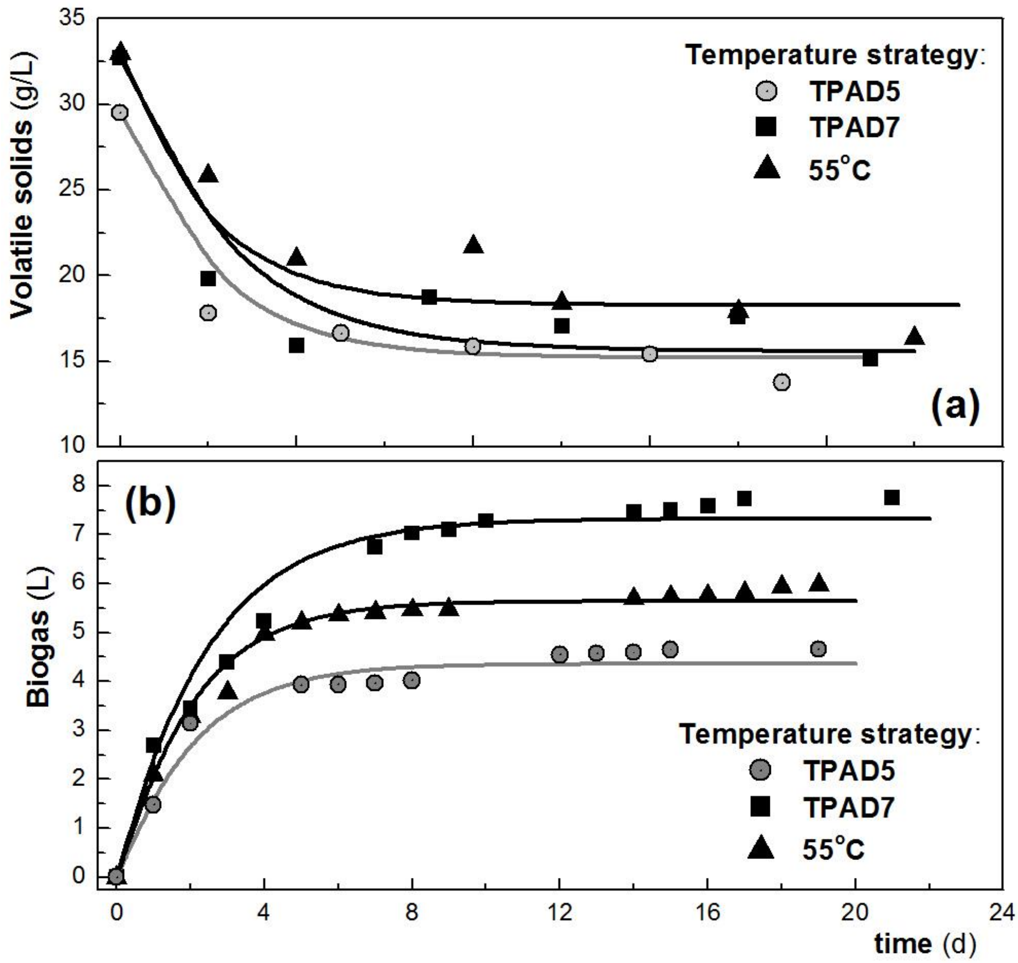

Figure 3. Kinetic modelling of TPAD. a) VS; b) Biogas production; • temperature-phased anaerobic digestion (5 days of thermophilic phase); ■ temperature-phased anaerobic digestion (7 days of thermophilic phase); ▲ 55 °C.

4. Conclusions

Mesophilic range, taking artichoke as a representative lignocellulosic substrate, showed to be a suitable temperature for anaerobic digestion regarding stability of the process and productivities (284.3 mL-CH_4/g-VS). However, the kinetic modelling shows a better maximum specific growth rate for T55 (0.0615 vs. 0.0308 d^{-1}). For the purpose of the study, thermophilic range is considered the best operative condition as it manages to reduce organic matter in an outstanding way, managing to remove up to

45.45 % of the TS. Despite this, productivities and absolute biogas production are very similar to M35 (6.0 L-biogas/reactor), which entails lower operative costs.

In TPAD configurations it was seen that the duration of the thermophilic stage is expectedly relevant, for longer periods result in higher degradations. Moreover, TPAD7 managed, not only to surpass biogas production of TPAD5 with up to 7.8 L vs. 4.7 L (0.05 vs. 0.03 kWh), but also exceed those productions from single-stage temperatures (maximum 6.1 L). The kinetic modelling showed how the non-biodegradable substrate is lower for TPAD (15.58 and 15.20 mg/L) than for any single-stage process (17.09, 16.67 and 18.28 mg/L). According to the results, TPAD, and more specifically TPAD7, is the proposed configuration for this kind of recalcitrant agricultural waste.

A growing implementation and optimization of agricultural waste as feedstock for AD, especially lignocellulosic substrates, would lead to a high energetic recovery from its organic fraction.

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Chapter 3

Kinetic approximation of asparagus biomethanization according to temperature strategies: Single-stages vs temperature-phased anaerobic digestion

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Kinetic approximation of asparagus biomethanization according to temperature strategies: Single-stages vs temperature-phased anaerobic digestion

Beatriz de Diego-Díaz[a], Francisco J. Peñas[a], Juana Fernández- Rodríguez[a]

[a] University of Navarra, Department of Chemistry, School of Sciences, Irunlarrea 1, 31009 Pamplona, Spain.

Abstract

Lignocellulosic waste presents low availability for anaerobic digestion (AD) but its structure and composition makes of it a potential feedstock for biogas production. Asparagus is a lignocellulosic vegetable that is highly produced worldwide but little research has been published related to AD of this substrate. Temperature of operation has been widely studied and proposed as a determinant parameter for AD process optimization. Herein, studies of temperature on AD of asparagus are presented. Three single-stages were chosen: mesophilic (35 °C –M35-), intermediate (42 °C –I42-) and thermophilic (55 °C –T55-). Results showed how M35 yielded higher stability to asparagus AD, with high productivities (408.0 mL-CH_4/g-VS), accumulated productions (8.1 L-biogas and 6.0 L- CH_4), and VS removal (62.06 %). However, T55 lasted less time and reached similar VS removals (63.81 %). On the other hand, I42 only showed positive VS elimination while the rest of the parameters did not show outstanding results.

Additionally, temperature-phased anaerobic digestion (TPAD) with thermophilic and mesophilic phases was carried out on asparagus. Two configurations were studied: one with 5 days (TPAD5) and another one with 7 days (TPAD7) at 55 °C, followed in both

cases by a second phase at 35 °C. Both showed through kinetic modelling a considerably higher maximum specific growth rate (TPAD7: 0.0956 and TPAD5: 0.1172 d^{-1}) than single-stage temperature processes. Moreover, all CH_4:biogas ratio for single-stage conditions were improved by TPAD with productions of CH_4 of 84 and 79 % of total biogas for TPAD7 and TPAD5, respectively.

These results could contribute to the limited knowledge on biological degradation of asparagus through anaerobic digestion.

Keywords: lignocellulosic waste; asparagus; single-stage temperature; temperature-phased anaerobic digestion (TPAD); kinetic modelling

1. Introduction

Anaerobic digestion (AD) is a biological process in which organic substrates are treated and broken-down. This technology not only allows a controlled disposal of organic matter and its stabilization, but also biogas production. Hence, AD manages to recover a considerable amount of the energy present in waste (energetic valorisation). Nevertheless, the composition of some organic substrates poses an obstacle for its hydrolysis. The high content in lignocellulose, for instance, makes of the waste a very recalcitrant substrate that is difficult to be attacked by the degrading machinery of anaerobic microbiota. Among them, those derived from the agricultural and forestry industries as well as overall plant biomass can be mentioned.

Lignocellulose is a complex polymer with great biomethanization potential due to its structure and composition. Moreover, it is the most abundant renewable material on earth, which makes of it a preferred source of molecules to be broken-down into chemicals, biofuels, materials, etc. However, due to its strong bonded and packed structure (phenolic and non-phenolic), lignin is the fraction that shows more resistance to be decomposed, hindering as well the break-down of the other two components of lignocellulose, namely cellulose and hemicellulose. On top of this, the high content in sulphur and oxygen is a drawback for the production of liquid additives and fuels, and it is, therefore, why lignin residue is usually directed to combustion (Ge et al., 2016; International Lignin Institute, 2018; Kleinert and Barth, 2008).

As aforementioned, the crystalline structure of lignocellulose can pose an obstacle to its biological degradation. This is so especially because its low exposure to microbial mechanisms results in retarded biomass transfer and intermediate products accumulation for its further break-down, which can lead to inhibition (Shi et al., 2013; Yang et al,. 2015). Despite all this, as greenhouse gas emissions and the shift towards greener forms of energy are a main environmental concern nowadays, the use of this vast amount of lignocellulosic waste can be implemented as feedstock for large-scale AD reactors. Additionally, Spanish legislation like the National Plan for Waste Research 2008-2015 (BOE, 2009), which is still in force, promotes biological treatment pathways like AD and/or composting due to their sustainability as opposed to combustion. The European Union promotes the implementation of small-scale biogas plants in the agro-food and beverage industry because it can report benefits like energy savings, reduction of environmental impact and carbon footprint, and reduction of waste management costs. In fact, Directive 2009/28/EC establishes a common legislative framework for the use of energy from renewable sources in order to limit greenhouse gas emissions and to promote cleaner transport (European Parliament and of the Council, 2009). Consequently, improvement of AD on this type of waste is highly important and temperature phases and co-digestion, among others, have been reported to be the main strategies (Ji et al., 2017).

Among the parameters of operation, temperature is a relevant condition that has commonly been used as a way of optimizing AD. Temperature ranges are classified as follows: psychrophilic (< 20 °C), mesophilic (20-45 °C), thermophilic (45-60 °C) and hyperthermophilic (> 60 °C). The most widely used ranges are mesophilic and thermophilic, being the optimal microbial growth temperature set at 35 °C and 55 °C, respectively. Nevertheless, intermediate temperatures (41-45 °C) are also object of study and industrial implementation (Sakar et al., 2009). The advantages of high temperatures, such as thermophilic range, include higher microbial growth, better conversion rates, more efficacious breakdown of substrates, higher velocities of degradation and shorter operating times, among others. However, also drawbacks are derived as, for instance, mild temperatures, such as mesophilic range, yield more stability, result from a lower accumulation of inhibitory intermediate compounds (De Vrieze et al., 2016).

Han and Dague (1997) reported that the advantages of combining two temperatures in the AD process of domestic primary sludge are manifold. Among them, separating biomethanization steps in two (first hydrolysis, at a higher temperature, and then the following phases at a lower temperature), potentially lowering operative costs, operating with different retention times and organic loads, controlling inhibition, speeding up early stages of the process, higher CH_4 productions, etc. (Wang et al. 2016). This configuration is called temperature-phased anaerobic digestion (TPAD) and it generally integrates a first thermophilic stage (optimal 55 °C) followed by a mesophilic one (optimal 35 °C). Until now, TPAD has been widely studied in batch and semicontinuous regimes, and especially to optimize and compare single-stage processes (Aslanzadeh et al., 2014; Chu et al., 2008; Fernández-Rodríguez et al., 2016 and 2015; Kim et al., 2011).

In this study, asparagus was chosen as representative of agricultural lignocellulosic waste and its treatment and optimization through AD is tackled. Asparagus consumption is high worldwide, being China the principal producer of this vegetable and accounting with up to 88 % of the global cultivation (Chen et al. 2014). However, the edible part of asparagus is small and the rest of it is discarded as waste. This, along with the rapid economic growth and the structural agricultural adjustment of the country, is translated in big amounts of residue not only from asparagus but also from fruit and vegetables in general. For instance, 1.3 million tonnes per day of waste are generated in China, while the second largest producer barely reaches 895 t/day. This, as reported by the literature, and due to its composition, can be used as animal feed, composting, feedstock for bioenergy or even for pulping and papermaking (Garcia-Peña et al. 2011; Ji et al. 2017) and, more importantly, AD (Chen et al., 2014). In fact, in spite of the large global scale of the asparagus and the potential as lignocellulosic biomass for AD, it is difficult to find literature dealing with the topic. AD is a straight-forward process and its implementation is widely spread. Its optimization strategies are abundant and divers. Herein temperature approaches on asparagus waste AD are studied in single-stage: mesophilic range (35 °C), intermediate temperature (42 °C) and thermophilic range (55 °C). Also, TPAD studied, considering first thermophilic stage followed to second mesophilic stage (T55:M35) were performed.

2. Materials and methods

2.1 Experimental system

Experimental procedures were performed in accordance to the methods described by Holliger et al. (2016). Each continuous stirred-tank reactor (CSTR) was a sealed bottle (V_T = 1.0 L and V_W = 0.6 L) with a biogas collecting port to which a 5L-fluorinated ethylene propylene bag (SKC) was attached. To guarantee homogeneity, magnetic stirring was used and each digester was placed in a thermostatic bath filled with oil in order to avoid evaporation of the heating fluid. Reactors were set up in accordance to total solids (TS, ≃ 6 %) operating in the wet range (4-10 %).

Digesters were filled up with 0.2 L of adapted inoculum (1/3 of the V_W) and 0.05 L of the nutrient solution based on that proposed by Sevillano et al. (2011).

The conditions studied were mesophilic (35 °C, identified as M35), intermediate (42 °C, identified as I42) and thermophilic (55 °C, identified as T55) ranges. Temperature-phased anaerobic digestion, first thermophilic stage followed by a second mesophilic stage (TPAD-T55:M35), were additionally carried out to compare the performance with single stages. Two TPAD systems were picked out in accordance to preliminary studies of thermophilic first stage: one with 5 days of thermophilic phase (TPAD5) and another one with 7 days (TPAD7).

2.2 Analytical Methods

Analytical methods were performed in accordance with the Standard Methods (APHA, 2012). The samples from reactors were subjected to the following procedures: soluble and total chemical oxygen demand (sCOD and tCOD, respectively), pH, alkalinity, total solids (TS) and volatile solids (VS). Samples for sCOD were first centrifuged and then the supernatant was filtered through 0.45 μm and 0.2 μm filters (Starstedt). A spectrophotometer (Agilent Technologies 8453) was used for tCOD and sCOD determination. Alkalinity was performed in diluted samples (1:50) in deionized water with a final point of pH 4.3. The samples of the solid wastes were leachate in water (proportion 1:5) during 1 h in order to obtain the soluble analytical parameters.

Biogas production was quantified with a gasometer and its content in CH_4 was determined by bubbling biogas through an alkaline solution in a gasometer (Holliger et al., 2016). In addition, a biogas analyzer (Geotech Biogas5000) was utilized to determine biogas composition.

2.3 Substrate Characterization

Asparagus employed in this paper were locally harvested. The inoculum, which was an already digested sludge from an anaerobic reactor from a municipal wastewater treatment plant, did not significantly contribute with organic matter to the system and was previously adapted to each of the herein studied temperatures. Both were characterized and results are shown in Table 1.

Table 1. Characterization of substrate and inoculum employed for this study.

Waste	tCOD (gO_2/L)*	sCOD (gO_2/L)*	TS (%)	VS (%)***	pH	Alkalinity ($mgCaCO_3/L$)
Inoculum	7.50	5.50	1.10	1.00	6.60	900
Asparagus	115.03**	40.84**	10.47	9.54	6.08**	895

*Standard error of 0.2 gO_2/L (equipment error)
** Measured with diluted leachate of the sample (1:10)
*** Percentage from the total fraction

2.4 Statistical Analysis

Statistical analyses were carried out in order to estimate the value of the experimental data. All statistical analyses were executed using Excel software 2010 (Microsoft, USA), through the Data Analysis AppIn. The following equations are the ones applied for each analysis:

- Correlation coefficient (ρ) yields information about the statistical relationship between two variables (biogas and VS), according with:

$$Correl\ (X, Y) = \frac{\sum(x-\bar{x})(y-\bar{y})}{\sqrt{\sum(x-\overline{x})^2 \sum(y-\overline{y})^2}}$$

Where x = each value of variable 1; $\bar{x}$ = average value of variable 1; y = each value of variable 2; $\overline{y}$ = average value of variable 2.

- Asymmetry coefficient (AC) establishes the symmetry degree in a probability distribution of a random variable, according with:

$$\frac{n}{1(n-1)(n-2)}\sum\frac{(x-\bar{x})^3}{s}$$

Where n = size of the sample; $x =$ each value of the variable; $\bar{x}$ =average value of the variable; $s =$ standard deviation.

2.5 Kinetic modelling

The kinetic adjustment for this study was already successfully employed in de Diego-Díaz et al. (2018), based in VS consumption (S) and biogas formation (B), where:

$$-\mathrm{d}S/\mathrm{d}t = \mu_M\,(S_M - S)\,(S - S_\infty)\,/\,(S_0 - S_\infty)$$

$$-\mathrm{d}B/\mathrm{d}S = \alpha$$

μ_M: maximum specific growth rate of biomass; S_M: maximum theoretical substrate concentration if initial biomass, S_∞: non-biodegradable substrate concentration; S_0: initial substrate concentration; α: biogas yield coefficient.

3. Results and Discussion

In this paper, temperature approaches on asparagus waste AD are studied in single-stage: mesophilic range (M35), intermediate temperature (I42) and thermophilic range (T55). Also, TPAD (T55:M35) in two different configurations was performed (TPAD5 and TPAD7). The characterization of digesters at set-up point was performed and it is shown in Table 2.

Table 2. Characterization of reactors at set-up point of the experiments.

Waste	tCOD (gO_2/L)*	sCOD (gO_2/L)*	TS (%)	VS (%)**	pH	Alkalinity ($mgCaCO_3/L$)
M35	62.05	36.31	3.5	2.3	7.27	600
I42	55.37	22.65	3.67	3.18	7.31	1650
T55	35.80	22.59	5.58	3.54	6.66	800
TPAD7	55.37	22.65	3.67	3.18	7.31	1650
TPAD5	51.60	19.95	5.14	4.56	7.14	1100

*Standard error of 0.2 gO_2/L (equipment error)
** Percentage from the total fraction

3.1 Effect of single-stage temperature on asparagus

Temperature studies showed how different conditions can provide advantages and disadvantages to the process attending to different parameters (Table 3). Biogas production for the three conditions was fairly good, between 5.5 and 8.1 L. These results are higher than those obtained for rice straw (2.4-5.3 L), which also has a high content in lignocellulose, as demonstrated by Chandra et al. (2012). Additionally, the pace of the production is in accordance with the expected results, as there is an initial high production due to the hydrolytic phase and the initial contact of the microbial community with an accessible amount of organic matter that is susceptible of being used. Furthermore, Figure 1 serves as proof of the adaptation of the inoculum, as there is no lag phase, which would have meant that the microbiota is not acclimated to the operative conditions of the experiments. Biogas production is considerably higher for M35, while I42 and T55 have similar absolute generations, 32.4 % and 28.9 % lower than M35, respectively. This higher production could have been due to a higher stability of the process, as supported by pH control that was always easier for M35 than for I42 and T55.

It is also worth-noting that between days 16 and 17 of M35 there was a production peak as can be seen in Figure 1. This event could be due to the progressive break-down of organic matter. Sometimes, the recalcitrance of the substrate hinders activity of the bacterial community that can only degrade attackable matter. The crystalline structures take longer to be accessible, especially under lower temperatures such as M35 at which thermodynamics are not as favorable. Therefore, this sudden higher production of biogas (1.1 L) can be explained through the presence of newly released intermediate substrates that trigger a high activity in the microbiota that can further degrade the feedstock.

Attending to CH_4 content, it was M35 the one that managed to yield the highest CH_4:biogas production ratio with an absolute generation of CH_4 that surpassed 74.43 %. The same was seen for T55, for which CH_4 generation was 61.7 % of the cumulate biogas produced. Both percentages are in accordance with the expected biogas composition, considering the recalcitrance of the substrate. Moreover, previous studies on lignocellulosic substrates have shown a cumulate CH_4 content of 55-65 % (Negi et al., 2018). However, I42 only managed to produce a biogas with 49.5 % of CH_4. This could be related with the fact that 42 °C is generally not an optimal growth temperature, thus arresting and lowering the efficiency of the process.

On the contrary, organic removal was similar to that obtained for M35 and T55 (mean elimination of 63.4 %). These results improve those reported by Chen et al. (2014) for the same substrate (54.3 %)

Figures 1 and 2 show that there is a clear correlation between biogas production and VS elimination. The former starts arresting around day 6-8 and the latter does the same at day 6. Interestingly, TS removal for T55 is almost 3-fold that from M35 and I42, which has to do with thermodynamics at this temperature that is able of breaking down not only organic substrates but inorganic as well.

Attending to productivities related to VS removal, M35 is once again the condition that yielded the best results, followed by T55. CH_4/VS-eliminated for M35 reached 408.0 mL/g (2.7 and 2.0-fold that from I42 and T55, respectively), indicating that the stability of the reactor is key to an efficient process in which the resources, namely the organic waste, are well utilized. Moreover, M35 is the only that surpasses the efficiency reported elsewhere for asparagus AD (Chen et al., 2014).

Table 3. General results for single-stage temperature studies on asparagus anaerobic digestion.

	M35	I42	T55
TS removal (%)	22.29	23.19	62.72
VS removal (%)	62.06	64.47	63.81
COD consumed (%)	29.85	24.04	28.03
Biogas (L)	8.1	5.5	5.8
CH_4 (L)	6.0	2.7	3.6
Biogas/TS removed (mL/g)	1736.0	1074.4	275.2
Biogas/VS removed (mL/g)	548.2	301.9	323.3
CH_4/TS removed (mL/g)	1292.0	531.5	169.8
CH_4/VS removed (mL/g)	408.0	149.4	199.4
Time (≃days)	17	10	8

3.2 Temperature-phased anaerobic digestion on asparagus

TPAD studies were performed with two configurations: TPAD7 and TPAD5, 7 days and 5 days in thermophilic range, respectively. Those configurations were chosen because previous studies in our laboratories showed that this two were the most appropriate when compared to others in which the thermophilic phase was fixed between 3 and 10 days.

The initial phase (thermophilic) showed general better results for TPAD7. Biogas production was almost the same for both configurations as can be seen in Table 4. However, CH_4 production and CH_4:biogas ratio were higher for TPAD7-T probably due to the fact that a longer hydrolytic phase involves more degradable intermediate substrates. This, together with a longer period for CH_4 production, makes of TPAD7 a preferred configuration regarding productions and solids removals. To this regard, TS elimination was up to 1.4 times more efficacious than for TPAD5. On the other hand, TPAD5-T VS removal was close to that from TPAD7-T, showing both of them a good performance in very early stages of the process. What is more, TPAD7-T yielded a VS elimination (55.53 %) that was only 12 % lower than the whole single-stage temperature M35 (62.06 %), which took 17 days as opposed to 7 of TPAD7-T.

In addition, it is worth-noting that solids were mainly removed for both configurations in the thermophilic phase, which was expected, as higher temperatures favor hydrolysis from a thermodynamic point of view. On the other hand, for TPAD5-M only 9.01 % of the remaining VS were eliminated while for TPAD7-M up to 18.27 % of them were removed in this second stage. This can also be seen in Figure 3, as the biogas production arrests for TPAD5 after the thermophilic phase, while for TPAD7 the process can be considered to still be operating until day 14. Additionally, VS graph shows how TPAD5 can no longer degrade the substrate, while TPAD7 shows a slow pace tendency that still manages to eliminate the organic matter present in the reactor.

The black box description, taking into account the entrance and the exit flows of the processes, shows a scenario that also has to be studied. In spite of TPAD7 potentially requiring more operative costs, biogas and CH_4 productions are higher than those for TPAD5. For productivities, values are slightly higher for TPAD5; however, they are fairly close to those showed by TPAD7, especially regarding VS. Additionally, this configuration (247.6 mL-CH_4/g-VS), as well as TPAD5 (252.7 mL-CH_4/g-VS), demonstrated to have better productivities than T55 (199.4 mL-CH_4/g-VS). As described by the literature, TPAD manages to improve CH_4 content in a 26-50 %. This is in accordance with our studies only regarding I42 and T55, as TPAD5 and TPAD7 managed to increase CH_4:biogas ratio in 23-41 % (Ge et al., 2011)

Nevertheless, TPAD on other food wastes has previously shown to be, for instance, more efficacious in removing VS, with eliminations that reached 75 % (Gaby et al., 2017). TPAD for asparagus stabilization can be, therefore, further optimized through different approaches. Its high content in recalcitrant macromolecules such as lignocellulose hinders total degradation of a substrate that can yield further biogas production and lower organic content in the final effluent. On the other hand, as little has been done with this type of waste, the results presented in this study are a starting point for a deeper understanding of the treatment of asparagus residue.

Table 4. General results for TPAD studies on asparagus anaerobic digestion.

	TPAD7		TPAD5		Black box	
	TPAD7-T	TPAD7-M	TPAD5-T	TPAD5-M	TPAD7	TPAD5
TS removal (%)	45.40	6.81	33.33	11.22	49.12	40.81
VS removal (%)	55.53	18.27	52.14	9.01	63.66	56.58
COD consumed (%)	21.55	48.01	17.37	54.53	59.21	62.43
Biogas (L)	4.6	0.4	4.5	0.4	5.0	4.9
CH_4 (L)	3.9	0.3	3.6	0.3	4.2	3.9
Biogas/TS removed (mL/g)	332.5	315.2	440.7	148.0	331.2	387.1
Biogas/VS removed (mL/g)	313.7	166.4	317.8	289.6	294.9	314.7
CH_4/TS removed (mL/g)	280.8	245.5	350.0	135.9	278.1	310.8
CH_4/VS removed (mL/g)	264.9	129.6	252.5	265.8	247.6	252.7
Time (≃days)	7	16	5	9	21	14

3.3 Statistical analysis

Statistical analyses were performed on VS elimination and biogas production under every condition herein studied in order to relate operative variables and monitored parameters. As expected, correlation coefficient for biogas and VS ($\rho_{biogas,VS}$) showed a very strong relationship in every operating condition (> |0.9065|), which indicates that biogas production is, obviously, a consequence of VS elimination. The fact that the relationship is inverse is also logical because when VS decrease, cumulate biogas increases.

Asymmetry coefficients (AC) on VS are positive and most of the conditions have a value above 2. This is attributed to a longer right tail in the distribution of the values, meaning that there are more low values than high ones. This has to do with a rapid hydrolysis in early stages that considerably reduces VS in the beginning of the process, while the rest of it shows VS that decrease little by little. The opposite argument occurs for biogas production, whose AC is negative because its left tail is the one with more values.

Attending to different conditions correlation, biogas generation was statistically studied and results show how, for each temperature combination, cumulate biogas are highly correlated (> 0.9495). Interestingly, TPAD studies are the most highly correlated (0.9966) which make sense as both TPAD7 and TPAD5 operate at the same temperatures (35 °C and 55 °C). However, the differences between almost all processes are significant (α > 0.05) which means that despite the expected correlation, each configuration is different.

Table 5. Statistical analysis for VS and biogas at different conditions. ρ=correlation coefficient. AC=asymmetry coefficient.

	M35	**I42**	**T55**	**TPAD7**	**TPAD5**
$\rho_{biogas.VS}$	-0.9065	-0.9758	-0.9397	-0.9922	-0.9918
AC_{biogas}	-1.218	-2.536	-2.488	-2.706	-2.648
AC_{VS}	2.378	2.167	1.786	2.280	1.992
	M35/I42	**M35/T55**	**I42/T55**	**TPAD7/TPAD5**	-
$\rho_{x.y\ (biogas)}$	0.9501	0.9495	0.9936	0.9966	-

3.4 Kinetic modelling

The results attending to VS removal and biogas generation for the kinetic model adjustment are expressed in Table 6. Additionally, experimental and predicted data are shown in Figures 1-4 and they happen to be concordant between each other thanks to the chosen kinetic model. Furthermore, combined regression coefficient of biogas production and organic matter elimination supports these fit with a value as high as 0.9711.

Table 6. Fitting coefficients for kinetic modelling of organic matter consumption and biogas generation in the biomethanization of asparagus under different temperature configurations.

System	S_∞ (mg/L)	S_M (mg/L)	μ_M (d^{-1})	α (L/g-VS)	R^2 (for VS)	R^2 (for biogas)
M35	7.62	222.00	0.0124	0.4626	0.9393	0.9589
I42	9.04	220.00	0.0652	0.2654	0.9924	0.9502
T55	9.94	215.97	0.0311	0.2956	0.9856	0.9493
TPAD7	10.76	129.37	0.0956	0.3029	0.9948	0.9689
TPAD5	12.60	129.26	0.1172	0.3129	0.9876	0.9900

From the exposed results, it is worth-mentioning that maximum specific growth rate (μ_M) is notably high in TPAD processes, reaching this parameter 0.0956 d^{-1} and 0.1172 d^{-1} for TPAD7 and TPAD5, respectively. Therefore, it can be hypothesized that TPAD processes are thermodynamically favorable for microbial growth. This is probably due to a heterogeneous synergic population that is able to adapt to different temperatures, as different species with different optimal growth temperatures take part in the process in specific moments.

Also, productivity of biogas (α) is predicted to be better for TPAD (mean value of 0.3079 L-biogas/g-VS) in comparison to single-stage temperature processes (1.04-1.16-fold higher than I42 and T55), except for M35, which showed the highest value with up to 0.4626 L-biogas/g-VS.

On the other hand, theoretical VS removal ($1–S_{\infty}/S_0$) was higher for single-stage systems, reaching 68.1 % for M35. This is related to the non-biodegradable substrate (S_{∞}), which also showed to be the lowest in this condition (7.62 mg-VS/L). From these data, it can be concluded that process stability highly affects its success. However, the time of operation should be borne in mind, as costs for industrial operation are very relevant. Therefore, regardless the amount of VS that the system is able to degrade in the future, the lowest amount of it should be achieved in the minimum amount of time.

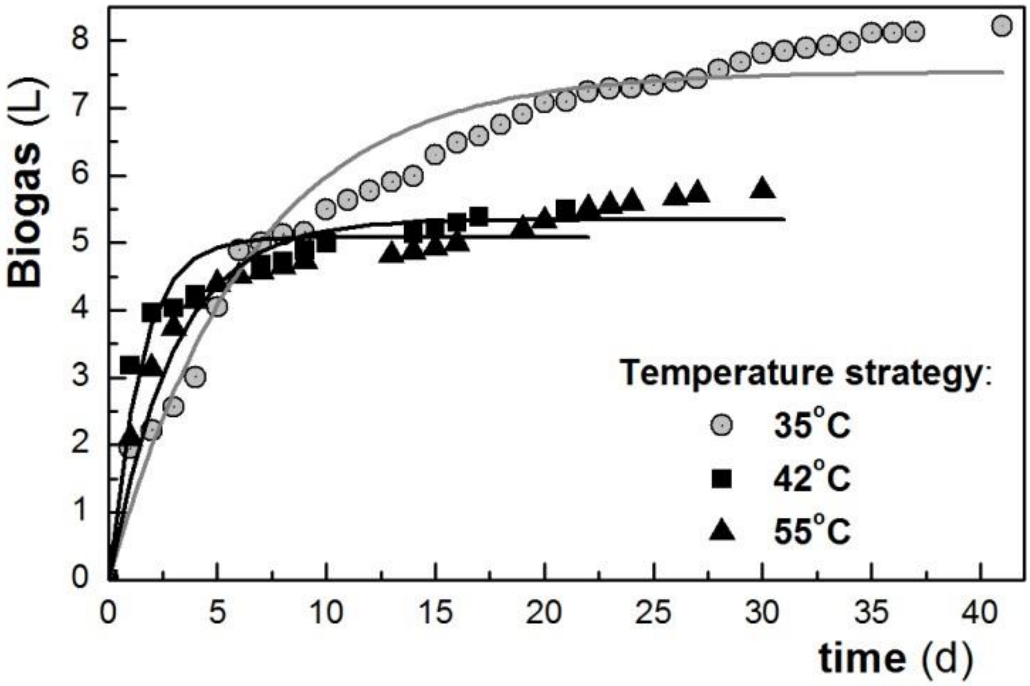

Figure 1. Kinetic adjustment of biogas production for single-stage temperature. • 35 °C; ■ 42 °C; ▲ 55 °C.

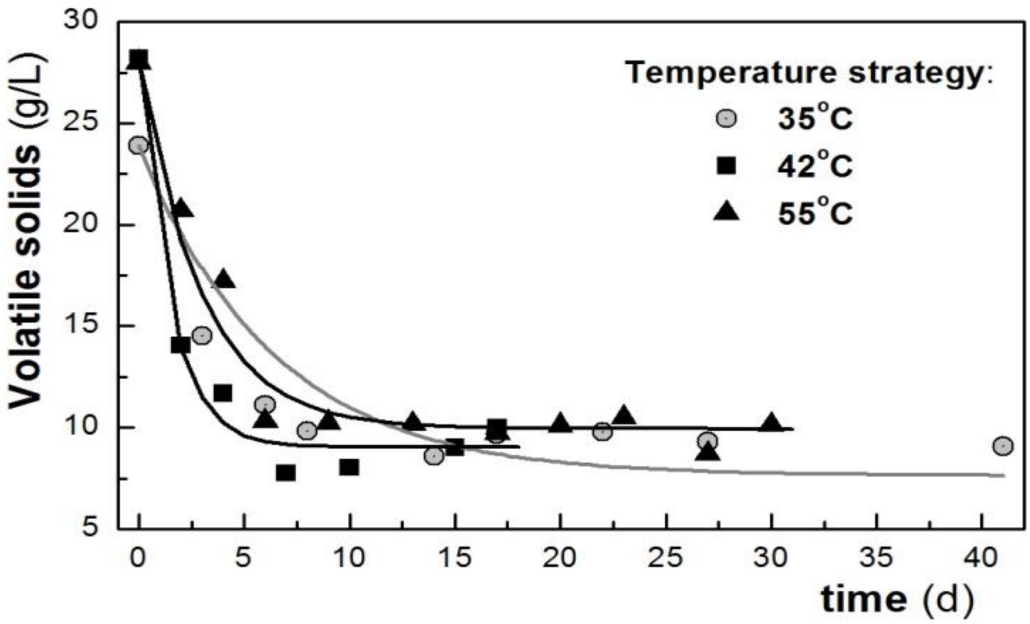

Figure 2. Kinetic adjustment of VS removal for single-stage temperature. • 35 °C; ■ 42 °C; ▲ 55 °C.

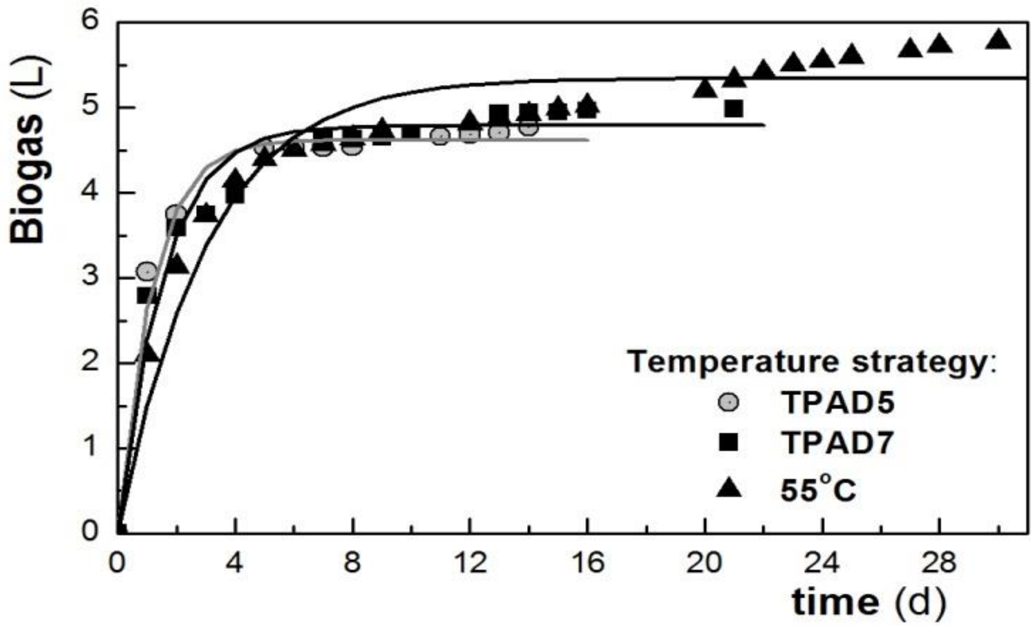

Figure 3. Kinetic adjustment of biogas production for TPAD. • temperature-phased anaerobic digestion (5 days of thermophilic phase); ■ temperature-phased anaerobic digestion (7 days of thermophilic phase); ▲ 55 °C.

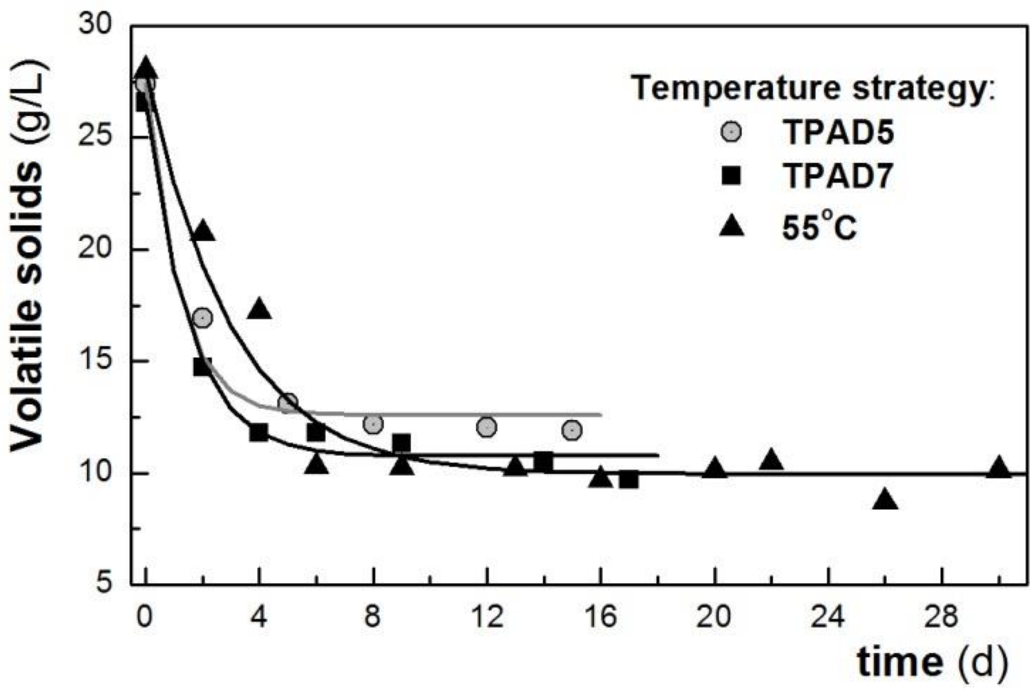

Figure 4. Kinetic adjustment of VS removal for TPAD. • temperature-phased anaerobic digestion (5 days of thermophilic phase); ■ temperature-phased anaerobic digestion (7 days of thermophilic phase); ▲ 55 °C.

4. Conclusions

Single-stage anaerobic digestion of asparagus showed better overall results for mesophilic condition (35 °C -M35-) compared to thermophilic (55 °C –T55-) and intermediate ranges (42 °C –I42-). Despite VS removal being similar to the three conditions (mean value of 63.45 %), productivities and cumulate productions were the highest for M35 (548.2 mL-biogas/VS, 408 mL-CH_4/VS, 8.1 L-biogas and 6.0 L-CH_4). Additionally, M35 yielded the best CH_4:biogas ratio, 0.74. All these, along with the easier pH control of the process and the lowest energetic consumption, allows hypothesizing that M35 is an operative condition that provides a higher stability and feasibility to AD handling a lignocellulosic waste like asparagus. On the other hand, it should be borne in mind that the process took considerably longer than T55 (17 vs. 8 days), which also reverts potential costs. Furthermore, TS removal was more successful for T55 (2.8 times higher than M35 and I42).

Attending to TPAD strategies, both configurations -7 days and 5 days in thermophilic-, yielded similar results, which did not significantly improve single-stage temperature studies. Nevertheless, kinetic modelling showed an outstanding maximum specific growth rate for TPAD7 and TPAD5 (0.0956 and 0.1172 d^{-1}, respectively) when compared to M35 (0.0124 d^{-1}), I42 (0.0652 d^{-1}) and T55 (0.0311 d^{-1}). Additionally, TPAD improved CH4:biogas ratio of all single-stage temperature, reaching for cumulate productions 84 and 79 % for TPAD7 and TPAD5, respectively.

Taking into account the potential of the lignocellulosic biomass of asparagus for biogas production, the presented results can complement the scarce literature addressing its anaerobic digestion.

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Chapter 4

Kinetics of anaerobic co-digestion at different temperatures on lignocellulosic substrates of the agricultural industry: asparagus and artichoke

Kinetics of anaerobic co-digestion at different temperatures on lignocellulosic substrates of the agricultural industry: asparagus and artichoke

Beatriz de Diego-Díaz[a], Francisco J. Peñas[a], Juana Fernández- Rodríguez[a]

[a] University of Navarra, Department of Chemistry, School of Sciences, Irunlarrea 1, 31009 Pamplona, Spain.

Abstract

Recalcitrant lignocellulosic wastes are difficult to degrade through biological pathways and co-digestion of several substrates of these characteristics could be the solution for their efficient break-down. To this aim, the effect of temperature (35 °C, 42 °C and 55 °C) on co-digestions of artichoke and asparagus studies was assessed. Two configurations were studied: one with a higher proportion of asparagus than artichoke and the vice versa (1Art/2Asp and 2Art/1Asp, respectively).

Overall results showed how both configurations improved to certain extent the performance of the substrates alone, which were previously studied. Especially, productivities that reached 404.1 mL-CH_4/g-VS at 35 °C and 127.5 mL-CH_4/g-VS at 42 °C for 2Art/1Asp, and 351.6 mL-CH_4/g-VS at 55 °C. Results indicate that the most suitable configuration would be 2Art/1Asp at 35 °C, as asparagus poses a bottleneck to the system despite kinetic modelling showing that the non-biodegradable substrate is always lower for 1Art/2Asp. Also kinetic modelling showed that the theorical efficiency (α) of the porposed system is one of the highest with up to 0.5483 L-biogas/g-VS.

Keywords: anaerobic digestion, artichoke, asparagus, co-digestion, lignocellulosic substrates.

1. Introduction

Anaerobic digestion (AD) is a natural-occurring process in which organic matter is partially oxidized and stabilized in the absence of oxygen. This leads, not only to waste treatment, but also to biogas production, product derived from microbial metabolism. Bacteria and Archaea are able to synergically break-down organic waste; however, not every substrate is equally susceptible to degradation. Lignocellulosic compounds like the derived from the agricultural industry, forestry or plant biomass, are more recalcitrant and, therefore, hamper their degradation through biological pathways.

Lignocellulose has been reported to be the most abundant renewable material on earth, thus being a potential supply of organic molecules that can be converted into marketable compounds such as chemicals, materials and biofuels. Within the lignocellulosic structure, lignin is the most refractory and hinders the overall degradation because of its phenolic and non-phenolic chemically stable arrangement (Kleinert and Barth, 2008). This adds up to the presence of sulfur and oxygen, which poses problems for the production of fuels or liquid additives (International Lignin Institute, 2018). Consequently, lignin waste is mostly directed to combustion. Biological treatments are difficulted by retarded mass transfer that keeps the microbial community from degrading it because of the crystalline structure (Shi et al., 2013). Despite this, due to its high potential for biogas generation, public organisms encourage biological pathways. For instance, the European Union supports the development of small-scale biogas plants for the agro-food and beverage sector due to the savings in energy and waste management costs, and the reduction in carbon footprint and environmental damage. Directive 2009/28/EC states the legislation as to establish limitations in GHG emissions (European Parliament and of the Council, 2009). Therefore, the optimization of AD on abundant and recalcitrant wastes is of high relevance. Co-digestion, among others, has been described to be one of the best approaches towards AD improvement (Ji et al., 2017).

Anaerobic co-digestion (AcoD) yields a great variety of advantages for process optimization from which the most worth-noting are the nutrient equilibrium attained through the combination of different substrates and lower costs derived from handling various wastes. Both result in higher biogas productions and productivities, as well as better relative CH_4 productions. However, the literature still poses the question of what is the most suitable combination and proportion of substrates, which at the moment remains at the level of specific individual experiments (Alatriste-Mondragón et al., 2006; Ji et al., 2017)

Among the fruit and vegetable sector wastes, artichoke and asparagus have already shown to be eligible for AD treatment in few studies (Chen et al., 2014; de Diego-Díaz et al. 2018c, 2018d; Fabbri et al., 2014; Ros et al., 2013), despite of the significant global production. These have a high content of lignocellulose that is potentially useful for biogas production and have also shown to be a good enhancer of the process when combined with other substrates such as pepper and other types of sludge from fruit and vegetables (Ros et al., 2013). On the one hand, artichoke production reaches up to 1.79 million tonnes but approximately 50 % of it is discarded as waste (FAO, 2014a). On the other hand, asparagus consumption worldwide situates this vegetable as one of the most highly demanded, being China the responsible of 88 % of its production

(Chen, 2014; FAO, 2014b). The growing interest for asparagus relies on the challenge of managing the residue of its cultivation, mainly composed of lignocellulosic stems.

The optimization of AD is also dependent on operative parameters such as temperature. This can be classified as psychrophilic (< 20 °C), mesophilic (20-45 °C), thermophilic (45-60 °C) and hyperthermophilic (> 60 °C). Among them, the most widely used are mesophilic and thermophilic, which have their optimal microbial growth at 35 °C and 55 °C, respectively. Both ranges present advantages and drawbacks. Among them, mesophilic yields a very stable performance while the degradation-production rates are slower than for thermophilic conditions. On the other hand, processes at high temperatures can be considered faster as the hydrolytic phase is overcome earlier; but this condition provides a more unstable and inhibitive environment that increases the probabilities of process arrest or failure (Fernández-Rodríguez et al., 2013).

However, intermediate temperatures (41-45 °C) are also of interest at laboratory and industrial scale and the literature has already shown effective substrate degradations reaching even a 70 % of total organic carbon removal. Temperature is not the sole factor that affects reactor performance, as substrate does have a great impact. Therefore, it is highly interesting to assess this temperature range because it could be suitable for the treatment of a specific substrate (de Diego-Díaz et al., 2018a; Sakar et al., 2009).

This study tackles treatment of artichoke and asparagus in AcoD configurations through AD: one with a higher proportion of asparagus than artichoke (1Art/2Asp) and the vice versa (2Asrt/1Asp). Considering the importance of temperature as a determinant operative condition, three ranges were studied: mesophilic (35 °C –M35-), intermediate (42 °C –I42-) and thermophilic (55 °C –T55-).

2. Materials and Methods

2.1 Experimental system

Co-digestion assays were carried out in continuous stirred-tank reactors (CSTR) in accordance with Holliger et al. (2016). Each reactor was a sealed bottle with a total volume of 1.0 L and a working volume of 0.6 L. The bottles had a port in order to collect biogas in 5L-fluorinated ethylene propylene bags (SKC). To keep homogeneity within the digesters, magnetic stirring was employed. Also, these were immersed in a thermostatic bath with oil to prevent evaporation of the heating fluid. Reactors were set up in accordance to total solids (TS), around 6 %, as to work within the wet range (4-10 % TS). Each digester was filled with 0.05 L of a nutrient solution based on that utilized by de Diego-Díaz et al. (2018a) and 0.2 L of inoculum (1/3 of the working volume of the digester). This fraction did not significantly contribute to the total organic matter of the reactors.

Three different temperature conditions were studied on co-digestion studies: mesophilic range (M35), an intermediate temperature (I42) and thermophilic range (T55). The AcoD configurations for the two lignocellulosic substrates were: one part of artichoke waste and two parts of asparagus waste (1Art/2Asp) and the vice versa (2Art/1Asp) in order to assess the effect of the concentration of substrates in AcoD.

2.2 Analytical Methods

All analytical methods were carried out according to standardized methods (APHA, 2012). The controlled parameters were total and soluble chemical oxygen demand (tCOD and sCOD, respectively), pH, alkalinity, total solids (TS) and volatile solids (VS). Samples for sCOD were centrifuged and the supernatant was then filtered through 0.45 µm and 0.2 µm filters (Starstedt). tCOD and sCOD were quantified in a spectrophotometer (Agilent Technologies 8453) and alkalinity was measured in a 1:50 dilution in deionized water to final point of pH 4.3.

Biogas generation was quantified by bubbling it in a gasometer, and its CH_4 composition was measured in an alkaline gasometer (Holliger et al. 2016). Also, a biogas analyzer (Geotech Biogas5000) was used to determine the composition.

2.3 Substrates and initial characterization

Artichoke and asparagus of this assessment were harvested locally and the inoculum was digested sludge from an industrial anaerobic digester of a municipal wastewater treatment plant. This was adapted to each respective temperature through previous incubation. The characterization of the three is shown in Table 1 as well as reactors at set-up point.

Table 1. Characterization substrate and inoculum used in this study and reactors at digester set-up.

System	Substrate	tCOD (gO_2/L)*	sCOD (gO_2/L)*	TS (%)	VS (%)***	pH	Alkalinity ($mgCaCO_3/L$)
-	Inoculum	7.50	5.50	1.10±0.05	1.00±0.06	6.60	900
-	Artichoke	72.59**	24.68**	13.87±0.37	12.17±0.33	5.86**	1305
-	Asparagus	115.03**	40.84**	10.47±0.17	9.54±0.09	6.08**	895
M35	1Art/2Asp	76.08	16.07	5.91	5.20	6.87	550
	2Art/1Asp	87.52	24.81	4.89	4.69	6.97	600
I42	1Art/2Asp	64.26	13.71	4,81	4.18	7.09	800
	2Art/1Asp	78.08	26.49	5.12	4.58	7.29	750
T55	1Art/2Asp	56.24	15.60	4,90	4.26	7.11	850
	2Art/1Asp	65.38	20.34	5.24	4.69	6.93	850

*Standard error of 0.2 gO_2/L (equipment error)
** Measured with 1:10 dilution of the sample
*** Percentage from the total fraction

2.4 Statistical analysis

In order to study correlations, statistical significances and distribution of the experimental data, statistical studies were performed with Microsoft Excel software, which uses the following equations:

Correlation coefficient (ρ), which allows the determination of the statistical relationship between two variables:

$$Correl\ (X, Y) = \frac{\sum(x-\bar{x})(y-\bar{y})}{\sqrt{\sum(x-\overline{x})^2 \sum(y-\overline{y})^2}}$$

Where x: each value of variable 1; $\bar{x}$=average value of variable 1; y= each value of variable 2; $\overline{y}$= average value of variable 2.

Asymmetry coefficient (AC), which establishes the degree of symmetry in a probability distribution of a random variable:

$$\frac{n}{1(n-1)(n-2)}\sum\frac{(x-\bar{x})^3}{s}$$

Where n= sample size; x: each value of the variable; $\bar{x}$=average value of the variable; s: standard deviation.

2.5 Kinetic modelling

Kinetic adjustment in this paper used a model that was previously used in de Diego-Díaz et al (2018b). This considers substrate consumption (S) and biogas generation (B), being:

$$-\mathrm{d}S/\mathrm{d}t = \mu_M\,(S_M - S)\,(S - S_\infty)\,/\,(S_0 - S_\infty)$$

$$-\mathrm{d}B/\mathrm{d}S = \alpha$$

S_M: maximum theoretical substrate concentration if initial biomass, S_∞: non-biodegradable substrate concentration; S_0: initial substrate concentration; μ_M: maximum specific growth rate of biomass; α: biogas yield coefficient.

3. Results and Discussion

3.1 Effect of co-digestion 1Art/2Asp

Co-digestion containing a higher proportion of asparagus was performed at the three temperatures of the study and the results are reflected in Table 2. The most interesting is biogas production, for which M35 and I42 produced a similar amount (6.7 and 6.6 L, respectively) but T55 rose up to 9.4 L. This is also worth mentioning because this high production even surpassed the highest of asparagus alone (8.1 L at M35) (de

Diego-Díaz et al., 2018d), meaning that the presence of artichoke waste yields a positive effect on asparagus anaerobic treatment. Moreover, 1Art/2Asp at the other two temperatures, 6.6 L at I42 and 9.4 L at T55, also improved the production of asparagus alone (5.7 L mean production of I42 and T55). The benefits of thermophilic ranges compared to milder temperatures are manifold. Among them: higher microbial growth rates, more efficient conversion rates, effective and faster substrate degradations, etc. (De Vrieze et al., 2016).

On the other hand, CH_4 content was not remarkable, especially when compared to monodigestion. However, the proportion comparing monodigestions and 1Art/2Asp followed the same pattern, being the highest for M35, then for T55 and lastly for I42. The latter is explained by the fact that an intermediate temperature may not yield optimal results as the optimal microbial growth is generally established at 35-37 °C and 55 °C for the mesophilic and thermophilic ranges, respectively. It is, nonetheless, worth mentioning that the content of CH_4 of the biogas for M35 is still within the values previously reported by the literature for recalcitrant lignocellulosic substrates (53-70 %) (Negi et al., 2018; Yang et al., 2015). Despite a lower CH_4 relative production, its productivity was highly optimized through co-digestions for T55 (1.8-fold) with 351.6 mL-CH_4/g-VS. This result also surpasses that described in the literature, where the best productivity was 242.3 mL-CH_4/g-VS for a pretreated asparagus waste with NaOH, which is also close to the 221.0 mL-CH_4/g-VS of M35 (Chen et al., 2014). This can lead to the hypothesis that co-digestion is a more efficacious process than an alkaline pretreatment that industrially could entail very high costs. Biogas productivity for T55 was also highly improved in a 42 % (772.4 mL-biogas/VS).

Regarding solids removal, monodigestion had previously shown better VS eliminations than those resulting from 1Art/2Asp (63.45 % and 50.7 % mean consumption of three temperatures, respectively). However, TS removal for both M35 and I42 was enhanced through this co-digestion configuration reaching 34.32 % and 24.13 %, respectively.

Attending to the speed of the process 1Art/2Asp yielded similar results to those of monodigestion. For M35 it was slightly improved (16 days vs 17 days of monodigestion), while for I42 and T55 the difference with monodigestion was one day longer for the co-digestion process.

Table 2. General results for AcoD of 1Art/2Asp.

	M35	**I42**	**T55**
TS removal (%)	34.32	24.13	24.88
VS removal (%)	56.92	47.13	47.65
COD consumed (%)	39.16	44.03	56.15
Biogas (L)	6.7	6.6	9.4
CH_4 (L)	3.9	1.2	4.3
Biogas/TS removed (mL/g)	551.8	884.5	1286.4
Biogas/VS removed (mL/g)	378.0	520.8	772.4
CH_4/TS removed (mL/g)	322.6	174.0	585.5
CH_4/VS removed (mL/g)	221.0	102.5	351.6
Time (≃days)	16	11	9

3.2 Effect of co-digestion 2Art/1Asp

A configuration of co-digestion with a higher proportion of artichoke than asparagus was also performed and results are shown in Table 3. Interestingly, for this study, 2Art/1Asp managed to improve the cumulate biogas production of artichoke monodigestion (de Diego-Díaz et al., 2018c) at all the three conditions with 7.0 L, 6.7 L and 6.3 L for M35, I42 and T55, respectively. However, the most remarkable improvement was for I42, for which the generation was almost 2 L higher than at monodigestion (4.8 L). For M35 and T55 this cumulate biogas production did not significantly change compared to the individual ones It can be hypothesized that inhibitors accumulation prevents microorganisms from continuing to consume organic matter, despite being resources for the biogas-producing machinery enough for high productions, but not for as many solids elimination as desired.

Once again, M35 for 2Art/1Asp, as well as for artichoke monodigestion, yielded the highest biogas production of all three conditions. This has to do with stability of the process, as a mild temperature, despite being slower, avoids intermediate products accumulation and, thus, inhibition. This was supported by the pH control of M35, which was notably easier throughout the experiments than for I42 and T55. Also, alkalinity was higher for M35, meaning that the system is more capable of facing changes result of the presence of newly broken-down products.

Nevertheless, the cumulate production of CH_4 was similar at M35 (5.1 L for 2Art/1Asp and 5.2 L for artichoke monodigestion) while for I42 and T55 was notably lower for 2Art/1Asp. This, therefore, means that the relative CH_4 generation is also worse for co-digestion, as overall biogas production is higher as previously stated.

Regarding biogas productivities, 2Art/1Asp showed outstanding results, ranging from 438.2 mL-biogas/g-VS for T55 to 552.9 mL-biogas/g-VS for M35. Artichoke monodigestion only reached 360.1 mL-biogas/g-VS as the maximum biogas productivity, for T55. This allows stating that a small amount of a different residue, such as asparagus, already improves the treatment of artichoke waste. Moreover, the considerable improvement of I42 (284.4 mL-biogas/g-VS for artichoke alone and 519.4 mL-biogas/g-VS for 2Art/1Asp) could potentially be attributed to the balance that co-digestion provides. A more heterogeneous substrate leads to a more balanced and synergic microbial population, thus improving its activity even at non-ideal growth temperatures. On the other hand, CH_4 productivity per VS consumed at this condition was the same, meaning that despite co-digestion biogas enhancement, those in charge of the methanogenesis cannot face the non-optimal temperature of growth. This is supported by the fact that I42 showed the lowest productivity of the three conditions of study (127.5 mL-biogas/g-VS). The most worth noting improvement was for M35, that managed to produce 404.1 mL-CH_4 per g-VS consumed, which is more than 70 % higher than for artichoke monodigestion (284.3 mL-biogas/g-VS).

Again, solids removal was not remarkably improved as the mean elimination of the three conditions for 2Art/1Asp was 47.85 % and the one for artichoke monodigestion 53.77 %. However, for co-digestion the removal increased as the temperature of operation rose, which is in accordance with break-down thermodynamics. Lastly, time of operation can mainly be considered to have been improved for T55, as it was reduced from 17 days for artichoke monodigestion to 7 days for 2Art/1Asp.

Table 3. General results for AcoD of 2Art/1Asp.

	M35	I42	T55
TS removal (%)	11.97	23.95	31.92
VS removal (%)	44.99	47.16	51.39
COD consumed (%)	40.87	31.08	17.64
Biogas (L)	7.0	6.7	6.3
CH_4 (L)	5.1	1.7	3.1
Biogas/TS removed (mL/g)	1993.1	915.3	631.2
Biogas/VS removed (mL/g)	552.9	519.4	438.2
CH_4/TS removed (mL/g)	1456.6	224.6	304.4
CH_4/VS removed (mL/g)	404.1	127.5	211.3
Time (≃days)	10	7	7

3.3 Comparison of both co-digestion systems

Results for both co-digestion configurations, namely 1Art/2Asp and 2Art/1Asp, have been discussed comparing with monodigestions of the relatively most abundant wastes, respectively. Despite having co-digestions shown general better results than monodigestions, it is also interesting to establish similarities and differences between both co-digestion systems to determine which one would be the most suitable for the treatment of these recalcitrant substrates.

Regarding temperature studies, for 2Art/1Asp M35 and I42 yielded better results than T55. On the contrary, 1Art/2Asp showed the best performance at T55. Moreover, from both configurations, it was 1Art/2Asp that was best overall at T55. On the other hand, for M35 and I42, it was 2Art/1Asp the best combination. Attending to productivities, and relative production of CH_4 it was 2Art/1Asp at M35 the most suitable. This finds an explanation in the more stable process that a mild temperature provides. Furthermore, CH_4 productivity is in accordance with the literature for AcoD of lignocellulosic wastes including agricultural (304-655 mL-CH_4/g-VS) (Abouelenien et al., 2014; Pages-Díaz et al., 2014; Xu and Li, 2012). Additionally, results somehow state that a higher proportion of asparagus hinders the treatment due to a less accessible structure that only the thermophilic range manages to degrade.

3.4 Statistical analysis

Statistical analyses of TS, VS and biogas were performed in order to mathematically determine the relationship between sets of experimental data in AcoD at different temperatures. Correlation coefficient (ρ) showed a high relationship for TS and VS (> 0.7770) for each process and co-digestion configuration (Table 4). Regarding asymmetry coefficient (AC), all values are positive. Interestingly, the lowest values are for M35 in both co-digestions. It can be hypothesized that lower ACs relate to slower hydrolytic phases because more values that are far from the average are found in the left tail of the distribution. Therefore, a mild temperature like M35 always shows a slower initial stage while I42 and T55 have higher ACs due to a thermodynamically more favorable condition that speeds up hydrolysis. Additionally, there is a contrast with monodigestion studies in which artichoke, for instance, showed negative values at M35 and T55. This can lead to the hypothesis that co-digestions somehow improve the speed of the process, mainly at the beginning, which generally considered a bottleneck.

Correlation coefficient for biogas and VS between both co-digestions is very strong (> 0.9266). Despite this, statistical significance (α=0.05) proved that they are significantly different from one another. It is also interesting to point that there is a bigger difference between ρ of biogas and ρ of VS for M35 than for I42 and T55. This can be related with the longer period of time that this process takes, as the VS consumption has a more different pace than biogas formation.

Table 4. Statistical analysis for TS, VS and biogas at different conditions. ρ=correlation coefficient. AC=asymmetry coefficient.

Substrate	Analysis	M35	I42	T55
1Art/2Asp	$\rho_{TS.VS}$	0.8957	0.9059	0.9001
	AC_{TS}	0.7678	1.7456	1.2502
	AC_{VS}	1.0649	2.7432	2.4383
2Art/1Asp	$\rho_{TS.VS}$	0.7770	0.9366	0.9169
	AC_{TS}	0.3364	2.0108	2.2744
	AC_{VS}	1.2128	2.1655	2.7220
1Art/2Asp	$\rho_{x.y\ (biogas)}$	0.9278	0.9935	0.9457
2Art/1Asp	$\rho_{x.y\ (VS)}$	0.9608	0.9679	0.9266

3.5 Kinetic modelling

The fitting results of the kinetic modelling are shown in Table 5 and relate VS removal with biogas production. Experimental and predicted data are represented in Figures 1-4 and show the close agreement between them thanks to the proposed model. This is supported by the regression coefficients that quantitatively show that estimated and experimental results fit successfully. Furthermore, the combined R^2 of organic matter consumption and biogas production hits 0.9544 for 1Art/2Asp and 0.9576 for 2Art/1Asp. Interestingly, maximum specific growth rate (μ_M) at each temperature is always higher for 2Art/1Asp except for I42, where 1Art/2Asp reaches 0.0396 d^{-1} and 2Art/1Asp 0.0384 d^{-1}. The biggest difference of all is for T55 in which μ_M is up to 2.3 times higher for 2Art/1Asp (0.0477 d^{-1}) than for 1Art/2Asp (0.0204 d^{-1}). A higher amount of asparagus can relate to a lower microbial growth as the access to an apparently more recalcitrant substrate can hinder the expansion of the community. Regarding non-degradable substrate (S_∞), both co-digestion configurations had lower values as temperature of operation rose (M35 > I42 > T55), being the lowest value of all 12.91 mg/L (1Art/2Asp). This value contrasts with the aforementioned microbial growth rate, which under this temperature condition was better for 2Art/1Asp. However, the difference is not significant (13.51 mg/L) and the theoretical elimination VS ($1–S_\infty/S_0$) for 2Art/1Asp is higher than for 1Art/2Asp (52.0 % vs 49.5 %), which is in accordance with being asparagus a bottleneck of the process.

Table 5. Fitting coefficients for kinetic modelling of organic matter consumption and biogas generation in the biomethanization of artichoke and asparagus AcoD under different temperature configurations.

System	Substrate	S_∞ (mg/L)	S_M (mg/L)	μ_M (d^{-1})	α (L/g-VS)	R^2 (for VS)	R^2 (for biogas)
M35	1Art/2Asp	14.22	159.04	0.0135	0.3776	0.9521	0.9387
	2Art/1Asp	17.05	150.49	0.0150	0.5483	0.9187	0.9568
I42	1Art/2Asp	13.70	165.58	0.0396	0.4989	0.9869	0.9637
	2Art/1Asp	15.11	165.98	0.0384	0.4902	0.9784	0.9300
T55	1Art/2Asp	12.91	151.70	0.0204	0.6800	0.9395	0.9593
	2Art/1Asp	13.51	163.02	0.0477	0.4155	0.9727	0.9616

Compared to monodigestion configurations of the substrates, both co-digestion configurations yielded better S_∞ for all conditions of artichoke. This scenario was not the same for asparagus that showed lower value in monodigestion than co-digestion. On the contrary, predicted productivities (α) for asparagus monodigestion were highly improved through both configurations of co-digestion with up to 0.6800 L/g-VS in the case of 1Art/2Asp at T55.

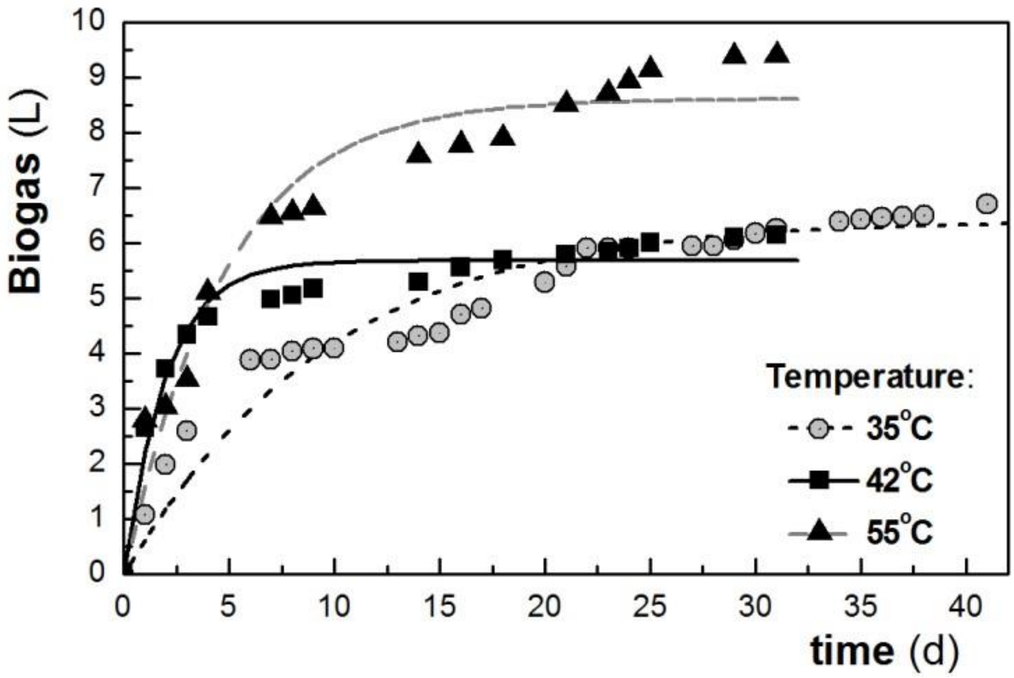

Figure 1. Kinetic modelling for biogas production of 1Art/2Asp at three operative conditions. • 35 °C; ■ 42 °C; ▲55 °C.

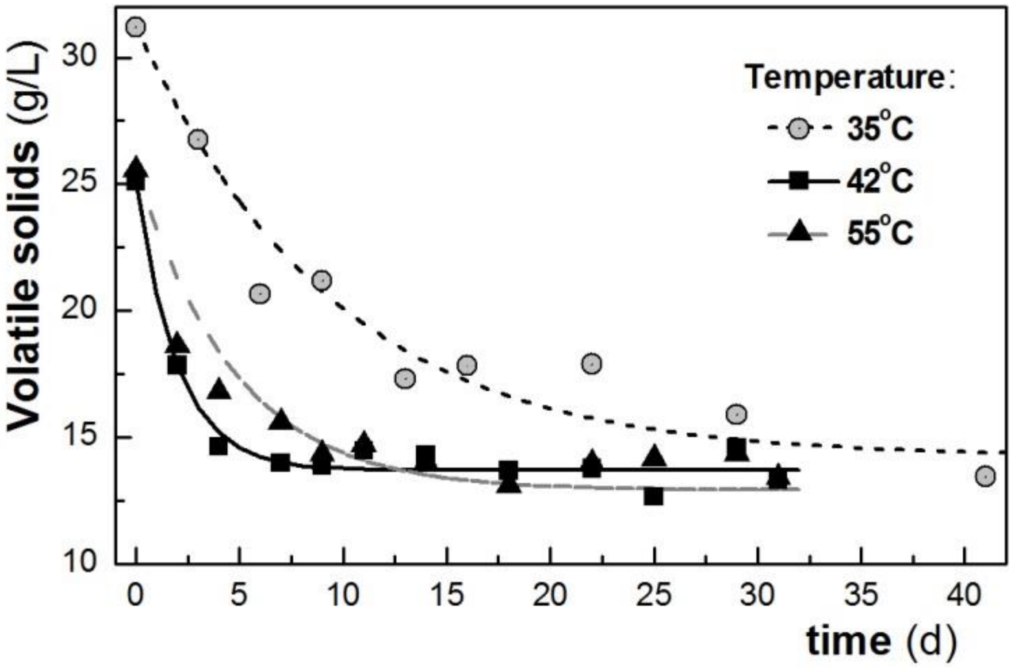

Figure 2. Kinetic modelling for VS consumption of 1Art/2Asp at three operative conditions. ● 35 °C; ■ 42 °C; ▲ 55 °C.

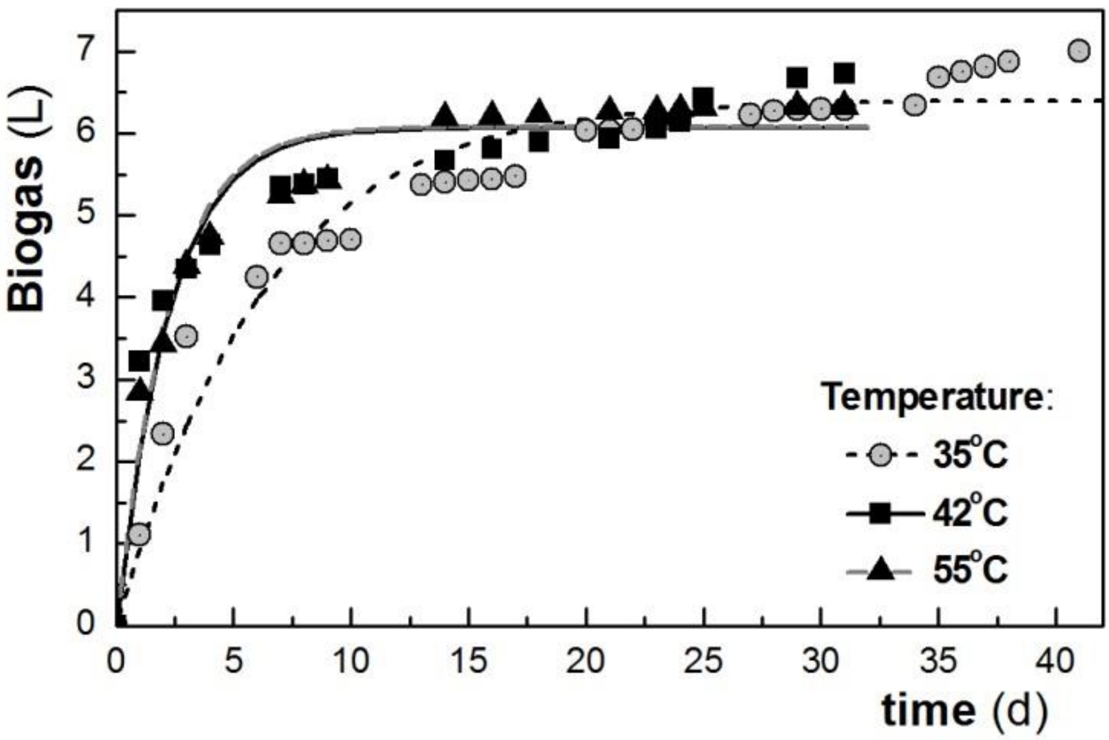

Figure 3. Kinetic modelling for biogas production of 2Art/1Asp at three operative conditions. ● 35 °C; ■ 42 °C; ▲55 °C.

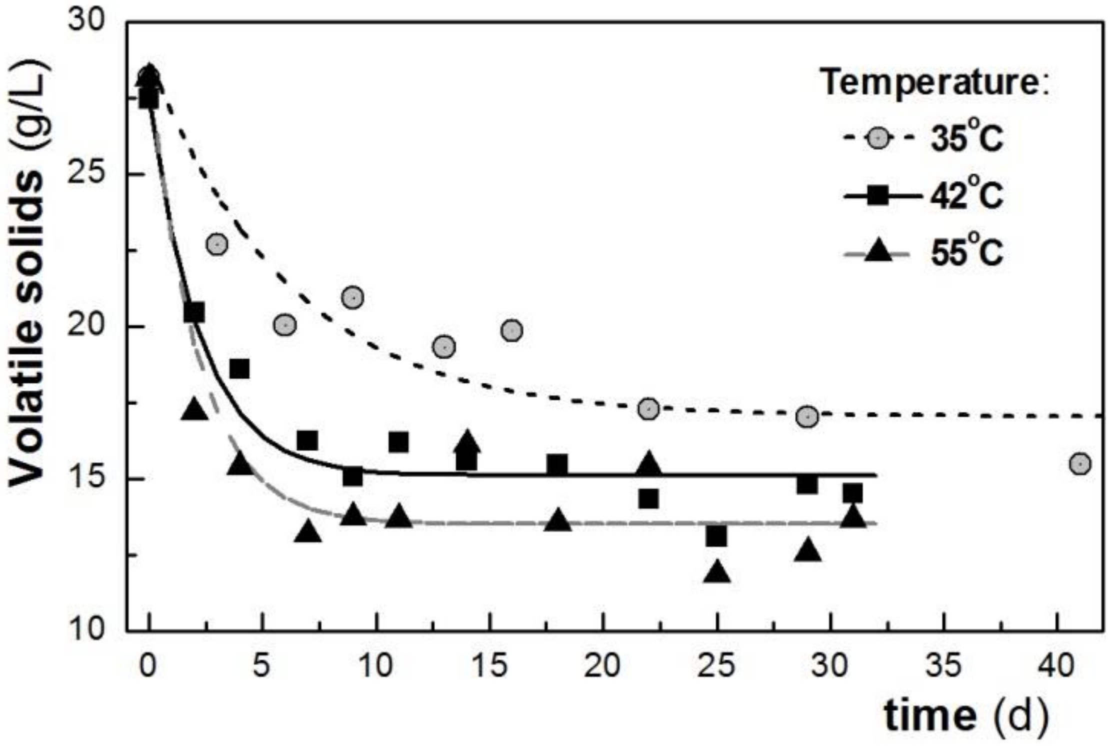

Figure 4. Kinetic modelling for VS consumption of 2Art/1Asp at three operative conditions. ● 35 °C; ■ 42 °C; ▲ 55 °C.

4. Conclusions

Lignocellulosic substrates such as artichoke and asparagus require of a break-down process optimization to yield improved solids and organic removal, as well as an efficient biogas production. Both co-digestion configurations have somehow improved monodigestions of most abundant substrates for each case. Artichoke monodigestion was more efficacious in removing solids than 2Art/1Asp, both total and volatile. However, this AcoD configuration was remarkably efficient as shown by its high productivities and biogas cumulate generation for any of the three operative conditions: M35, I42 and T55. Moreover, M35 and I42 not only showed better performance than artichoke alone but also the best of all the herein considered studies (asparagus and artichoke monodigestions, and 1Art/2Asp and 2Art/1Asp). This made clear that asparagus can hamper the process when compared to artichoke as it is more refractory and can hinder AcoD operation. On the other hand, 1Art/2Asp managed to overall yield the best results for T55 of all the considered experiments with up to 9.4 L-biogas, 772.4 mL-biogas/g-VS and 351.6 mL-CH_4/g-VS. Consequently, this AcoD system was also more efficient than the most abundant substrate, asparagus, in monodigestion.

Attending to the aforementioned observations and taking into account the potential industrial application, the most suitable configuration would be 2Art/1Asp at M35. This would result in lower operative costs and energy that could be derived from reactor heating and the handling of different substrate types.

Acknowledgements

This work was supported by University of Navarra (Research Plan PIUNA project 2015-06) and the Ministry of Economy and Competitiveness of Spain (Project CTQ2014 – 59312–P). The authors also like to thank the Friends of the University of Navarra Inc. for the grant of Beatriz de Diego-Díaz.

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Section III: Pretreatments and microbiology studies in anaerobic digestion

Chapter 5

Biomethanization of solid wastes from the alcoholic beverage industry: Malt and sloe. Kinetic and microbiological analysis

Published in Chemical Engineering Journal

Diego-Díaz B, Fernández-Rodríguez J, Vitas AI, Peñas FJ. Biomethanization of solid wastes from the alcoholic beverage industry: Malt and sloe. Kinetic and microbiological analysis. Chemical Engineering Journal, 2018, 334: 650-656.
http://dx.doi.org/10.1016/j.cej.2017.10.075

Chapter 6

Two-year microbial adaptation during hydrogen-mediated biogas upgrading process in a serial reactor configuration

Published in Bioresource Technology

Treu L, Kougias PG, Diego-Díaz B, Campanaro S, Bassani I, Fernández-Rodríguez J, Angelidaki I. Two-year microbial adaptation during hydrogen-mediated biogas upgrading process in a serial reactor configuration. Bioresource Technology, 2018, 264: 140-147. https://doi.org/10.1016/j.biortech.2018.05.070

Supplementary Information

Two-year microbial adaptation during hydrogen-mediated biogas upgrading process in a serial reactor configuration

L. Treu[1], P.G. Kougias[1*], B. de Diego-Díaz[1,2], S. Campanaro[3], I. Bassani[1], J. Fernández-Rodríguez[2] and I. Angelidaki[1]

[1] Department of Environmental Engineering, Technical University of Denmark, Kgs. Lyngby DK-2800, Denmark

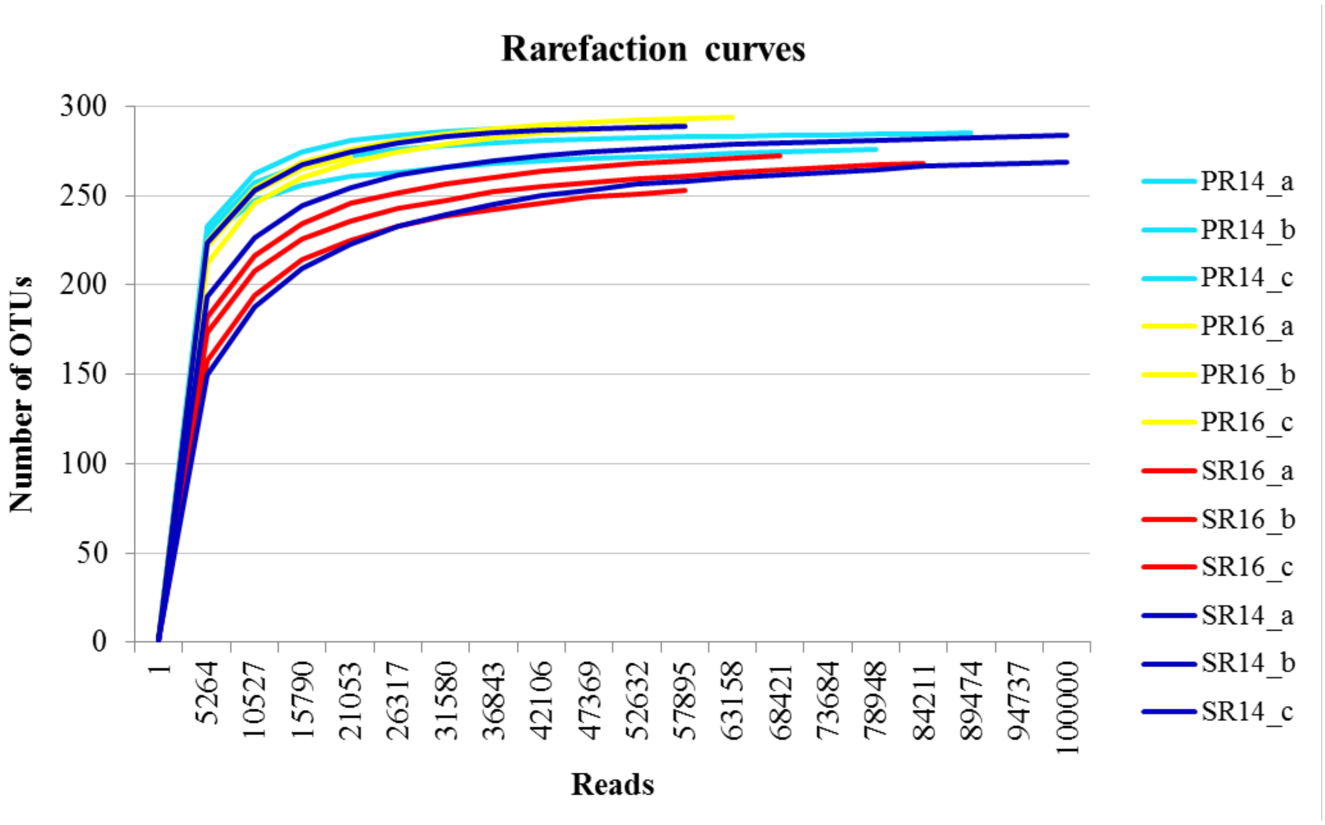
[2] Department of Chemistry, University of Navarra, Spain

[3] Department of Biology, University of Padua, Via U. Bassi 58/b. 35131 Padova, Italy

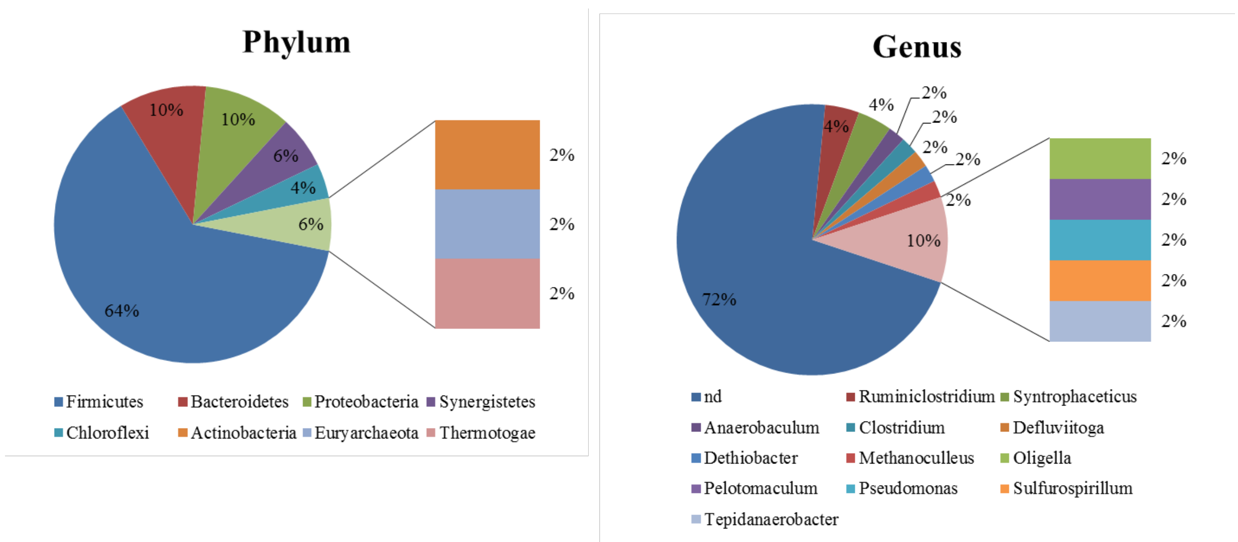
[*]Corresponding author: Panagiotis G. Kougias, Department of Environmental Engineering, Technical University of Denmark, Bld 113, 2800 Lyngby, Denmark, E-mail address: panak@env.dtu.dk, Tel.: +45 45251454

Table S1. Cattle manure characteristics

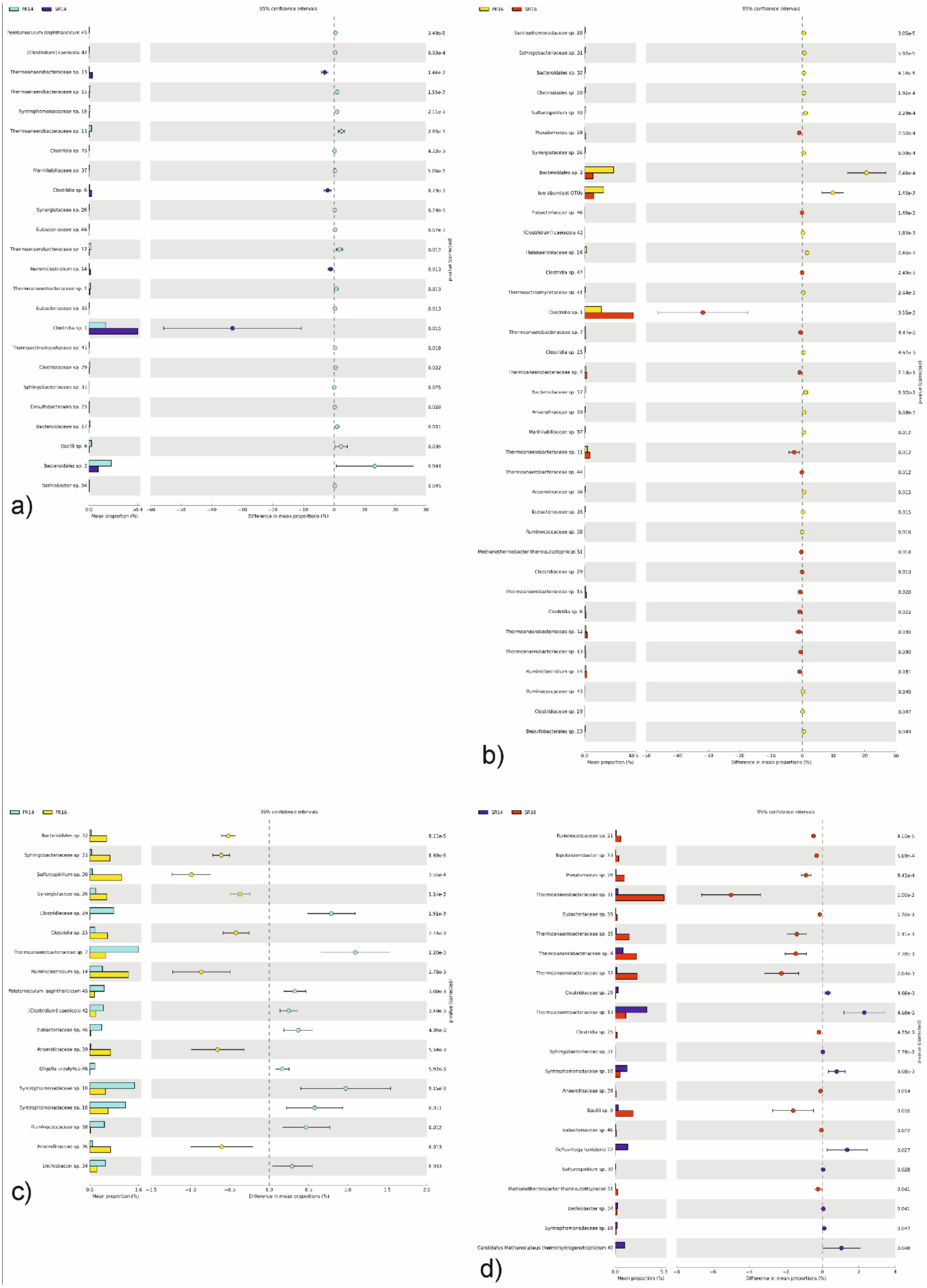
Parameter	**Unit**	**Values**
pH	-	7.43±0.02
Total solids (TS)	g/L	47.6±1.7
Volatile solids (VS)	g/L	34.6±1.4
Total Kjeldahl Nitrogen (TKN)	g-N/L	3.05±0.17
Ammonium Nitrogen (NH_4^+)	g-N/L	2.10±0.05
Total Volatile Fatty Acids (VFA)	g/L	6.80±0.5



Supplementary Figure 1. Alpha diversity calculated as number of OTUs is represented as rarefaction curves.



Supplementary Figure 2. Taxonomic overview of the most relevant OTUs. The number of OTUs assigned to each phylum (left) and genus (right) calculated as percentage of the selected OTUs (51) considering all samples is presented.



Supplementary Figure 3. Statistical comparisons of OTUs relative abundance between the two periods (2014 and 2016) in the primary reactor (PR) are presented in A) and B) respectively. The same analyses performed for the secondary reactor (SR) are presented in C) and D).

Section IV: Influence of external substances. Pesticides

Chapter 7

Effect of crop pesticides on anaerobic co-digestion of agricultural wastes at different temperature configurations

Water Research. Under Review

Effect of crop pesticides on anaerobic co-digestion of agricultural wastes at different temperature configurations

Beatriz de Diego-Díaz[a], María Eugenia Tapia[a], Francisco J. Peñas[a], Juana Fernández-Rodríguez[a]

[a] University of Navarra, Department of Chemistry, School of Sciences, Irunlarrea 1, 31009 Pamplona, Spain.

Abstract

Anaerobic co-digestion (AcoD) of agricultural wastes can present many sources of inhibition. Crop fields normally are treated with chemical substances to increase agricultural productions: pesticides. Thus, AcoD of agricultural waste should include this kind of substances; like mancozeb and tefluthrin. In this study, three temperatures of operation on agricultural AcoD were assessed: mesophilic (35 °C-M35-), intermediate (42 °C-I42-) and thermophilic (55 °C-T55-) ranges. M35 yielded the highest biogas and CH_4 cumulate (6.7 L/reactor and 5.2 L/reactor) and relative productions (77.6 %), as well as eliminations (65.0 % volatile solids-VS- removal). The effect of pesticides was assessed in different configurations: mancozeb alone, tefluthrin alone, the combination of both and the combination of half of each pesticide, comparing in all cases with the process without pesticides. Overall results indicated that mancozeb barely had an effect on AcoD, if so it was positive. Interestingly, tefluthrin highly improved AcoD, especially relative CH_4 productions (81.2 % for M35, 57.7 % for I42 and 65.4 % for T55) and almost all parameters for T55 (8.0 L biogas and 347.2 mL-biogas/g-VS). The combination of both pesticides, on the other hand, happened to hinder the process at every temperature of operation. From

the derived results, it can be concluded that inhibition takes place when mancozeb and tefluthrin are present together and it can be elucidated that no nutritional scarcity has to do with the enhancement of them alone.

Keywords: mancozeb; tefluthrin; pesticide; co-digestion; temperatures (35 °C, 42 °C, 55 °C)

1. Introduction

Agriculture is a worldwide sector that plays an important role in society, economy and the environment. However, its production can be threatened by plagues that have to be controlled. Modern agriculture makes a wide use of pesticides that protect seeds and the harvesting process from all sorts of pathogenic organisms such as virus, bacteria or insects (Fabra et al., 1998). Their implementation has been, however, highly regulated by different international and national organisms such as the European Parliament. The Directive 2009/128/EC of the European Parliament and of the Council (2009) presented a plan for the sustainable use of pesticides directed to the member states that should establish national plans. Nevertheless, they are still widely used, being two of the most common mancozeb and tefluthrin.

Mancozeb is a universally used fungicide in the agricultural sector (Figure 1a). It is a manganese and zinc ethylene-bis-dithiocarbamate compound whose exact mechanism of action is yet to be fully understood. It is a selective fungicide that directly acts on the leaf surface on which it is administered, having the capacity of inhibiting the growth and proliferation of a wide range of different fungi such as *Alternaria, Botrytis, Cercospora, Colletotrichum, Mycosphaerella, Peronospora, Phythopthora, Rhizoctonia* and *Septoria*. Also, the broad assortment of crops makes of mancozeb a preferred pesticide among the sector. It does not penetrate the leaf and the multisite mechanism of action leads to enzyme and respiration inhibition, enzyme denaturalization, inactivation of sulphydryl groups, Krebs cycle disruption, ATP formation arrest and lipid membrane interference. This series of events induces the inhibition of spores' germination and the formation of the germination tube on the leaf surface, being the colonization process blocked (EPA, 1984; WHO, 1988).

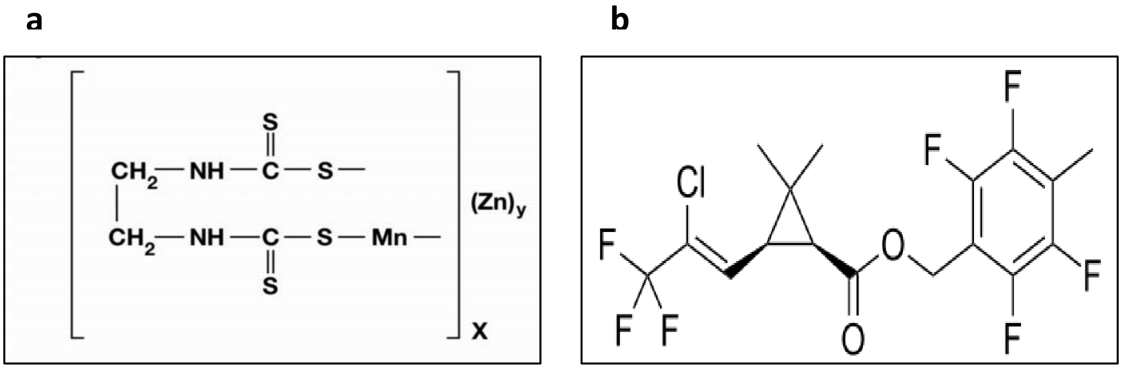


Figure 1. a) Mancozeb chemical structure. b) Tefluthrin chemical structure.

Additionally, others commonly used can be found such as tefluthrin (Figure 1b), an insecticide that targets a wide range of myriapods and insects from the orders dermaptera, lepidoptera, diptera and coleoptear, as well as their larvae. Tefluthrin is highly liposoluble, persistent and in vapor stage also has repellent activity. It is disposed directly on the earth along with the seeds to be harvested or prior to the seeding process (NCBI, 2017).

Anaerobic digestion (AD) is increasingly becoming the most feasible and efficient option for waste treatment and that generated from the agricultural sector has already shown to be valid as feedstock in various studies (Sawatdeenarunat et al., 2016). Among the advantages that can be mentioned, when compared for example with aerobic processes, low energy requirements and reduced sludge production are the most worth-mentioning (Chen et al., 2014). Additionally, environmentally and economic-wise, studies have shown that AD offers more benefits than composting and incineration, as it results on better carbon return on investment and lower impact for global warming potential, eutrophication potential and acidification potential (Oldfield et al., 2016).

However, AD presents high vulnerability to toxicants that can inhibit, or at least modify, the process. These are generally the cause of reactor upset and failure and studies have mainly been focused on inhibition mechanisms, factors affecting inhibition and overall problems throughout operation (Chen et al., 2008). Among the most frequently studied we find inorganic compounds such as ammonia, sulfide, heavy metals and light metal ions; and organics such as lignins, long-chain fatty acids, chlorophenols, etc. It is therefore important to study the effect of arising inhibitors or those that can be directly present in the feedstock of anaerobic reactors. Agricultural wastes have specific sources of inhibition due to its nature (lignins or resins, for example) and other external substances that are added to increase agricultural production, like the aforementioned pesticides: mancozeb and tefluthrin.

Temperature is an important parameter that can affect the process and it is generally carried out at different temperature ranges: psychrophilic (< 20 °C), mesophilic (20-45 °C) or thermophilic (45-60 °C). For the last two, 35 °C and 55 °C are considered optimal, respectively. Mesophilic conditions have generally shown to yield a more stable process but with slower degradation rates. On the other hand, higher temperatures, such as the thermophilic range, lead to increased break-downs of organic matter due to favored thermodynamics. However, the stability of the process and the high operative costs derived from energy requirements from reactor heating are two worth-mentioning disadvantages of this condition (Fernández-Rodríguez et al., 2013). Additionally, intermediate ranges are increasingly being considered. For instance, 42 °C has proven to yield high total organic carbon removals (de Diego-Díaz et al., 2018a). Therefore, the substrate and many other factors determine whether a temperature range is suitable or not (de Diego-Díaz et al., 2018b)

Also, the combination of various substrates has shown to be beneficial to the process, which is known as co-digestion. Co-digestion is beneficial attending to many aspects such as methane production optimization, inhibition avoidance, moisture and C/N balance, synergies establishment, enhancement of degradations, among others (Mata-Álvarez et al., 2011; Zamanzadeh et al., 2017). More specifically, agricultural-food wastes, which are highly generated worldwide, have been reported to be very efficacious when combined with different substrates. Nonetheless, the selection of the appropriate co-substrates is key to achieve the desired outcomes. Zamanzadeh et al. (2017), for instance, reflected how a mixture of food waste alone even had more positive effects than combined with cattle manure.

In this study the effect of pesticides (mancozeb and tefluthrin alone and combined) at three different temperatures (35 °C, 42 °C and 55 °C) on co-digestion of agricultural wastes (green pea, green bean, bean, carrot, cabbage and artichoke) is assessed.

2. Materials and Methods

2.1 Experimental system

Assays were carried out according to the methods described by Holliger et al. (2016). Batch digesters were set up as continuous stirred-tank reactors. Each digester was an airtight bottle (total volume of 1.0 L and working volume of 0.6 L). In the cap of the digesters two ports allowed biogas collection in a 5L fluorinated ethylene propylene bag (SKC, USA) and sampling. The digesters were placed in a thermostatic bath containing oil that prevented evaporation. They also were stirred magnetically in order to ensure homogeneous mixing. Reactors were arranged in accordance to TS (%), operating in the wet range (4-10 %). Biogas generation was quantified by bubbling it in a water gasometer and its content in CH_4 was determined by NaOH displacement after being biogas bubbled in this alkaline solution (Holliger et al, 2016). Also a biogas analyzer was employed (Geotech Biogas-5000, UK).

Digesters contained 0.2 L of an adapted inoculum collected from the anaerobic sludge treatment line of a wastewater treatment plant, and 0.05 L of the nutrient solution, as previous studies showed the best performances proposed in de Diego-Díaz et al. (2018a). Inoculum adaptation was carried out in accordance with Holliger et al. (2016), by incubation at the respective temperature and removal of residual biomass.

The operative conditions assessed were mesophilic (M35), intermediate (I42) and thermophilic (T55) ranges, with temperatures of 35 °C, 55 °C and 42 °C, respectively.

For the reactors, in which the effect of pesticides was assessed, these were added according to their commercial use in the crop field (Table 1). The aim of this is to simulate a scenario in which all the substance (mancozeb or tefluthrin) used in the agricultural activity would get in the anaerobic digester.

Table 1. Data for pesticide addition calculation.

Vegetable	kg vegetable/ha
Carrot	52000
Cabbage	16000
Bean	9300
Green Bean	16300
Green pea	6300
Artichoke	11300
Mean value	18533
Mancozeb (kg pesticide/ha)	Tefluthrin (kg pesticide/ha)
2.25	12.5

2.2 Analytical Methods

All analytical methods were carried out according to the Standard Methods (APHA, 2012). Chemical oxygen demand (COD) was measured (Agilent 8453, USA) in samples that had been digested at 150 °C for 2 hours (Velp Sci, Italy). Soluble COD (sCOD) required centrifugation at 13000 rpm for 10 min (VWR Micro Star 12, USA) and later filtering through 0.45 µm and 0.2 µm (Starstedt, Germany). Alkalinity was carried out in deionized water with a 1:50 dilution. Composition of pesticides was analyzed with a CHN-900 analyzer. On the other hand, trace elements (like Mg, Ca, Fe, Mn, Zn) were analyzed by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) on an Agilent 7500a ICP-MS instrument. Calibration standards for ICP-MS analysis were prepared from multi-element stock solutions (Custom Grade Standard, Inorganic Ventures).

2.3 Substrate Characterization

Commercial substrates, artichoke, green pea, bean, carrot and cabbage and green bean were harvested in Spain. All of them were characterized (Table 2). The herein studied configurations can be identified as expressed in Table 3. Firstly, the co-digestion substrate was assessed at the three aforementioned temperatures (35 °C –M35-, 42 °C –I42- and 55 °C –T55-) and were utilized as reference to compare the effect of pesticides. These were studied in four different ways: mancozeb alone (-M), tefluthrin alone (-T), the combination of both pesticides (-MT) and the combination of both but with half concentration of each pesticide (-MTa). All these were again operated at the three temperatures.

Table 2. Characterization of substrates used for this study.

Substrate	Substrate (scientific name)	TS (%)	VS (%)**	Total COD (gO_2/L)*	Soluble COD (gO_2/L)*	pH	Alkalinity ($mgCaCO_3/L$)
Inoculum	-	1.10	1.00	7.50	5.50	6.60	900
Artichoke	*Cynara scolymus*	10.45	9.86	56.43	29.88	7.03	1150
Green pea	*Pisum sativum*	24.02	23.21	56.14	25.93	6.98	970
Bean	*Vicia faba*	17.82	17.13	42.03	22.48	8.21	1200
Carrot	*Daucus carota*	7.39	6.80	49.37	20.85	6.76	1050
Cabbage	*Brassica oleracea var. gemmifera*	15.52	14.21	54.98	26.53	8.34	1410
Green bean	*Phaseolus vulgaris*	13.58	12.74	45.40	23.71	7.52	1320

*Standard error of 0.2 gO_2/L (equipment error)
**Percentage from the total fraction

Table 3. Configuration identifiers in this study.

Configuration	35 °C	42 °C	55 °C	Mancozeb (mg/L)	Tefluthrin (mg/L)
Co-digestion	M35	I42	T55	-	-
Co-digestion + Mancozeb	M35-M	I42-M	T55-M	57.7	-
Co-digestion + Tefluthrin	M35-T	I42-T	T55-T	-	309.7
Co-digestion + Mancozeb + Tefluthrin	M35-MT	I42-MT	T55-MT	57.7	309.7
Co-digestion + ½ Mancozeb + ½ Tefluthrin	M35-MTa	I42-MTa	T55-MTa	28.8	154.8

2.4 Pesticide characterization

Table 4 shows the composition of the commercial formulation of the pesticides: mancozeb and tefluthrin. Both have a higher proportion of C when compared to H and N, being mancozeb the one that has an overall higher content in C, H and N. This indicates that tefluthrin formulation has a more heterogeneous composition than mancozeb, in which up to 29 % is formed out of these three elements. The other elements studied were interestingly distributed, as the proportion of each of them follows a similar pattern between both pesticides, being Mg the most abundant.

Table 4. Pesticide characterization of main elemental components (expressed in percentage –CHN- and in ppm –trace elements-). RSD: relative standard deviation (n=2).

	Mancozeb	RSD	Tefluthrin	RSD
C*	17.18	± 0.30	6.80	± 0.40
H*	2.58	± 0.07	1.82	± 0.14
N*	9.24	± 0.10	0.06	± 0.00
Mg	325200.76	± 11.42	92230.77	± 1.94
Al	72562.54	± 39.16	18639.54	± 0.03
Ca	26477.85	± 11.62	17016.20	± 10.92
Fe	7184.29	± 16.72	9227.53	± 1.76
K	6817.31	± 6.86	4292.99	± 2.31
S	1038.32	± 4.48	892.56	± 3.40
Na	451.91	± 19.88	404.04	± 2.96
Mn	191.87	± 6.28	257.75	± 14.04
Ti	88.21	± 60.10	247.42	± 3.09
P	55.23	± 9.48	257.47	± 6.55
Sr	40.26	± 8.52	84.50	± 7.93
Ba	28.43	± 38.19	61.11	± 6.82
Zn	11.13	± 85.77	46.67	± 16.22

*expressed in %

3. Results and Discussion

The effect of mancozeb and tefluthrin (alone and combined) on the co-digestion of agricultural-food wastes will be exposed in the following sections. Firstly, the co-digestion alone at the three operative conditions and then in the presence of pesticides.

3.1 Co-digestion at different temperatures

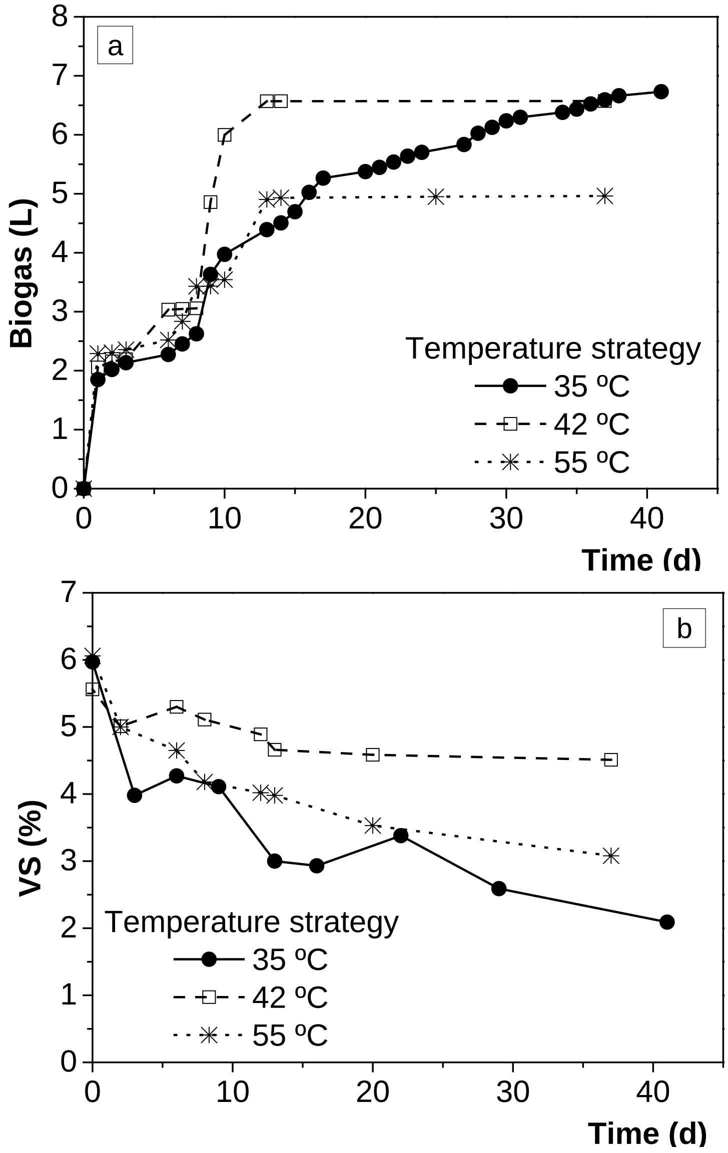


Figure 2. a) Cumulative biogas production (L) of co-digestion at three temperatures; b) VS (%) of co-digestion; • 35 °C; □ 42 °C; ⋇ 55 °C.

Studies of temperature are shown in Table 5 and Figure 2. Results showed a general better performance for M35. The stability of the process and the little demanding pH control was remarkable for this condition. This is an important aspect to be considered, especially when scaling up the process as inhibition from pH can arrest the whole biomethanation process. Also, M35 managed to produce the highest amount of biogas with up to 6.7 L, 1.35-fold the generation for T55. Also, VS removal reached

64.99 % of elimination while the other conditions only managed to remove 18.88 % and 49.17 % for I42 and T55, respectively. However, the process at M35 is a bit longer than the other two conditions as VS removal arrests after more than I42 and T55. Additionally, biogas production keeps going in a steady fashion while the other two temperatures stop increasing the cumulative production at day 13.

Similarities in the curves behavior of M35 and I42 were contrasted statistically as to see whether the processes were the same making use of the significance level expressed as α, which has also been applied in previous studies (de Diego-Díaz et al., 2018b). This refers to the probability of dismissing a null hypothesis when it is true. For instance, when $\alpha=0.05$, it means that there is a risk of a 5 % of concluding that a difference does exist when there is no actual difference. Results show how both conditions are significantly different ($\alpha < 0.05$) for VS and biogas ($\alpha=0.023$ and $\alpha=0.003$, respectively).

Biogas composition showed the highest content in CH_4 for M35 (77.2 %), which once again has to do with the stability of a slightly slower process than higher temperatures that release intermediate products more rapidly, leading to their accumulation and arrest of the full degradation (Li et al., 2017). However, T55 also yielded a very positive CH_4 production (65.5 %) while I42, a non-optimal microbial growth temperature, only reached 41.9 %. This study, therefore confirms the suitability of the two other optimal conditions, 35 °C and 55 °C, both of which surpass the CH_4 relative production of a similar food waste that yielded 60-62.2 % for mesophilic and thermophilic ranges at pilot and full-scale (Cavinato et al., 2013).

Attending to productivities, these are considerably good. I42 shows the highest values for gas production per VS consumed (1043.2 mL-biogas/g-VS and 436.7 mL-CH_4/g-VS). Nonetheless, taking into account the importance of solids removal in AD, this system reaches high gas productions barely eliminating solids, as it was mentioned before. This is why M35 can still be considered the most suitable as it shows an outstanding balance between organic matter consumption and its conversion into biogas (289.1 mL-biogas/g-VS and 223.0 mL-CH_4/g-VS). Actually, M35 is the one that yields the highest CH_4 volume per VS_{added}, 145.0 mL-CH_4/g-VS_{added}, which is in accordance with the literature as this operative condition has shown to yield 76-430 mL-CH_4/g-VS_{added} for different configurations of food waste co-digestions (Di Maria et al., 2016; Li et al., 2017).

Table 5. Overall results for co-digestion at the three operative conditions of temperature.

	M35	I42	T55
TS removal (%)	20.26	24.08	33.85
VS removal (%)	64.99	18.88	49.17
COD consumed (%)	35.75	54.53	50.62
Biogas (L)	6.7	6.6	5.0
CH_4 (L)	5.2	2.8	3.3
Biogas/TS removed (mL/g)	1206.1	725.4	376.0
Biogas/VS removed (mL/g)	289.1	1043.2	277.6
CH_4/TS removed (mL/g)	930.6	303.6	246.4
CH_4/VS removed (mL/g)	223.0	436.7	181.9
Time (≃days)	18	13	13

3.2 Co-digestion in presence of pesticides

After the description of co-digestion results, the effect of pesticides addition to the systems (M35, I42 and T55) will be discussed.

3.2.1 Effect of mancozeb

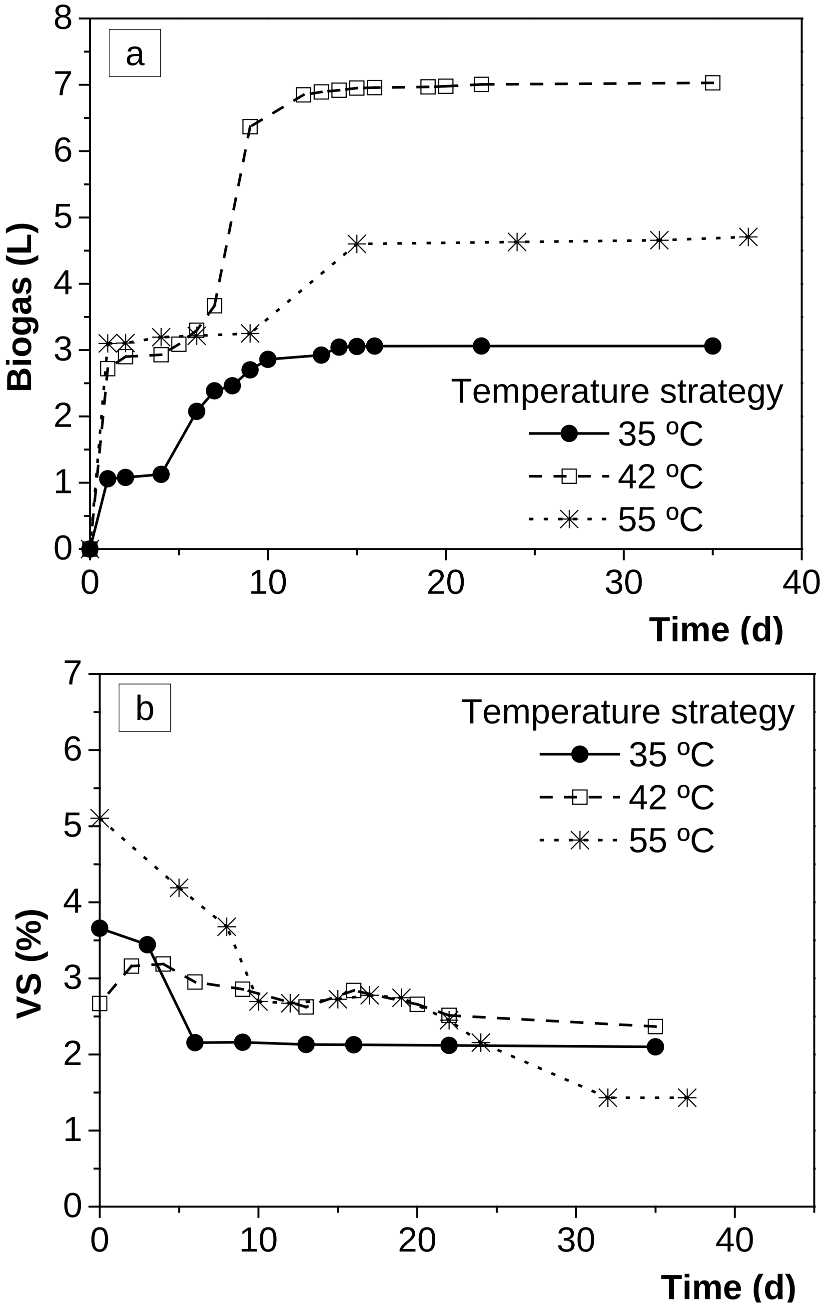


Figure 3. Evolution of co-digestion in the presence of mancozeb at three different temperatures. a) Cumulate biogas (L); b) VS (%); • 35 °C; □ 42 °C; ⁎ 55 °C.

Co-digestion in the presence of mancozeb (M35-M, I42-M and T55-M) showed in general a positive scenario (Table 6 and Figure 3), especially regarding solids elimination and productivities when compared to the processes in the absence of it.

At T55-M waste stabilization was the most successful of all the three operative conditions. Up to 71.95 % of VS and 58.17 % of TS were eliminated in this process. Also COD was reduced in a 37.41 %. These data, in comparison with the other two temperatures, are very positive and show a high efficacy of the stabilization process which is one of the main objectives of AD. Moreover, studies with lower success of organic matter removal resulted in digestates that were eligible for their agricultural application as compost (Cavinato et al., 2013). On the other hand, regarding productivities and cumulate productions, T55-M yielded worse performances than T55, being even surpassed by I42-M.

For I42-M, on the contrary, mancozeb happened to have a very positive impact, managing to improve biogas cumulate production (7.0 L, 1.1-times higher), time of operation (12 days), VS elimination (25.12 %, 1.3-times higher) and, more interestingly, productivities (1475.3 mL-biogas/g-VS and 575.1 mL-CH_4/g-VS, 1.4 and 1.3-times higher, respectively). The most worth noting is that 42 °C is not an optimal temperature of growth. Therefore, this enhancement can be attributed to the addition of mancozeb that provides elements such as manganese and zinc, which are very abundant in this pesticide. These have shown to be key in the operation of AD, affecting microbial metabolism through enzymatic activity (Choong et al., 2016; Huang et al., 2017).

Mesophilic conditions yielded a clearly lower cumulate production but this has to do with the initial set-up of the reactors, as M35 had a slightly higher content in organic matter than M35-M. Despite this, eliminations were also more efficacious without mancozeb and, as opposed to 55 °C, productivities were somehow improved (327.3 mL-biogas/g-VS and 236.3 mL-CH_4/g-VS). These, nonetheless, are still in accordance with what is expected from this type of substrate, allowing hypothesize that mancozeb would not be a significant drawback in the AD of agricultural-food waste. Li et al. (2017), for instance, reported productivities in various stages of a mesophilic reactor of 234-350 mL-CH_4/g-VS.

It is, however, important to mention that mancozeb did not have an outstanding impact in the composition of biogas at any operative condition, as this had a 1.1-fold lower mean content in CH_4 in the presence of mancozeb.

Table 6. Overall results for co-digestion in presence of mancozeb at the three operative conditions of temperature.

	M35-M	**I42-M**	**T55-M**
TS removal (%)	24.58	8.00	58.17
VS removal (%)	42.60	25.12	71.95
COD consumed (%)	12.38	9.61	37.41
Biogas (L)	3.1	7.0	4.7
CH_4 (L)	2.2	2.7	2.9
Biogas/TS removed (mL/g)	519.98	3640.74	229.88
Biogas/VS removed (mL/g)	327.3	1475.3	213.6
CH_4/TS removed (mL/g)	373.3	1419.1	143.8
CH_4/VS removed (mL/g)	236.3	575.1	133.6
Time (≃days)	13	12	15

3.2.2 Effect of tefluthrin

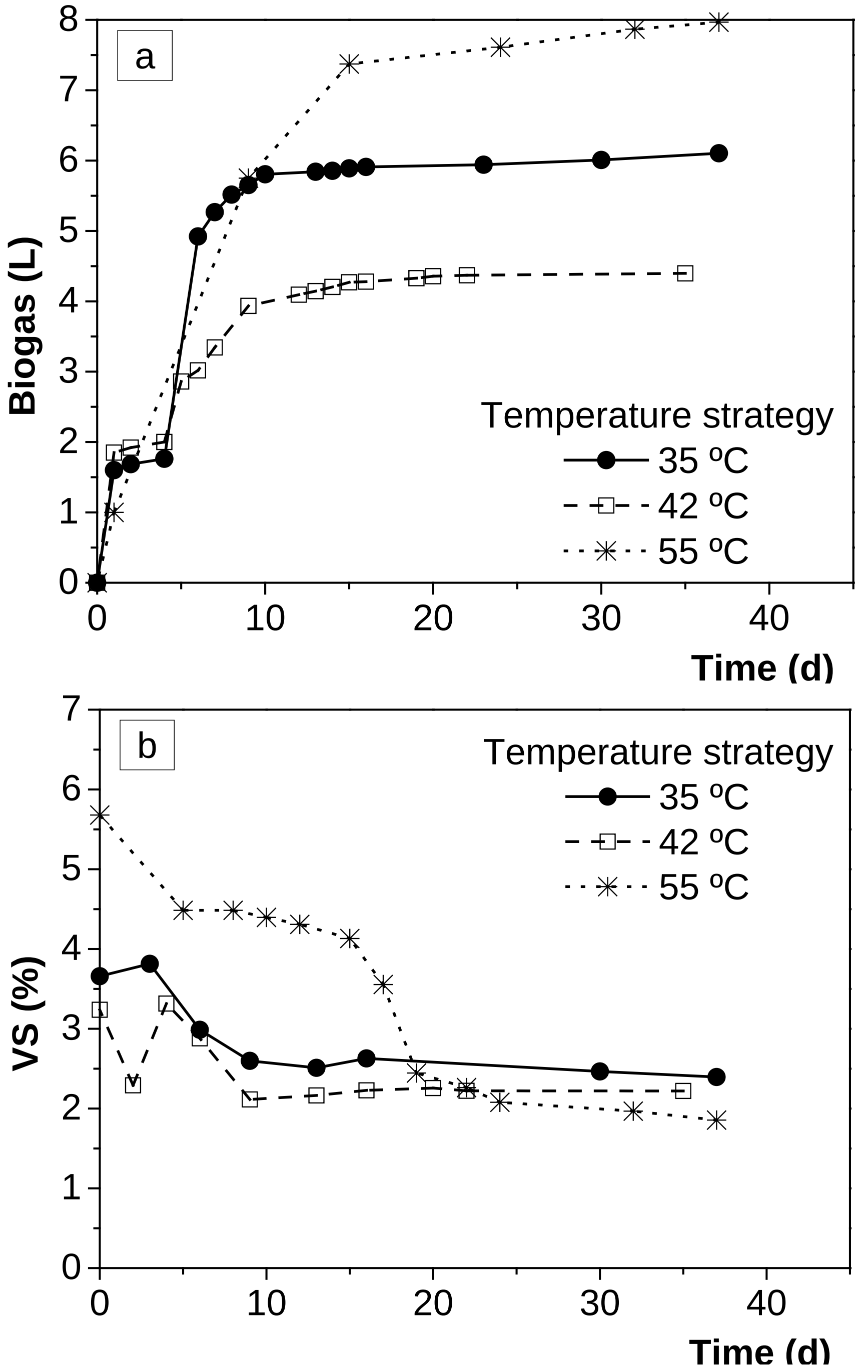


Figure 4. Evolution of co-digestion in the presence of tefluthrin at three different temperatures. a) Cumulate biogas (L); b) VS (%); • 35 °C; □ 42 °C; ⁎ 55 °C.

Co-digestion studies in presence of tefluthrin were performed (M35-T, I42-T and T55-T) and results are shown in Figure 4 and Table 7. This pesticide, similarly to mancozeb, improved some of the herein assessed parameters as will be further discussed in this section. However, again it was for T55-T for which the enhancement was more remarkable. VS elimination reached 67.33 %, 1.4-times higher than T55. Along with higher cumulate productions, both for biogas (8.0 L, the highest of the whole study) and CH_4 (5.2 L), productivities reached very positive values: 347.2 mL-biogas/g-VS and 227.1 mL-CH_4/g-VS. Moreover, relative CH_4 production (65.4 % of total biogas) was almost the same as T55 (66.0 %) and higher than that yielded by T55-M (61.7 %). This allows stating that tefluthrin may have a more positive effect or, at least, a lower impact than mancozeb on anaerobic co-digestion of agricultural-food waste.

Regarding I42-T, results should not be dismissed. It showed high stability of the process and the pH maintenance was not as demanding as I42 or T55-T. Additionally, biogas productivities (720.0 mL-biogas/g-Vs), relative CH_4 production (57.72 % vs 42.42 % for I42-T and I42, respectively) and solids removal (31.44 % of VS) can be considered as fairly good, but considering higher operative costs, a milder temperature would be advised.

For M35-T, the most worth noting enhancement was that of the relative CH_4 production, which reached 81.2 % of the total cumulate biogas. This is not only the highest for this condition but also the highest of all the configurations and temperatures in this paper. Moreover, it surpasses the average established by Xu et al. (2018) of 60-70 % composition of CH_4. On the other hand, organic eliminations are not outstanding, possibly meaning that it is used more efficaciously and slowly for successful conversion into CH_4. Additionally, once again M35 yielded the highest productivities (716.9 mL-biogas/g-VS and 581.8 mL-CH_4/g-VS).

Table 7. Overall results for co-digestion in presence of tefluthrin at the three operative conditions of temperature.

	M35-T	I42-T	T55-T
TS removal (%)	9.93	23.42	57.00
VS removal (%)	37.20	31.44	67.33
COD consumed (%)	24.31	27.93	55.83
Biogas (L)	6.0	4.4	8.0
CH_4 (L)	5.0	2.5	5.2
Biogas/TS removed (mL/g)	2472.3	686.2	347.1
Biogas/VS removed (mL/g)	716.9	720.0	347.2
CH_4/TS removed (mL/g)	422.0	2101.4	150.4
CH_4/VS removed (mL/g)	581.8	415.6	227.1
Time (≃days)	10	9	15

3.2.3 Combined effect of tefluthrin and mancozeb

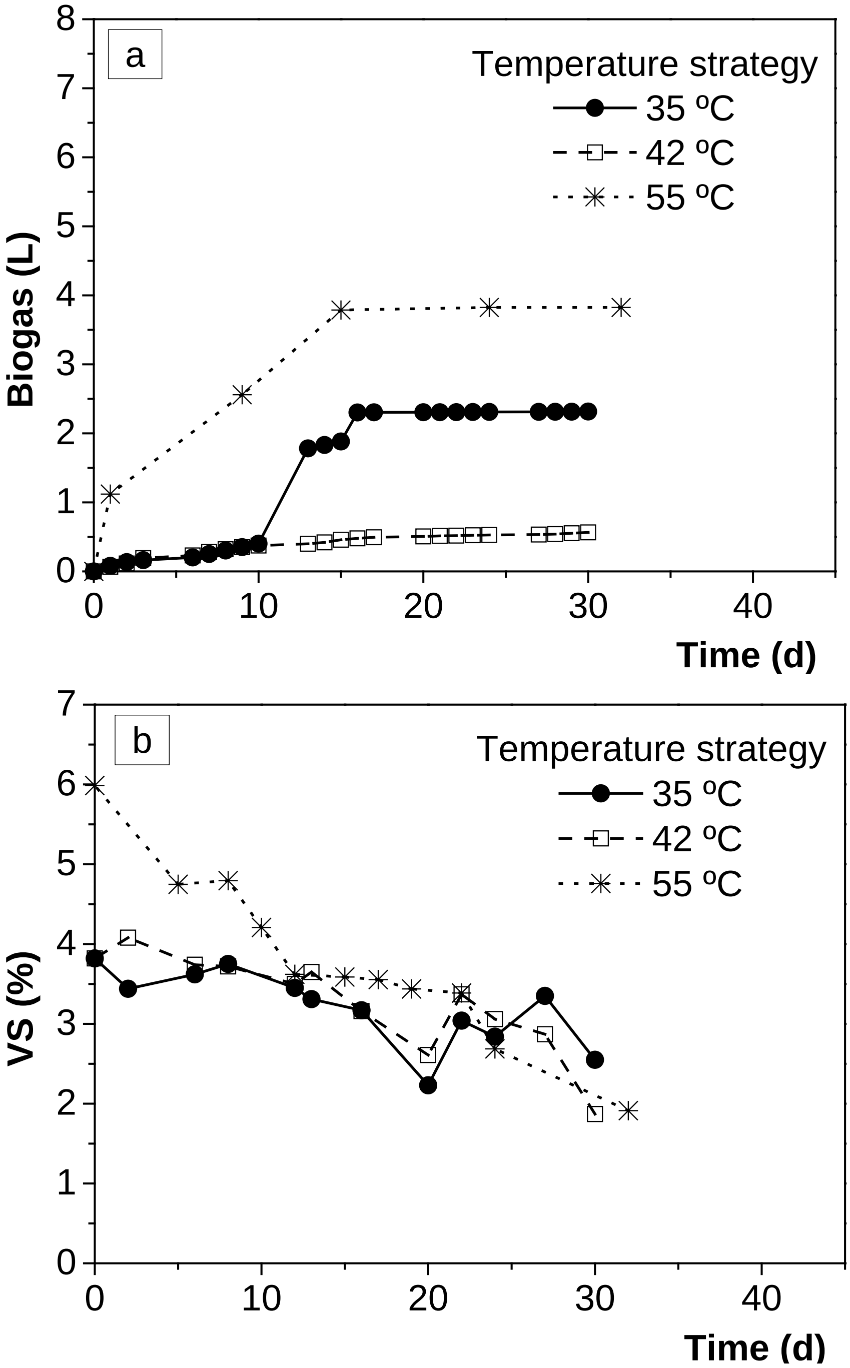


Figure 5. Evolution of co-digestion in the presence of mancozeb and tefluthrin at three different temperatures. a) Cumulate biogas (L); b) VS (%); • 35 °C; □ 42 °C; ⁎ 55 °C.

This section describes the effects encountered for the addition of mancozeb and tefluthrin combined. This can potentially take place in the agricultural activity as crop fields may require the utilization of both. Moreover, the combined effect of two substances can lead to an enhanced effect or to a diminished one due to intermediate inhibition within the anaerobic digester. Therefore, both pesticides in the same concentrations previously studied were added to the reactors (M35-MT, I42-MT and T55-MT), in order to be degraded together. Results are shown in Figure 5 and Table 8.

Once again, biogas production was the highest at T55-MTwith 3.8 L (1.7 times that from M35-MT and up to 6.3 times that from I42-MT). This is due to the faster reaction rate that this condition provides. However, all cumulate biogas productions were surprisingly low when compared to the previously discussed conditions, being the lowest from the whole study for each respective temperature.

Also, it is worth-mentioning that I42-MT yielded a remarkably low cumulative biogas, barely 0.6 L, which is the lowest biogas production from all conditions and configurations here studied. Attending to organic matter removal, however, I42-MT showed being efficacious when eliminating TS, VS and COD (40.93 %, 54.17 % and 61.78 %, respectively). This can be translated into an ineffective use of organic matter by the system, as this was not properly used for biogas production, as seen for the productivity data in Table 7. Inhibition or arrest in matter degradation are feasible reasons that support this, as a microbial growth at non-optimal temperature can lead to scarce or incomplete break-down of molecules.

The three conditions showed an overall scenario that was clearly hampered by the presence of both pesticides combined. Even relative CH_4 generation was worsened

(46.9 % for M35-MT, 26.2 % for I42-MT and 22.3 % for T55-MT). This is justified by the higher sensitivity that methanogenic archaea present. These are more easily affected by the process performance, leading to an inefficient final transformation into CH_4 (Appels et al., 2008). To this regard, previous studies have shown that CH_4-producing microbial community can be inhibited due to the presence of other toxicants from a 50 % up to a 100 % (Bayr et al., 2012; Siles et al., 2010). Additionally, the time of the process was not improved, being especially clear the increase in days for I42-MT (15 days vs 13 days for I42, 12 days for I42-M and 9 days for I42-T).

In short, comparing results with the other configurations, for 35 °C the process is not enhanced through the presence of any pesticide, only slightly with mancozeb. On the other hand, for 42 °C it is the experiment with mancozeb that generates the highest amount of biogas. Finally, for 55 °C it is tefluthrin that improves biogas production, being the highest generation of all conditions. As opposed to the individual pesticides, the combined effect of mancozeb and tefluthrin diminishes the action on the system when compared to the systems with only one substance. This is probably due to inhibition of the process through summed effects of both pesticides.

Table 8. Overall results for co-digestion in presence of mancozeb and tefluthrin at the three operative conditions.

	M35-MT	I42-MT	T55-MT
TS removal (%)	23.00	40.93	58.62
VS removal (%)	33.25	54.17	68.05
COD consumed (%)	32.23	61.78	46.91
Biogas (L)	2.3	0.6	3.8
CH_4 (L)	1.1	0.1	0.9
Biogas/TS removed (mL/g)	354.1	48.5	163.4
Biogas/VS removed (mL/g)	303.9	42.6	24.4
CH_4/TS removed (mL/g)	166.0	12.7	36.4
CH_4/VS removed (mL/g)	142.5	11.2	34.8
Time (≃days)	17	15	15

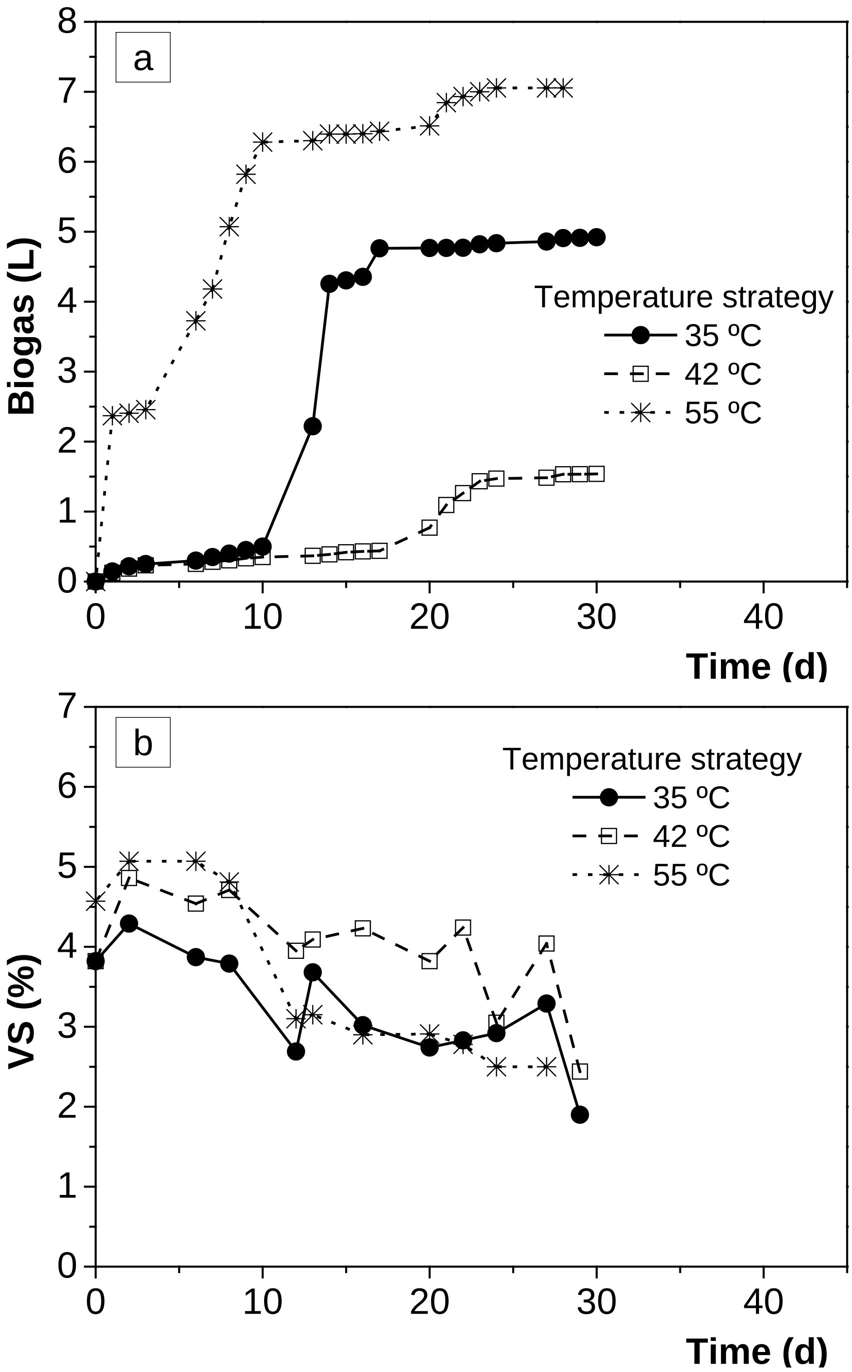


Figure 6. Evolution of co-digestion in the presence of half of the generally applied concentration of mancozeb and tefluthrin at three different temperatures. a) Cumulate biogas (L); b) VS (%); • 35 °C; □ 42 °C; ⁎ 55 °C.

As the sum of both pesticides could have inhibited the enhancement of the process or hindered the positive effects of them, assays at a lower concentration (half of each respective concentration) were carried out (M35-MTa, I42-MTa and T55-MTa). Results

(Figure 6 and Table 9), however, do not show a remarkable amelioration in comparison with the effects of pesticides added individually. Also, comparing both concentrations of pesticides and their effect at each operative condition, significant differences ($\alpha < 0.05$) were seen as statistically indicated for biogas production throughout the process: α=8.200E-06, α=0.002, α=0.031 for 35 °C, 42 °C and 55 °C, respectively.

Only T55-MTa (7.1 L) managed to generate a similar amount of biogas as T55-T (8.0 L). Additionally, CH_4 relative generation was higher for T55-MTa than for T55-MT, which also happened at 42 °C, while 35 °C yielded a slightly better performance to this regard for M35-MT.

It is also worth-mentioning that the three operative conditions show a late beginning of the process in spite of having used an adapted inoculum. This shows that the combination of both pesticides is incompatible when enhancing the process. Moreover, the duration of it is considerably higher than in the other configurations herein assessed. Additionally, the fact that half concentration of them yielded better results in general than the normal combined concentration, supports the hypothesis that a nutritional problem is not involved.

Table 9. Overall results for co-digestion in presence of half of the generally applied concentration of mancozeb and tefluthrin at the three operative conditions.

	M35-MTa	I42-MTa	T55-MTa
TS removal (%)	19.83	20.55	42.53
VS removal (%)	50.26	36.13	45.30
COD consumed (%)	24.01	30.15	45.35
Biogas (L)	4.9	1.5	7.1
CH_4 (L)	2.2	8.5	3.3
Biogas/TS removed (mL/g)	872.52	246.47	529.65
Biogas/VS removed (mL/g)	427.2	185.7	568.0
CH_4/TS removed (mL/g)	391.8	135.9	244.2
CH_4/VS removed (mL/g)	191.8	102.4	261.9
Time (≃days)	17	23	21

4. Conclusions

Co-digestion of vegetable wastes absent of pesticides has shown to be an efficacious process and temperature studies reveal that M35 is a more stable and efficacious process than I42 or T55, with biogas generations that surpass 6.7 L. On the other hand, tefluthrin and mancozeb added individually on the co-digestion substrate have shown to boost processes, with high productivities when compared to the reference assays. Derived from the obtained results, it can be hypothesized that manganese and zinc present in the commercial formulation of mancozeb and tefluthrin could have an effect in microbial activity, especially attending to enzymatic performance. On the other hand, the combined effect of the pesticides at two different concentrations hinders the action of them added individually. Finally, results show how for every condition M35 is overall the most suitable temperature from two points of view: analytical performance of the processes and the energy saving compared to the others temperature ranges.

Acknowledgements

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Chapter 8

Preliminary scale-up studies on anaerobic digestion of agricultural wastes in the presence of pesticides

Preliminary scale-up studies on anaerobic digestion of agricultural wastes in the presence of pesticides

Beatriz de Diego-Díaz[a], María Eugenia Tapia[a], Francisco Javier Peñas[a], Juana Fernández-Rodríguez[a]

[a] University of Navarra, Department of Chemistry, School of Sciences, Irunlarrea 1, 31009 Pamplona, Spain.

Abstract

Anaerobic digestion has widely shown to be a suitable approach for agricultural wastes valorization. The presence of pesticides that come along with this type of substrates have shown to not pose an obstacle. However, further studies in larger anaerobic reactors may yield a different scenario.

This study covers the use of two widely used pesticides, mancozeb and tefluthrin, in the anaerobic treatment of four different types of substrates: sloe, malt, artichoke and asparagus. Results indicate that the effect of each pesticide is dependent on the type of substrate. Sloe and malt showed a better performance when mancozeb was present (109.4 and 88.2 mL-CH_4/g-VS, respectively). On the other hand, artichoke and asparagus yielded better results for tefluthrin (222.1 and 330.1 mL-CH_4/g-VS, respectively). Additionally, organic removals were more efficacious on sloe and malt (average of 59.12 % of VS) than for artichoke and asparagus (average of 54.34 % of VS), due to the lignocellulosic structure of the latter substrates. In comparison with previously reported studies on these wastes, scale-up (from 1-L to 5-L reactors) showed to have an expected negative effect, especially attending to pH control and risk of failure. However, and in spite of further optimization required, results indicate that the process is viable with these substrates, this reactor volume, and the presence of both mancozeb and tefluthrin.

Keywords: anaerobic digestion; pesticide; mancozeb; tefluthrin; scale-up

1. Introduction

Anaerobic digestion (AD) has been widely studied at laboratory and pilot scale. This has allowed the implementation of this microbiological process at industrial scale, with high success around the world that is supported by the high number of operative plants treating many types of organic waste.

However, results of scientific research are almost always biased when processes are scaled-up. The factors affecting a larger digester are manifold (seasonality, waste conditions, inhibitors, technical issues, etc.) and the operative control should be more meticulous, especially regarding specific parameters that can potentially lead to reactor arrest or failure. For instance, the development of the microbial community after the start-up phase, once the operative conditions are already settled, takes longer periods of time to reach the level of richness, diversity and evenness required (Goux et al., 2016).

A worth-noting aspect that can highly affect the performance of the process is the substrate composition. This not only refers to the possible changes that the seasonality of the agricultural sector entails, but also to the substances that can have an effect on the treatment such as pesticides that come along with the agrifood residue. Mancozeb and tefluthrin applied individually have previously shown to not have a negative effect on anaerobic co-digestion (AcoD) of several vegetables (green bean, pea, carrot, bean, artichoke and cabbage). Moreover, if any effect was registered, this was positive, especially attending to CH_4 productivities. Additionally, these experiments showed how, overall, the best operative condition was the mesophilic range (35 °C), especially attending to productivities and CH_4:biogas productions (de Diego-Díaz et al., 2018a. Chapter 7).

Nevertheless, these experiments were carried out in small laboratory scale reactors (total volume, 1.0 L; working volume, 0.6 L). Therefore, the conclusion that these two pesticides alone do not involve a major problem should be supported by larger reactors.

In this study, the scaling-up of the reactors used throughout the whole project (0.6 L) to a working volume of 3.0 L is assessed. The utilized substrates are also the ones that play a relevant role in this thesis, namely sloe, malt, artichoke and asparagus. These will be treated in the presence of the aforementioned pesticides as to determine whether the previously found conclusions also apply when the process is scaled-up.

2. Materials and Methods

2.1 Experimental system

Batch experiments were performed in accordance with the methods described by Holliger et al. (2016) in continuous stirred-tank reactors. These had a total volume of 5.0 L and a working volume of 3.0 L. Two ports allowed the collection of biogas, in 10-L fluorinated ethylene propylene bags (SKC, USA), and sampling. Reactors were placed in a closed thermostatic chamber with a constant temperature of 35 °C. Mechanical stirring at 30 rpm was kept constant to guarantee homogeneous mixing and solubilisation. Digesters were set up attending to total solids (TS) and operating in the wet range (4-10 % of TS).

Biogas was quantified by water displacement in a gasometer, and CH_4 through NaOH displacement (Holliger et al, 2016). A biogas analyzer was also utilized (Geotech Biogas-5000, UK).

Reactors contained 1.0 L of adapted inoculum (1/3 of reactor volume) and 0.25 L of a nutrient solution containing elements as described in de Diego-Díaz et al. (2018b. Chapter 1). The adaptation of the inoculum was done again as described by Holliger et al. (2016), this is, through previous incubation at 35 °C and elimination of residual biomass.

Pesticides were added individually in each reactor, as previous studies concluded that the combination of mancozeb and tefluthrin in different concentrations has a negative effect on the process. These were added in accordance to their commercial use on the crop field as described elsewhere (de Diego-Díaz et al., 2018a. Chapter 7).

2.2 Analytical Methods

Analytical methods were implemented as advised by the Standard Methods (APHA, 2012). Soluble chemical oxygen demand (sCOD) was determined (Agilent 8453, USA) in samples that had been digested at 150 °C for 2 hours (Velp Sci, Italy) after centrifugation (13000 rpm for 10 min, VWR Micro Star 12, USA) and filtering (0.45 µm and 0.2 µm, Starstedt, Germany). Pesticide composition analyses were carried out and described by de Diego-Díaz et al. (2018a. Chapter 7).

2.3 Substrate Characterization

Commercial substrates, sloe, malt, artichoke and asparagus were harvested in Spain and characterized as shown in Chapters 2 (artichoke), 3 (asparagus) and 5 (sloe and malt). Each reactor of this study can be identified as described in Table 1.

Table 1. Configuration identifiers in this study.

Pesticide/Substrate	Sloe	Malt	Artichoke	Asparagus
Mancozeb	sloe-M	malt-M	art-M	asp-M
Tefluthrin	sloe-T	malt-T	art-T	asp-T

3. Results and Discussion

The following sections will describe the results (Tables 2 and 3) derived from this study attending to the effect of each pesticide, type of substrate and scale-up (from 1-L to 5-L reactors).

3.1 Effects of pesticide

Mancozeb and tefluthrin yielded overall different results but showed to be dependent on the substrate as will be described in further sections. In general terms, the presence of mancozeb was more positive than tefluthrin, especially attending to cumulate productions (both biogas and CH_4). However, each parameter should be assessed individually.

For sloe and malt, mancozeb was clearly the best option, especially for malt where the differences between both pesticides were more significant. Malt-M was the most efficacious in removing solids (77.45 % VS removed). Moreover, this was the highest elimination from all the configurations studied and up to 63 % higher than that achieved by malt-T (48.88 %). Additionally, cumulate biogas productions were 2.8 and 2.4-fold higher for malt-M (15.3 L-biogas and 10.0 L-CH_4) when compared to malt-T (5.4 L-biogas and 4.1 L-CH_4) for biogas and CH_4, respectively. Despite this, the quality of the biogas produced was better for malt-T, which contained up to 75.9 % of CH_4 as opposed to malt-M, which contained 65.6 %.

Sloe-M, as aforementioned, was better than sloe-T. Nonetheless, solids removals were more efficacious for sloe-T, with up to 60.65 % elimination of VS (56.17 % for sloe-M). Cumulate biogas and CH_4 generations reached 14.6 L and 9.5 L, yielding a CH_4:biogas ratio of 0.65, which is slightly better than sloe-T, 0.59.

It should be mentioned that biogas productions were remarkably low for these two substrates, especially for sloe-T (0.7 L-biogas and 0.4 L-CH_4), which was the lowest of all configurations, and malt-T (5.4 L-biogas and 4.1 L-CH_4). This led to a notably low efficiency of the process as represented by productivities (Tables 2 and 3). Process failure can be adduced.

Artichoke and asparagus, on the other hand, showed better results when tefluthrin was present, as opposed to mancozeb. Firstly, VS removal for art-T reached 54.38 %, 1.14 times higher than the elimination achieved by art-M. Despite a higher amount of cumulate biogas and CH_4 generated for art-M (36.9 L-biogas and 20.2 L-CH_4), art-T yielded a better quality biogas with up to 60.8 % of CH_4. Moreover, art-T productivities represented a highly efficient process, with 222.1 mL-biogas/g-VS and 365.4 mL-CH_4/g-VS, which is translated into a 1.3 and 1.4 times a more productive operation than art-M. Despite this yields not reaching those reported by Fabbri et al.

(2014) in 450-mL reactors (working volume), ranging from 271.8 to 408.6 mL-CH_4/g-VS, the achieved results in this study for asparagus are appropriate considering the scale-up effect.

Lastly, for asparagus AD only solids eliminations were more effective for asp-M (60.65 %) than for asp-T (56.47 %), but the rest of the parameters showed to be remarkably better for the latter than for the former. Asp-T reached 33.4 L-biogas and 21.5 L-CH_4, being this the highest cumulate CH_4 of all the configurations, and reaching a 64.35 % of the biogas produced. It is worth-mentioning that asp-M barely yielded 6.7 L-biogas and 3.3 L-CH_4, almost as low as malt-T. Additionally, asp-T yielded, not only higher productivities than asp-M (95.9 mL-biogas/g-VS and 46.5 mL-CH_4/g-VS), but the highest of every experiment, reaching 513.0 mL-biogas/g-VS and 330.1 mL-CH_4/g-VS. Moreover, it surpassed the previously reported productivity from AD on pretreated asparagus, 242.3 mL-CH_4/g-VS (Chen et al., 2014).

Table 2. General results for AD on sloe, malt, artichoke and asparagus in the presence of mancozeb (-M).

	Sloe-M	Malt-M	Art-M	Asp-M
TS removal (%)	52.93	75.73	42.51	52.49
VS removal (%)	56.17	77.45	47.56	60.65
sCOD removal (%)	31.63	20.42	5.45	12.77
TOC removal (%)	40.83	55.75	22.12	45.51
Biogas (L)	14.6	15.3	36.9	6.7
CH_4 (L)	9.5	10.0	20.2	3.3
Biogas/TS (mL/g)	162.6	123.5	243.5	96.7
Biogas/VS (mL/g)	167.5	134.5	281.3	95.9
CH_4/TS (mL/g)	106.2	81.0	133.2	46.9
CH_4/VS (mL/g)	109.4	88.2	153.9	46.5
Time (≃days)	56	52	56	56

Table 3. General results for AD on sloe, malt, artichoke and asparagus in the presence of tefluthrin (-T).

	Sloe-T	Malt-T	Art-T	Asp-T
TS removal (%)	54.45	44.75	48.78	48.24
VS removal (%)	60.65	48.88	54.38	56.47
sCOD removal (%)	24.09	29.37	9.25	22.04
TOC removal (%)	28.42	51.77	62.98	27.42
Biogas (L)	0.7	5.4	26.0	33.4
CH_4 (L)	0.4	4.1	15.8	21.5
Biogas/TS (mL/g)	7.2	74.2	352.5	523.9
Biogas/VS (mL/g)	7.0	75.7	365.4	513.0
CH_4/TS (mL/g)	4.2	56.4	214.2	337.1
CH_4/VS (mL/g)	4.2	57.4	222.1	330.1
Time (≃ days)	13	59	56	52

3.2 Effect of substrate

Overall, eliminations were higher for sloe and malt (mean elimination of 59.12 % of VS) than artichoke and asparagus (mean elimination of 54.34 % of VS). This can be due to the highly recalcitrant structure of the two latter substrates, derived from their lignocellulosic nature. Therefore, a higher temperature of operation, such as the thermophilic range, could potentially lead to processes with a better performance. However, a higher temperature may result in a more unstable operation with a more demanding pH control and worse CH_4:biogas ratios. Artichoke, despite showing lower productivities and CH_4:biogas ratio than asp-M, has previously been reported to improve AD of other substrates in anaerobic co-digestion, increasing CH_4 content up to a 70 %. Hence, it can be hypothesized that the here studied configuration combined with other substrates may yield even better performances thanks to the potential and suitability of artichoke (Ros et al., 2013).

Interestingly, as aforementioned, asp-T yielded the highest productivities of all other substrates (513.0 mL-biogas/g-VS and 330.1 mL-CH_4/g-VS), which is within the range of previously found results on asparagus digestion in 1-L reactors (de Diego-Díaz et al., 2018d) at the same temperature (548.2 mL-biogas/g-VS and 408.0 mL-CH_4/g-VS)

In regard with the time of operation, all the substrates happened to reach the end of the process in the same range of time (52-59 days) (Figure 1). However, sloe-T arrested producing biogas at day 13. This could indicate failure in the operation of this substrate at initial stages due to acidification provoked by intermediate products release. Methanogenic archaea have a lower pH range of viability, while hydrolytic, acidogenic and acetogenic bacteria are able to face lower pH ranges. Therefore, the high VS elimination, despite de process arrest in sloe-T, could possibly find an explanation in the ongoing break-down activity of bacteria without biogas formation.

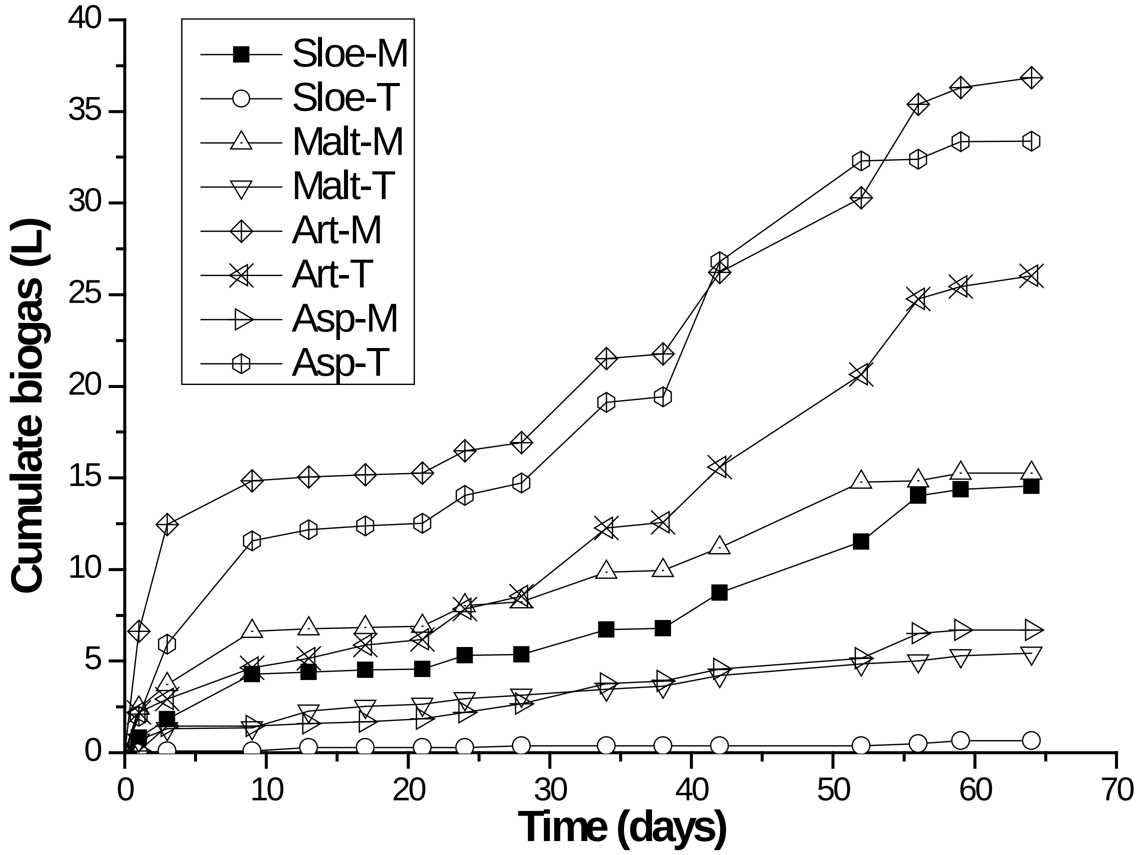

Figure 1. Cumulate biogas production with time for each substrate in the presence of pesticides. –M: manzoceb; -T; tefluthrin.

3.3 Effect of scale-up

Attending to overall performance, every studied configuration demanded a thorough control of pH as their acidification posed a high risk of reactor failure. This was one of the most remarkable differences between 1-L and 5-L digesters. Additionally, the tendency of biogas production for all reactors, except for sloe-T, differentiates two phases (Figure 1) that can be attributed to an initial hydrolytic, acidogenic an acetogenic phase, and a second one after a short arrest of production corresponding to the methanogenic stage. On the other hand, lower volume reactors described a logarithmic tendency. This could be related to the lower amount of organic matter available that is, thus, digested in a shorter period of time, while larger reactors, after the initial stages after start-up, still have accessible biodegradable substrates.

Scale-up has previously shown to have a negative effect on microbiological processes. André et al. (2018) reported that aspects such as hydric transfers, rheology, biochemical methane potential, inhibition, and microbial dynamics, should be paid attention to in order to fulfill industrial scale reliability and profitability. This has been observed in this study, especially regarding productivities, which were not only worse but also abnormally low in the cases of asp-M (95.9 mL-biogas/g-VS and 46.5 mL-CH_4/g-VS), sloe-T (7.0 mL-biogas/g-VS and 4.2 mL-CH_4/g-VS) and malt-T (75.7 mL-biogas/g-VS and 57.4 mL-CH_4/g-VS). However, the other configurations yielded results within the range of the expected as previously reported: 236.3-581.8 mL-CH_4/g-VS for the use of pesticides, 284.3 mL-CH_4/g-VS for artichoke, and 408.0 mL-CH_4/g-VS for asparagus (de Diego-Díaz et al., 2018a; de Diego-Díaz et al., 2018d; de Diego-Díaz et al., 2018e. Chapters 7, 2 and 3, respectively).

It is also interesting that sloe digestion had previously shown (de Diego-Díaz et al., 2018c) to be more easily digested in the mesophilic range (222.0 mL-biogas/g-VS) in comparison to the thermophilic (88.0 mL-biogas/g-VS), while malt showed the opposite scenario. However, in this scale-up study that has been performed at mesophilic range, sloe has shown very low productivities and VS removals despite being the preferred range in 1-L digesters. Malt, on the other hand, yielded results even better than sloe, for both mancozeb and tefluthrin.

4. Conclusions

The presence of pesticides has shown not to be a major problem in the performance of AD on agricultural wastes. However, the specific effect of each pesticide is dependent on the type of waste. The found results indicated that the presence of mancozeb yielded better performances on sloe and malt (109.4 and 88.2 mL-CH_4/g-VS, respectively) as opposed to tefluthrin, while artichoke and asparagus were better digested when tefluthrin (222.1 and 330.1 mL-CH_4/g-VS) was added.

The scale-up from 1-L digesters to 5-L digesters, despite having used different substrates, happened to be more demanding, as expected. This derived from the higher organic load in the reactors and the higher likelihood of process failure from inhibition. However, the successful performance in most configurations (sloe-M, malt-M, art-M, art-T and asp-T) sheds light to the further scaling-up of the process and potential operations in continuous or semicontinuous regimes, as these substrates, even in the presence of pesticides, have shown to be suitable for AD treatment.

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IV. General Discussion

The growing population worldwide and the increasing interest of lowering greenhouse gas emissions into the atmosphere are two of the main reasons for which waste treatment has become of capital importance. European, national and even regional organisms seek to implement regulations that care for the appropriate management of wastes. As previously seen and reported, the most effective approach is reducing residue generation (Oldfield et al., 2016). However, when this is not possible, its effective treatment is a crucial concern.

Anaerobic digestion (AD) is a well-documented technology and this project gives a quick insight into the various objects of study and optimization of the process. Its further implementation could give solution to a vast global amount of organic wastes generated.

Nevertheless, it should be noted that AD processes are still yet to be fully upgraded to their maximum levels. This has to do with the high complexity and the great number of factors that influence the operation. The former mainly relies on the fact that the microbial population responsible of degrading the organic matter is formed out of thousands of different species that interact between each other in different fashions. Moreover, these relationships are highly dependent on external aspects such as substrate composition (recalcitrance, crystallinity, degradation potential, nutritional balance), digestion temperature (growth rate, mass transfer, degradation rate, thermodynamics), pH (inhibition, process arrest and/or failure, microbial viability), alkalinity (stability, vulnerability to operational shifts), toxics/inhibitors, etc. (Gerardi, 2003).

This thesis dissertation has covered agricultural wastes that are relevant to the local economy in Spain and, more specifically, to Navarre. But these have also been chosen considering their high consumption as food all around the globe, especially artichoke and asparagus. However, it should be noticed that the herein presented studies pose hurdles as supported by the literature. Solid-state batch experiments present many experimental boundaries that are shared with lignocellulosic and difficult-to-solubilize substrates. Among them the most worth-mentioning are: high heterogeneity of the substrate that leads to low sampling representativeness, local inhibition in specific zones, and scale-up phenomena (André et al., 2018).

In this project, the optimization approaches that have been suggested are nutrient supplementation (Chapter 1), temperature-phased anaerobic digestion (Chapters 2 and 3), AcoD (Chapters 1, 4 and 7), thermal pretreatment (Chapter 5), *ex-situ* biogas upgrading from a microbiological point of view (Chapter 6), and pesticides effect and scale-up (Chapter 7 and 8). Summary tables can be found in Annex II.

In the following sections, the main results and hypotheses from the studies tackled in this project will be discussed trying to give light to an expanding technology.

1. Reactor design and process set-up

From an experimental point of view, the most common aspects of all the studies in this thesis are the employment of continuous stirred-tank reactors (CSTR) and an adapted inoculum. The advantage of CSTRs utilization is the guarantee of uniform and complete mixing, which allows microbial contact with the substrate. Furthermore, this stirring leads to homogeneous properties within the reactor such as temperature and density (Bhatia and Yang, 2017). Attending to CSTR arrangement, most of the reactors had a working volume of 0.6 L (total volume, 1.0 L; Figure 1a), the recommended by the literature (Holliger et al., 2016), and their operation was found to be similar, only differing in substrate type and digestion temperature. However, Chapter 8 allowed analyzing the effect of scale-up using digesters with a working volume of 3.0 L (total volume, 5.0 L; Figure 1b). From all the possible differences, the most worth-noting is pH control. From a qualitative outlook, larger CSTRs were more demanding and required a more thorough pH adjustment using NaOH, which was almost daily, even when compared to the most unstable experiments from 1-L reactors. To this regard, initial stages of AD took longer and methanogenesis was triggered in more advanced steps. Gallert et al. (2003) discussed that high VFA concentrations in the effluent does not necessarily lead to any problem in downstream handling, thus making this outflow suitable for direct disposal into any water body, always depending on the type of VFA accumulated (e.g. propionic acid generally entails process inhibition). Therefore, as long as the reactor does not fail to operate due to pH imbalance, the presence of more or less intermediate products could not entail a major problem.

Moreover, it has also been reported that organic matter removal decreased to some extent when the process was scaled-up, which agrees with what was found in the herein experiments (e.g. 70.7 % removal in 1-L reactors for sloe and 56.2 % for sloe with mancozeb in 5-L reactors). This could potentially have to do with high absolute organic load, as this takes longer to solubilize and be accessible for the microbial consortia, thus hindering its degradation. On the other hand, the scale-up experiments in this thesis did show better methane yields than those reported by the literature (e.g. 330.1 mL-CH_4/g-VS for asparagus with tefluthrin in 5-L reactors vs. 242.3 mL-CH_4/g-VS for asparagus according to Chen et al., 2014).

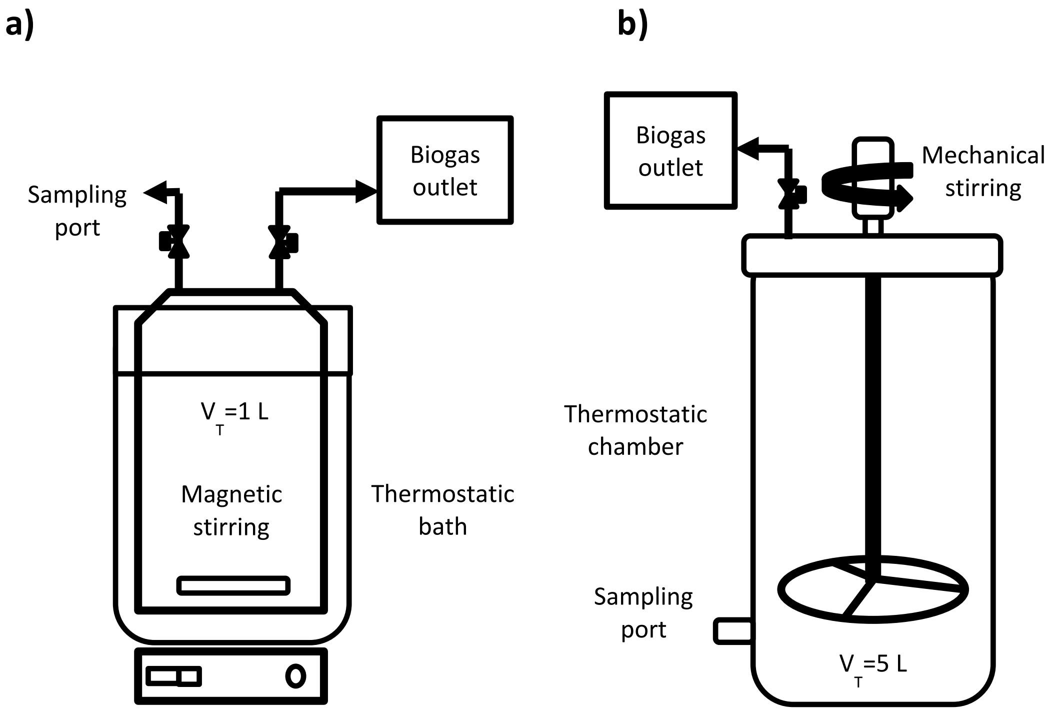

Figure 1. CSTR used in this study. a) 1-L reactors; b) 5-L reactors.

Biogas upgrading reactors used in Chapter 6 presented a different configuration, as sketched in Figure 2. It was the secondary reactor were H_2-mediated upgrading took place. The interest towards this approach is increasing, especially in those geographical areas were wind power plays an important role, generating even a surplus of energy that can be coupled to other forms of energy recovery like AD (Angelidaki et al., 2018). The strategy has widely shown to yield a suitable performance. The most remarkable aspect is the obtaining of highly upgraded biogas that can directly be used without any downstream process thanks to the 99 % of CH_4 from its composition.

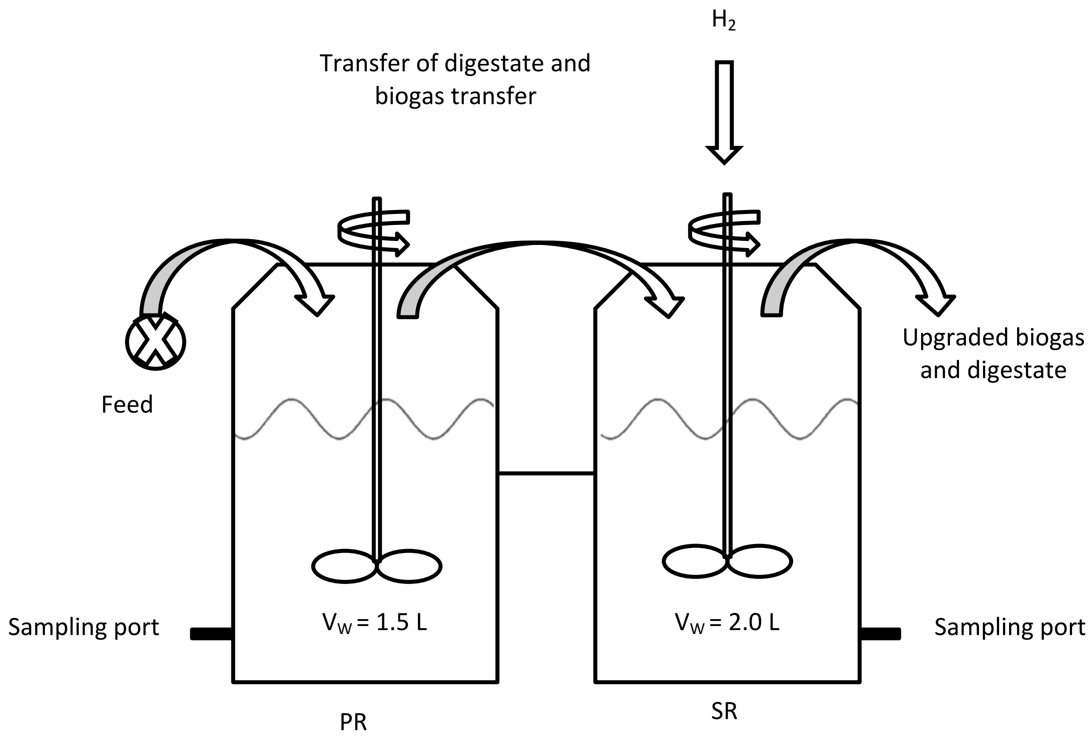

Figure 2. CSTRs used in Chapter 6 for *ex situ* biogas upgrading. PR: primary reactor. SR: secondary reactor.

2. General aspects of digestion temperature

Throughout the whole project, three different temperatures of operation attending to the classical ranges were utilized (mesophilic, 35 °C; intermediate, 42 °C; and thermophilic, 55 °C). Therefore, the different nature of the various substrates employed, allowed establishing common effects of temperature in each of the assessments.

Firstly, it can be overall stated that thermophilic range managed to produce the highest amounts of cumulate biogas. Moreover, organic matter elimination also tended to be more efficient at 55 °C reaching even 72 % of VS elimination for the case of AcoD in the presence of mancozeb pesticide (Chapter 7). However, the general trend in thermophilic range showed lower productivities, yields and relative productions. These lower efficiencies have been hypothesized to be attributed to a very strong starting-up of the hydrolytic phase. This step is considered to be a bottleneck of the AD process and the literature has expressed the interest of overcoming it without retarding the following stages (De la Rubia et al., 2013; Schnürer, 2016). Nonetheless, and taking into account the highly organic nature of the composition of the herein studied substrates, a rapid break-down of macromolecules can result in undesired inhibition for microbial consortia. Some cases, as reflected by Figure 3, lead to a difficult to control release of volatile fatty acids (VFA).

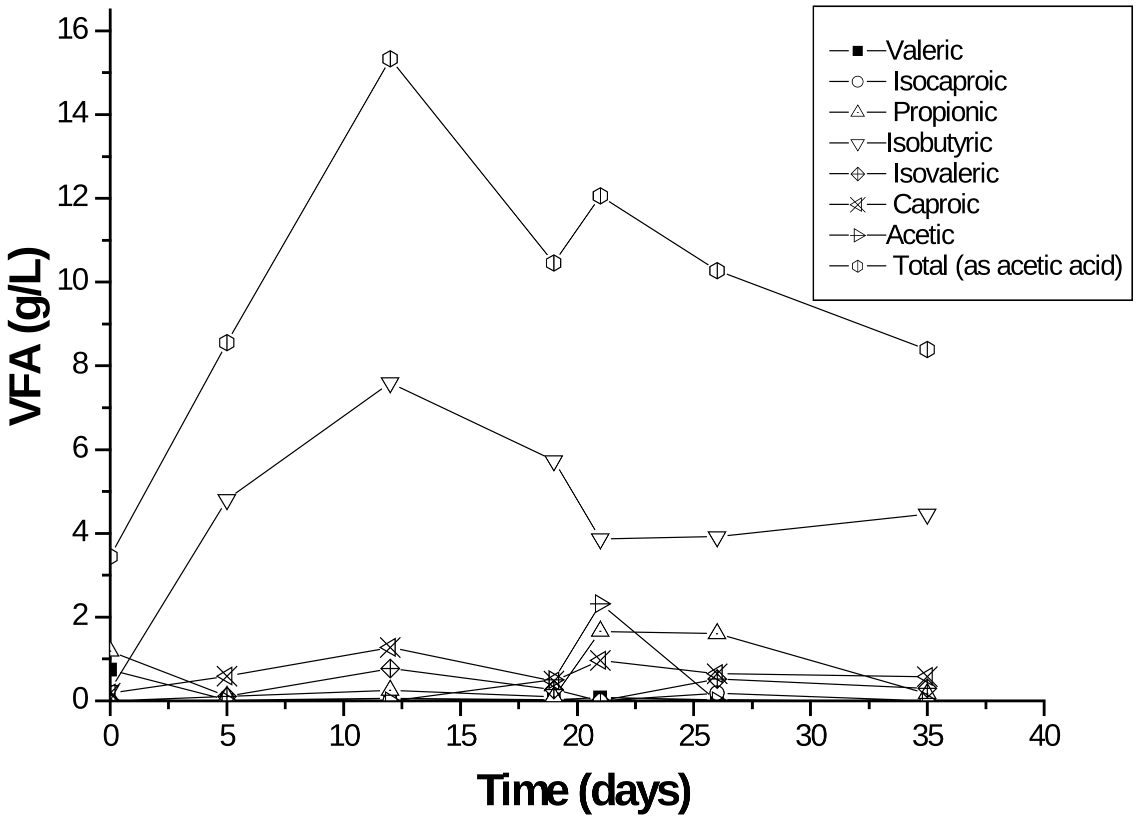

Figure 3. Evolution of VFA profiles for AcoD of 6 commercial vegetables at 55 °C (Chapter 7).

This is also supported by pH monitoring, which, due to a rapid acidification, its control is very demanding in the initial stages. To this regard, mesophilic reactors happened to stabilize at the convenient pH of operation (6.5-8.2) at an earlier stage than those at thermophilic conditions (Figure 4) (Goswami et al., 2016; Lee et al., 2009).

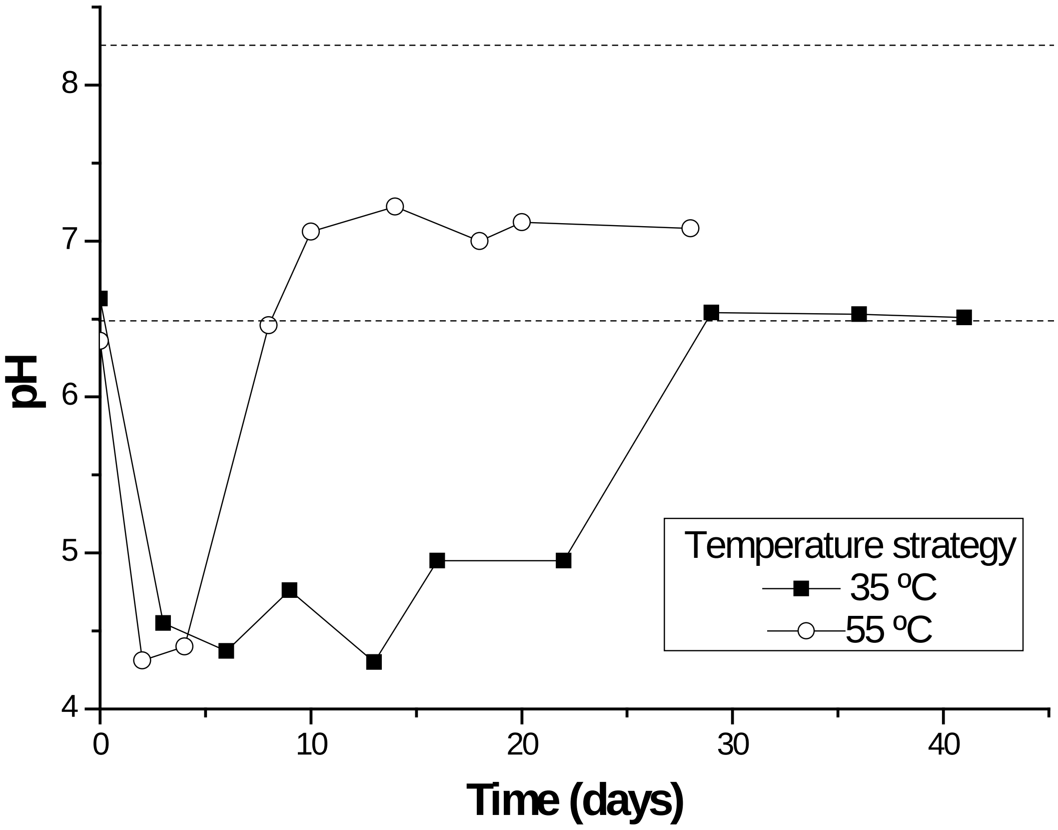

Figure 4. Example of pH monitoring throughout time for AcoD of 6 commercial vegetables at ■) 35 °C and o) 55 °C (Chapter 7). Dash lines indicate optimum range for methanogenesis.

The aforementioned is intimately related to the relative production of CH_4. The thermophilic reactors take longer to reach the methanogenic phase due to higher intermediate products accumulation, which leads to process inhibition and arrest, and non-optimal pH, being less stable than mesophilic range, for instance (Fernández-Rodríguez et al., 2013). Anaerobic bacteria that are involved in hydrolysis, acidogenesis and acetogenesis can face operative changes and non-optimal pH (4.5-8.5) to a certain extent. On the other hand, methanogenic microorganisms have more restrictive requirements and fail when conditions are not suitable for their activity (e.g. pH 6.5-8.2) (Goswami et al., 2016; Lee et al., 2009; Zoetemeyer et al., 1982). Hence, when VFA and other intermediate products accumulate and lower the pH within the digester, CH_4 production cannot take place and the composition of the biogas formed has a lower content in CH_4 and higher in other gases, product from microbial metabolic activity such as H_2. This was the case of AcoD of artichoke and asparagus (1Art/2Asp) for which CH_4 content barely reached 46 % of the total biogas produced (Chapter 4).

Temperature is, therefore, a determinant factor in microbial viability. Aside from the microbial analyses carried out in some parts of this thesis that have shown some limitations, the monitored biochemical parameters can also yield information to support hypotheses of reactor performance. For instance, the fact that higher temperatures have shown to lower richness in microbial variability can be translated into a more specialized community that is able of operating towards a very specific aim despite the low heterogeneity among species (Westerholm et al., 2017). On the one hand, this can be beneficial when the population is adapted to the loading of a constant substrate composition-wise. On the other hand, minimal changes in load composition or operative conditions (slight changes in temperature, pH, etc.) can result in treatment failure.

The opposite scenario was in general seen for mesophilic conditions. However, in comparison to 55 °C, the cumulate biogas production was adequate in spite of being slightly lower for each case. This is offset by the efficiency of the processes, as overall results yielded better productivities than for thermophilic reactors. This is translated into a more effective investment of microbial resources, as their activity of organic matter degradation leads to a better quality of biogas. Also technical resources can be considered to be better invested, as the somewhat longer operation times are balanced by an efficient degradation process with the desired byproducts (highly degraded substrates and good quality biogas) that also have to be less treated in downstream stages.

Lastly, as seen in most chapters, 42 °C was assessed as an intermediate digestion temperature. The overall performance of agricultural wastes at this condition was not remarkable, especially attending to CH_4 production which in some cases only was a 25 % of the total cumulate biogas generated (AcoD of 2Art/1Asp) (Chapter 4). However, this condition should not be dismissed and could be object of life cycle assessments, as its potential optimization may lead to lower operative costs in industrial plants in which working at 55 °C seems to be very costly. Moreover, nowadays there are plants that already are operating at supposedly non-ideal temperature ranges.

After a thorough analysis of the data collected in this project and the lower operative costs, mesophilic range, more specifically 35 °C, is the advised condition for the treatment of general agricultural wastes. Nevertheless, when the substrate is more recalcitrant due to its high lignin content (e.g. artichoke or asparagus), further approaches like AcoD or temperature-phased anaerobic digestion (TPAD) are advised to be implemented in order to fulfill the objectives pursued, such as effective lignocellulose break-down.

TPAD is carried out with an initial stage at a higher temperature than the second one (e.g. thermophilic and mesophilic range, or hyperthermophilic and thermophilic range). High temperatures yield a suitable scenario for initial hydrolysis, acidogenesis and acetogenesis through macromolecules break-down. This favors the process performance by releasing intermediate products that act as main substrates of the following stage, methanogenesis, at a lower temperature. This configuration allows speeding up the initial phases of AD that are considered a bottleneck in the process.

TPAD was performed on two recalcitrant substrates, namely asparagus and artichoke. Interestingly, the improvement, if any, was not remarkable. On preliminary studies, configurations of 10, 7, 5 and 3 days of thermophilic initial phase were performed. From these analyses, 7 and 5 days were considered the two best strategies as indicated by CH_4 yields, which reached 371.0-487.0 mL-CH_4/g-VS. Hence, these two were chosen to be implemented on artichoke and asparagus. The latter showed notable improvements on maximum specific growth rate of the microbial population. However, this did not result in outstanding differences with SPAD, only increasing CH_4:biogas ratio (84 % and 79 % for TPAD7 and TPAD5, respectively). This could be associated with low synergy among the microbial population or the outcompetition of specific groups that would not allow the successful performance of the process. On the other hand, TPAD on artichoke did yield different results between TPAD7 and TPAD5, showing that TPAD7 was the most suitable configuration on this substrate, especially attending to biogas production (7.8 L vs. 4.7 L). Moreover, kinetic modelling confirmed through the non-biodegradable substrate that TPAD is more efficacious than SPAD processes.

3. Optimization approaches of anaerobic digestion on agricultural wastes

Various optimization strategies have been proposed attending to different aspects of AD, always considering its high complexity. This makes very difficult to generalize this treatment process, leading to a certain extent to make of a specific problem (particular substrate, operational conditions, etc.) a case study. Nonetheless, some generic statements can be described, despite the diverse characteristics of the wastes herein employed. Productivities and organic removal can shed light into which procedure is the most convenient.

Focusing on VS elimination, from all the studies, thermal pretreatment on spent malt at 55 °C (72.6 % of VS removal) and AcoD of agricultural wastes at 55 °C in the presence of mancozeb (72.0 % of VS removal) were the most successful. However, this parameter ignores the nature of the substrate, as more lignocellulosic or crystalline substrates like asparagus or artichoke may already be enough difficult to degrade. Therefore, also the improvement of elimination, comparing the reference treatment with the optimization approach, should be considered. Among them, insignificant improvement of asparagus digestion at 35 °C through TPAD (1.03 higher removal), artichoke at 55 °C through AcoD with asparagus (2Art/1Asp, 1.02 higher removal) and spent malt at 55 °C via thermal pretreatment (1.09 higher removal), can be mentioned. It should not be dismissed that AcoD of various agricultural wastes showed to be improved when mancozeb (elimination at 55 °C and 42 °C were 1.46 times and 1.33 times higher, respectively) or tefluthrin (55 °C 1.37 times higher removal) were present. In spite of pesticides not being catalogued as an optimization approach, results clearly show that AD does not require of upstream processes that eliminate pesticides, as AD is actually improved through their presence.

Regarding process efficiency, CH_4 production per VS consumed is a very revealing parameter. The most outstanding productivity achieved was AcoD in the presence of tefluthrin at 35 °C, which reached 582 mL-CH_4/g-VS and the highest relative improvement (2.61 times the process without pesticide). Additionally, other processes were improved through the proposed strategies (Figure 5) from which the most worth-noting are the presence of nutrients, which made the productivity increase 1.93 times, showing a lack of nutrients, and asparagus through AcoD with artichoke (1Art/2Asp) that enhanced productivity 1.76 times. Figure 5 summarizes the most outstanding improvements in CH_4 productivities, comparing in each case the reference process and the optimization approach.

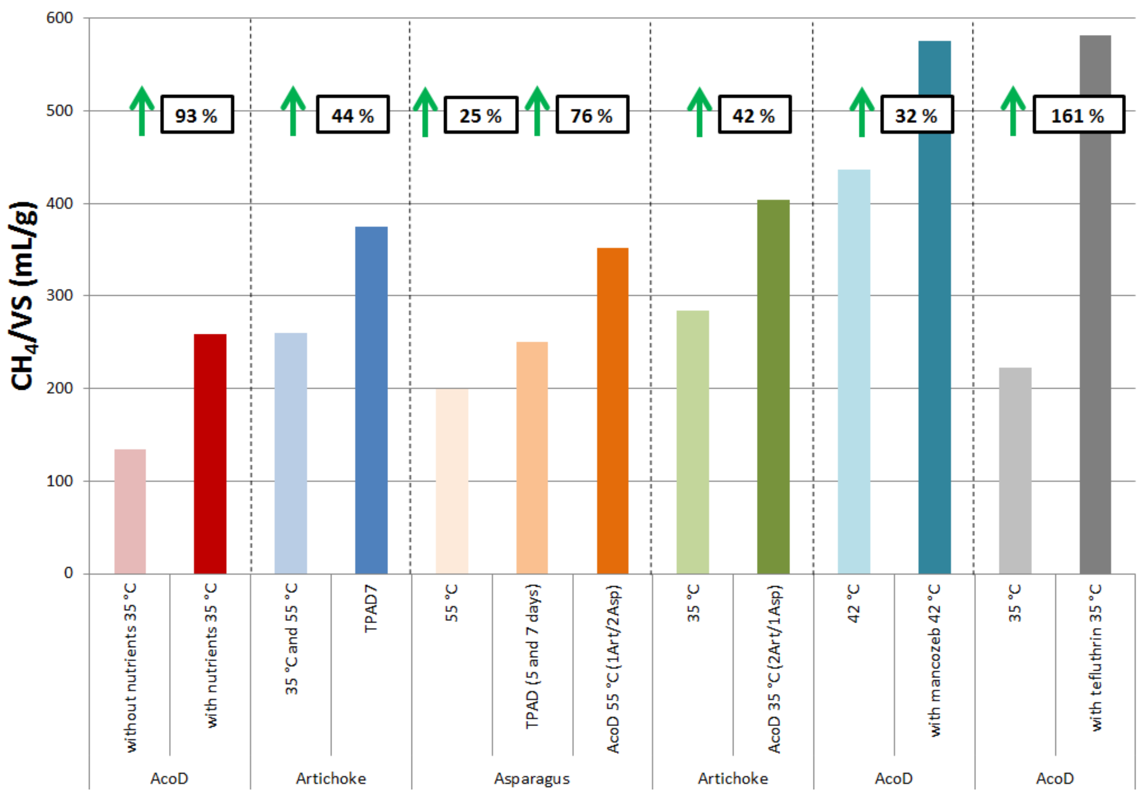

Figure 5. Best CH_4 productivity improvements attending to seven optimization approaches. Data in squares refer to the level of improvement (improved value/reference value).

It is also remarkable that each of the strategies here proposed gave rise to processes that were statistically different among them. In other words, despite the low or absent improvements observed for some cases, the approach and/or conditions did have an effect on each of the circumstances.

4. Substrate suitability for anaerobic digestion

Substrate composition has already shown to be determinant for a successful performance of the reactor. Not only the chemical structure (e.g. higher crystallinity in artichoke or asparagus), but also the composition (C/N balance, potential phenolic or sulfur release, lipids, proteins, carbohydrates, etc.) plays a role in the pace of the process (Mata-Álvarez et al., 2014). To a certain extent, the problems that arise from the waste nature are obligated object of study, as the available waste has to be treated one way or another and the solution should not be leaving it aside. Pretreatments have historically shown to be a feasible option for modifying the substrate nature without prescinding from its treatment and its energetic and agricultural valorization. However, in this project, the proposed thermal pretreatment has shown not to be the most suitable approach in two ways: firstly, process improvement, if any, was neither remarkable nor applicable to all wastes; secondly, industrial scale-up of a treatment phase at 80 °C would entail high operative costs with low cost effectiveness. Therefore, substrate suitability can be achieved by the combination of various substrates through AcoD.

Preliminary studies (data not shown) in the laboratory using commercial vegetables AcoD (as used in Chapters 1, 7 and 8) indicated that vegetables alone yield worse results in general than the combination of up to six of them. Moreover, more recalcitrant substrates such as asparagus and artichoke also showed to be more easily degraded when combined, surpassing even TPAD of the single substrates in some cases. Both TPAD and AcoD somehow improved artichoke and asparagus treatments, but advantages of AcoD over TPAD are the following: no need of substrate classification at the receiving point of the biogas plant, no need of control of operation for two different conditions (temperature, organic load and retention times), no need to operate thermophilic reactors, etc.

In short, the suitability of the waste is more a matter of its potential optimization rather than the consideration of its discarding. The profitability of the plant and the energy balance should, nevertheless, always be considered.

5. Microbial characterization

Anaerobic digestion fundamentals rely on the last term in microbial activity. Although this project did not aim to elucidate AD course through microbiological tools, a quick insight into it has been carried out. An initial approach towards bacterial identification was performed on reactors treating sloe and malt at different time points as to describe the possible effect of temperature and thermal pretreatment (Chapter 5). However, this was implemented with a commercial kit (Biomerieux API20A) and the technique roughly yielded information about some cultivable species. Moreover, some colonies may have not been picked up, and thus not identified, due to the arduousness and laboriousness that this method implies. For this complex system, API20A showed not to be suitable for the understanding of the microbial activity beyond AD. This statement is based on the following aspects:

- API20A only identifies cultivable colonies, which cover a very low percentage of the possibly present species within the digesters.
- API20A does not identify archaeal species.
- After initial culture, the selection of colonies to be identified is based on random criteria such as morphology or resources limitation, which is not an effective methodology for such a complex and vast community.

The international stay at the Technical University of Denmark allowed the performance of more specific studies in a specialized laboratory. For the purpose of this thesis, only the results described in Chapter 6 were included. Despite these not being comparable with the rest of the studies due to the reactor set-up, they yielded useful information that allowed hypothesizing about the possible explanation of biochemical parameters related to the microbiology of the processes. Moreover, the use of statistics in biological processes was made clear to be important and useful.

Molecular tools, more specifically metagenomic ones, allow determining not only the presence of microbial species but also their relative abundance and, thus their possible relationship between each other in accordance to their metabolism and possible activity within the reactor. Metagenomics based on the hypervariable V4 region of the 16S rRNA has been widely applied (Krause et al., 2008; Kröber et al., 2009; Schlüter et al., 2008) and our study represents an example of the valuable information that can be obtained from its sequencing.

The most interesting aspect of the study is the outcompetition of the predominant archaea of anaerobic digesters after a long time of operation. However, despite the convincing outcomes of the experiments and the accuracy of the sequencing, more conclusive results could be obtained. Microbiological identification using 16S rRNA is performed against public databases that account with operational taxonomic units (OTUs) that have been isolated from a different environment than those from an anaerobic reactor and probably grown under totally different conditions (Campanaro et al., 2016). Therefore, the build-up of a specific database is of high relevance and would allow the further unveiling of specific species of the biomethanization microbiome.

6. Final Remarks

Anaerobic digestion represents a suitable way of treating agricultural wastes in laboratory and large scale facilities, especially considering the safety of the process, the low environmental impact, and the valorization and recycling of waste. This project has covered many different aspects of AD and has shed light into features that had not been previously reported in the literature. However, further studies are recommended to fully unveil the process.

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V. Conclusions

1. Attending to the objective "**To study the influence of nutritional supplementation on anaerobic digestion of agricultural wastes":**

1.1 Temperature studies indicated that the mesophilic range is the most stable condition despite taking longer times to degrade the organic substrate. Also, the thermophilic range was the most productive regarding biogas generation and an intermediate temperature, 42 °C, is worth-studying.

1.2 Extra nutrients in AD of agricultural wastes positively affected the process at every temperature herein studied (mean value of 1.59 times more mL-CH_4/g-VS and 1.37 times higher biogas productions).

2. Attending to the objective **"To optimize anaerobic digestion on agricultural wastes with high content in lignocellulose":**

2.1 AD on artichoke showed the best performances at thermophilic range with high microbial growth rates and organic matter eliminations as well as biogas generations. However, mesophilic range yielded adequate results that also entail lower operative costs.

2.2 TPAD on artichoke improved its treatment when compared to single-stage, especially a configuration with 7 days in the thermophilic range.

2.3 AD on asparagus showed the best performance at mesophilic range with the best productions, productivities, organic eliminations and biogas quality.

2.4 TPAD on asparagus yielded similar results at the two configurations studied and no significant improvements were observed except for an increase in CH_4:biogas production. This increased from a mean content of 68.1 % to an 81.8 %.

2.5 Co-digestion of artichoke and asparagus improved monodigestions and the most suitable configuration is 2 parts of artichoke and 1 part of asparagus at mesophilic range.

3. Attending to the objective **"To study the performance and microbial populations of anaerobic digestion of agricultural wastes":**

3.1 AD on malt is more efficacious at thermophilic range whereas on sloe the preferred temperature is the mesophilic.

3.2 Thermal pretreatment has a positive effect on the treatment of sloe, improving productivities in a 43.7 % and 59.1 % for mesophilic and thermophilic range, respectively.

3.3 Kinetic modelling indicates that this positive effect of thermal pretreatment is not remarkable. The reduction of the non-biodegradable substrate, if any, only reached 8-12 %.

3.4 Biochemical identification tests allow the identification of some bacterial species from genders such as *Clostridium, Actinomyces* and *Propinibacterium*, which roughly confirm the progress of the process, especially hydrolysis, acidogenesis and acetogenesis.

3.5 Biogas upgrading in steady-state reactors can provide good quality biogas with up to 99 % of CH_4.

3.6 Anaerobic digesters at steady-state can shift microbial population with time. *Methanothermobacter thermautotrophicus* showed to be the most relatively abundant archaea (> 1 % of the population) in early stages of steady-state but was outcompeted by *Methanothermobacter thermautotrophicus* IN51 after two years.

4. Attending to the objective **"To study the effect of pesticides on the anaerobic digestion of local agricultural wastes":**

4.1 Tefluthrin and mancozeb added individually on the co-digestion substrate boosts the process of AD on various operative conditions.

4.2 Manganese and zinc present in the commercial formulation could have an effect in microbial activity, especially attending to enzymatic performance.

4.3 Process scale-up yielded acceptable results for most of the configurations but inhibition and low pH increase the possibilities of process failure.

4.4 Pesticides did not show to negatively affect the process and the effect of each of them was dependent on the substrate. Sloe and malt were better digested when mancozeb was present, while artichoke and asparagus yielded better results in the presence of tefluthrin.

VI. Annexes

Annex I: Detailed experimental design and analytical methods

1. Reactor set-up

Continuous stirred-tank reactors (CSTRs) were used in every section of the project. Most chapters employed 1-L CSTRs (total volume), which were filled up with 200 mL of inoculum and 50 mL of nutrient solution. In order to maintain homogeneity inside the reactors, substrate loading was in the wet range (4-10 % of total solids -TS-, aiming at 6 %). Water completed the working volume of 600 mL in each digester. Scale-up studies were performed in 5-L reactors (3 L of working volume). These were utilized in the same fashion as 1-L reactors (proportion of each component: inoculum, nutrient solution, TS, etc.). However, these were stirred mechanically instead of magnetically and located in a thermostatic chamber instead of a thermostatic bath. Reactors used at the Technical University of Denmark are described in Chapter 6.

The inoculum used for these experiments was sludge obtained from a local wastewater treatment plant that did not contribute with any organic matter susceptible of biomethanization. The adaptation was carried out according to Holliger et al. (2016) with previous filtering to remove solids and flocs. This consisted in gradual exposure of the inoculum to the desired temperature of each experiment, namely 35, 42 and 55 °C and kept incubated awaiting for its use. Also, glucose was added promptly to feed the inoculum.

The mineral solution was used to favor the process of biomethanization and its composition was based on that from Sevillano (2011). Modifications to it were carried out attending to previous experimental experiences. The mineral solution from Sevillano et al. (2011) showed to be suitable for biological systems but further information from the literature advised the addition of elements in a specific chemical form (e.g. $CuCl_2 \cdot 2\ H_2O$ instead of $Cu(CH_3COO)_2$), and some others that have shown to be key for anaerobic populations such as Mg and Ni. The final solution contained macronutrients (g/L: Na_2HPO_4, 2.44; KH_2PO_4, 1.52; $(NH_4)_2SO_4$, 1; $MgSO_4 \cdot 7\ H_2O$, 0.2; $CaCl_2 \cdot 2\ H_2O$, 0.05) and trace elements (mg/L: EDTA, 5; $FeSO_4 \cdot 7\ H_2O$, 2; $ZnSO_4 \cdot 7\ H_2O$, 0.1; H_3BO_3, 0.3; $CoCl_2 \cdot 6\ H_2O$, 0.2. µg/L $MnCl_2 \cdot 4\ H_2O$, 30; $CuCl_2 \cdot 2\ H_2O$, 10; $NiCl_2 \cdot 6\ H_2O$, 20; $Na_2MoO_4 \cdot 2\ H_2O$, 30).

2. Analytical methods

For pH, TS and VS (volatile solids), the reactor samples were directly characterized, while for alkalinity and chemical oxygen demand (COD) they were previously treated. For soluble COD, samples were centrifuged and the supernatant was filtered with 0.45 µm filters (Starstedt) (APHA, 2012). Characterizations of substrates and inoculum (alkalinity, pH, TS, VS, total and soluble COD, and TOC) were performed in triplicates.

- **Alkalinity and pH**

Alkalinity and pH were assessed with a pH-meter (Crisson GLP 22+) that was daily calibrated. In order to adjust pH to the optimal range (6.5-8.2), a solution of NaOH was utilized after daily control of the reactors when required.

- **CH_4 content**

In order to analyze CH_4 content NaOH gasometer system was used (Figure 1). The biogas sample was bubbled in an alkaline solution using $C_{27}H_{30}O_5S$ as indicator. This gasometer was connected to another one filled with water. The water volume displaced was used to determine CH_4 content.

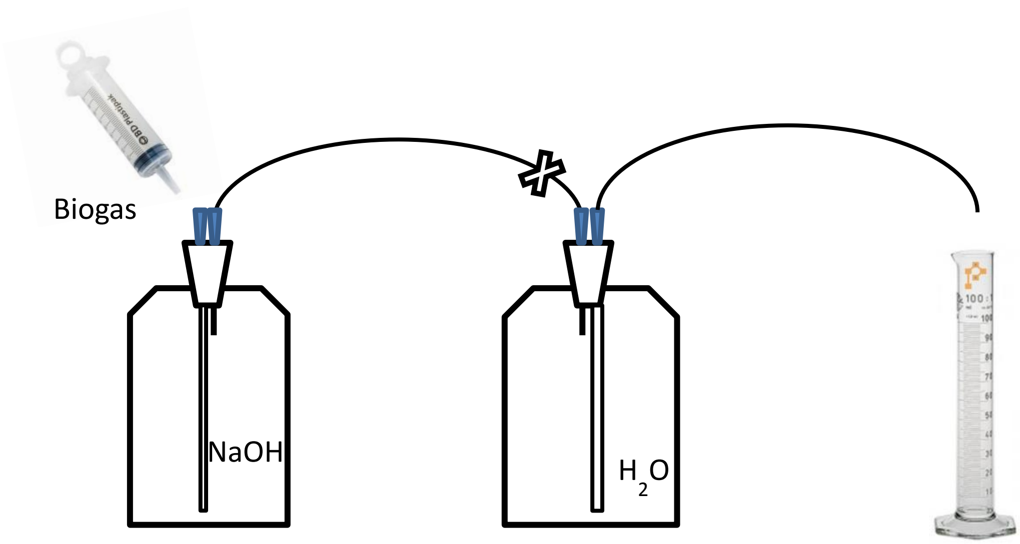

Figure 1. Alkaline gasometer sketch for biogas composition determination.

- **Totals solids (TS)**

TS determination is based on the evaporation of the water in the sample and the process is as follows. A crecible is weighted (W1) and then again with sample inside (W2) using an analytical balance (Mettler Toledo AB204. Precision of ± 0.01 g). A minimum of 5 g is advised and, therefore, the assay is only carried out once on reactor samples, as to not significantly alter the volume within it. However, it should be noticed that all analytical methods on the reactor are intimately related and this allows establishing a tendency with individual data without much error. The sample is to be dried at 105 °C for 24 hours (Memmert UE-200) or until there is total stabilization of weight (W3).

Total solids (%) = (W3-W1) / (W2-W1)*100

- **Volatile solids (VS)**

For VS determination, the residue from TS quantification is to be used. This is calcined at 550 °C (Hobersal HK-11) for 2 hours and weighted (W4).

Every weight determination for TS and VS is to be done once the sample is at room temperature but not long afterwards, as the weight of the sample can be altered.

Volatile solids (%) = (W3-W4) / (W2-W1) *100

- **Chemical oxygen demand (COD)**

Total and soluble COD was performed with a solution of $K_2Cr_2O_7$ of analytical grade (Scharlau), H_3PO_4 (Sigma Aldrich), H_2SO_4 (Panreac), Ag_2SO_4 (Panreac). $HgSO_4$ (Panreac) was also used to avoid halogen inhibition. Samples were incubated 2 hours at 150 °C in a COD digester (VELP Scientifica ECO16 Thermoreactor) and analyzed twice in a spectrophotometer (Agilent Technologies 8453) at 622 nm.

Each digestion tube included 3 mL of the digestion solution ($K_2Cr_2O_7$ solution), 2 mL of the sample (dilution of it as to obtain absorbances within the lineal range of the equipment, 0.1-1.1 a.u.) and a spatula tip of $HgSO_4$.

- **Total organic carbon (TOC)**

TOC determination is based on the thermal oxidation (680 °C) of a sample to CO_2. This is carried out by a TOC-L Shimadzu (Figure 2). This consists of an oven that is used to treat the samples and a NDIR detector (non-dispersive infrared sensor) to which the oxidized sample is taken through a carrier gas (zero air in this case) with a pressure of 200 kPa.

TOC-L Shimadzu uses a differential method to determine TOC, as inorganic carbon (IC) and total carbon (TC) are the one that the equipment is able to quantify. Each sample was determined using triplicates.

$$\mathbf{TOC = TC - IC}$$

Figure 2. TOC equipment (TOC-L Shimadzu).

- **Volatile fatty acids (VFA)**

VFA are analyzed through gas chromatography (Shimadzu GC-2010) (Figure 3). Samples require being first centrifuged (13000 rpm for 10 min) and then filtered through 0.20 µm filters (Starstead). Samples can be stored for later analysis at -20 °C with H_3PO_4. Sample preparation consists in the dilution with 1:10 H_3PO_4 and 6000 ppm C_6H_6O (internal standard) in a proportion of 5:4:1. The determination of up to eight VFA (namely CH_3COOH, $C_3H_6O_2$, $C_4H_8O_2$ $C_5H_{10}O_2$, $C_6H_{12}O_2$ and $C_7H_{14}O_2$) was achieved using 500 µL of each sample.

The optimized method and parameters for their detection are as follows:

- Capillar chromatography with Supelcowax 10 (Supelco) column and an 8.3 mL/min flux at 150 °C.
- Flame ionization detector (FID).
- Carrier gas is obtained from an ultrapure hydrogen generator (20H, Domnick Hunter). Its flux is set at 47.3 mL/min.
- Both detector and injector are at 200 °C.
- Pressure is set at 28 kPa.
- The utilized temperature ramp is 200 °C, 115 °C, and 200 °C.

Figure 3. Gas chromatograph (Shimadzu GC-2010).

- **Bacterial identification (metabolic tests)**

Bacterial characterization was assayed with a kit, API20A (Biomerieux), which is based on 20 metabolic tests that allow bacterial identification after isolation of each of the colonies. Samples were treated in a sterile environment and glycerol (10 %) was added to ensure a freezing process that did not alter the microorganisms. Each sample and their dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}) were plated (100 µL) on Schaedler (Oxoid) gelled media. After bacterial growth in an anaerobic atmosphere, colonies were picked up attending to their morphological differences and isolated on new Schaedler plates. After growth, API20A galleries were used according to the instructions of the manufacturer to identify each of the isolated colonies with the use of a specific software (Biomerieux APIweb^{TM}) that establishes a relationship between the qualitative results and a database.

- **Bacterial characterization (molecular analysis)**

In accordance with Chapter 6.

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Annex II: Summary Tables

Cells in grey represent overall best condition for each experiment.

Table 1. Main results obtained in chapter 1 (section I)

Configuration	AcoD					
	Without nutrients	With nutrients	Without nutrients	With nutrients	Without nutrients	With nutrients
Temperature	35 °C		42 °C		55 °C	
Volatile solids removal (%)	61.7	48.3	34.7	54.5	68.6	63.3
Cumulate biogas (L)	4.2	6.2	7.4	10.1	8.1	10.3
Cumulate CH_4 (L)	2.9	4.4	2.9	3.9	2.6	2.7
Biogas/VS (mL/g)	191.6	359.3	362.6	670.4	373.5	550.1
CH_4/VS (mL/g)	134.1	258.6	141.5	258.5	121.0	147.0

Table 2. Main results obtained in chapters 2-4 (section II)

Configuration	Monodigestion										AcoD					
Substrate	Artichoke					Asparagus					1Art/2Asp			2Art/1Asp		
Temperature	35 °C	42 °C	55 °C	TPAD7	TPAD5	35 °C	42 °C	55 °C	TPAD7	TPAD5	35 °C	42 °C	55 °C	35 °C	42 °C	55 °C
Volatile solids removal (%)	56.53	54.20	50.36	53.69	53.38	62.06	64.47	63.81	63.66	56.58	56.92	47.13	47.65	44.99	47.16	51.39
Cumulate biogas (L)	6.1	4.8	6.0	7.8	4.7	8.1	5.5	5.8	5.0	4.9	6.7	6.6	9.4	7.0	6.7	6.3
Cumulate CH_4 (L)	5.2	2.2	3.9	6.6	3.9	6.0	2.7	3.6	4.2	3.9	3.9	1.2	4.3	5.1	1.7	3.1
Biogas/VS (mL/g)	336.0	284.4	360.1	441.6	295.6	548.2	301.9	323.3	294.9	314.7	378.0	520.8	772.4	552.9	519.4	438.2
CH_4/VS (mL/g)	284.3	129.1	236.2	375.3	248.9	408.0	149.4	199.4	247.6	252.7	221.0	102.5	351.6	404.1	127.5	211.3

Table 3. Main results obtained in chapter 5 (section III)

Configuration	Monodigestion							
Substrate	Sloe		Pretreated Sloe		Malt		Pretreated Malt	
Temperature	35 °C	55 °C	35 °C	55 °C	35 °C	55 °C	35 °C	55 °C
Volatile solids removal (%)	70.7	72.3	66.2	53.1	72.5	66.6	56.8	72.6
Biogas/VS (mL/g)	222.0	88.0	319	140	95.0	113.0	103.0	64.0

Table 4. Main results obtained in chapter 6 (section III)

Substrate	Manure		
Sampling period	Before H_2 injection	2014 post H_2	2016 post H_2
Temperature	55 °C	55 °C	55 °C
Biogas production rate (mL/L·reactor-day)	387 ± 33	428 ± 9	355 ± 9
CH_4 production rate (mL/L·reactor-day)	259 ± 22	372 ± 12	344 ± 1
CH_4 (%)	66.9 ± 0.8	86.5 ± 2.8	98.7 ± 0.3

Table 5. Main results obtained in chapter 7 (section IV)

Configuration	AcoD														
	No pesticide			Mancozeb			Tefluthrin			Mancozeb+Tefluthrin			½ Mancozeb+ ½ Tefluthrin		
Temperature	35 °C	42 °C	55 °C	35 °C	42 °C	55 °C	35 °C	42 °C	55 °C	35 °C	42 °C	55 °C	35 °C	42 °C	55 °C
Volatile solids removal (%)	64.99	18.88	49.17	42.60	25.12	71.95	37.20	31.44	67.33	33.25	54.17	68.05	50.26	36.13	45.30
Cumulate biogas (L)	6.7	6.6	5.0	3.1	7.0	4.7	6.0	4.4	8.0	2.3	0.6	3.8	4.9	1.5	7.1
Cumulate CH_4 (L)	5.2	2.8	3.3	2.2	2.7	2.9	5.0	2.5	5.2	1.1	0.1	0.9	2.2	8.5	3.3
Biogas/VS (mL/g)	289.1	1043.2	277.6	327.3	1475.3	213.6	716.9	720.0	347.2	303.9	42.6	24.4	427.2	185.7	568.0
CH_4/VS (mL/g)	223.0	436.7	181.9	236.3	575.1	133.6	581.8	415.6	227.1	142.5	11.2	34.8	191.8	102.4	261.9

Table 6. Main results obtained in chapter 8 (section IV)

Temperature	35 °C							
Substrate	Sloe		Malt		Artichoke		Asparagus	
Pesticide	Mancozeb	Tefluthrin	Mancozeb	Tefluthrin	Mancozeb	Tefluthrin	Mancozeb	Tefluthrin
Volatile solids removal (%)	56.17	60.65	77.45	48.88	47.56	54.38	60.65	56.47
Cumulate biogas (L)	14.6	0.7	15.3	5.4	36.9	26.0	6.7	33.4
Cumulate CH_4 (L)	9.5	0.4	10.0	4.1	20.2	15.8	3.3	21.5
Biogas/VS (mL/g)	167.5	7.0	134.5	75.7	281.3	365.4	95.9	513.0
CH_4/VS (mL/g)	109.4	4.2	88.2	57.4	153.9	222.1	46.5	330.1

Annex III: Acronym List

AC: assymetry coefficient

AcoD: anaerobic co-digestion

AD: anaerobic digestion

ADP: adenosine diphosphate

APHA: American Public Health Association

ATP: adenosine triphosphate

BOD: biochemical oxygen demand

BOE: Boletín Oficial del Estado

COD: chemical oxygen demand

CSTR: continuous stirred-tank reactor

EBA: European Biogas Association

EGSB: expanded granular sludge blanket

FAO: Food and Agriculture Organization

GHGE: greenhouse gas emissions

HRT: hydraulic retention time

IC: inorganic carbon

ILI: International Lignin Institute

OLR: organic loading rate

PF: plug flow

PNIR: Plan Nacional de Investigación de Residuos

PR: primary reactor

SAO: syntrophic acetate oxidizing

SGBR: static granular sludge reactor

SPAD: single-phase anaerobic digestion

SR: secondary reactor

SRT: solids retention time

TC: total carbon

TOC: total organic carbon

TPAD: temperature-phased anaerobic digestion

TS: total solids

UASB: upflow anaerobic sludge blanket

USEPA: United States environmental protection agency

VFA: volatile fatty acids

VS: volatile solids

W-L: Wood-Ljungdahl

Ph.D. Thesis Errata

Page 83, Chapter 2:

VS removal only improved when compared with thermophilic digestion, as TPAD managed to eliminate slightly more than 53.00 % for both cases while T55 condition only reached 50.36 %.

Instead of:

VS removal only improved when compared with thermophilic digestion, as TPAD7 managed to eliminate 53.6 % while 35 °C condition only reached 50.99 %.

Page 209, Chapter 7, Table 9:

CH_4 for I42-MTa should be 0.8 L instead of 8.5 L

Page 262, Conclusions:

3.6 Anaerobic digesters at steady-state can shift microbial population with time. *Candidatus* M. thermohydrogenotrophicum showed to be the most relatively abundant archaea (> 1 % of the population) in early stages of steady-state but was outcompeted by *Methanothermobacter thermautotrophicus* after two years.

Instead of:

3.6 Anaerobic digesters at steady-state can shift microbial population with time. *Methanothermobacter thermautotrophicus* showed to be the most relatively abundant archaea (> 1 % of the population) in early stages of steady-state but was outcompeted by *Methanothermobacter thermautotrophicus* IN51 after two years.