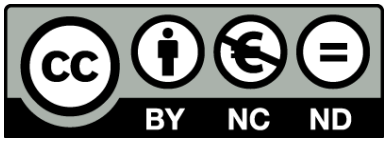
UNIVERSITAT DE BARCELONA

Strategies to improve anaerobic digestion of wastes with especial attention to lignocellulosic substrates

Xavier Fonoll Almansa



Universitat de Barcelona

Programa de doctorat d'*Enginyeria i Tecnologies Avançades*

STRATEGIES TO IMPROVE ANAEROBIC DIGESTION OF WASTES WITH ESPECIAL ATTENTION TO LIGNOCELLULOSIC SUBSTRATES

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El Dr. **JOAN MATA ÁLVAREZ**, catedràtic del Departament d'Enginyeria Química de la Universitat de Barcelona i el Dr. **JOAN DOSTA PARRAS**, professor agregat interí del mateix departament,

CERTIFIQUEN QUE:

El treball d'investigació titulat "**STRATEGIES TO IMPROVE ANAEROBIC DIGESTION OF WASTES WITH ESPECIAL ATTENTION TO LIGNOCELLULOSIC**" constitueix la memòria que presenta l'Enginyer Químic **Xavier Fonoll Almansa** per a aspirar al grau de Doctor per la Universitat de Barcelona. Aquesta tesi doctoral ha estat realitzada dins del programa de Doctorat "*Enginyeria i Tecnologies Avançades"*, en el Departament d'Enginyeria Química de la Universitat de Barcelona.

I perquè així consti als efectes oportuns, signen el present certificat a Barcelona, Setembre de 2015.

Dr. Joan Mata Álvarez i Dr. Joan Dosta Parras

Directors de la tesi doctoral

Mucha gente pequeña, en lugares pequeños, haciendo cosas pequeñas puede cambiar el mundo

Eduardo Galeano

Agradecimientos

Quiero poner en primer lugar el que quizá seas el capítulo más importante de la tesis puesto que esta tesis no se hubiera podido realizar sin la ayuda de las personas que voy a citar.

Esta tesis nunca se hubiera podido realizar de no ser porque los directores Joan Mata y Joan Dosta me dieron la oportunidad de trabajar junto con su equipo de investigación. Muchas gracias por todos los conocimientos transmitidos durante mi época de investigación.
Aun así, la tesis debería tener como tercer director a Sergi Astals, quien, desde que empecé, estuvo a mi lado como consejero y que también me transfirió, la mayoría de los conocimientos que tengo sobre el tema. Eres un crack!

Volia també agrair a la Fundació Crèdit Andorrà per la beca atorgada en 2012 per a poder realitzar la meva tesi doctoral. En especial voldria donar les gràcies a la Joëlle Bazile, per haver-me ates sempre tan amablement. Mai oblidaré el dia que em vas trucar per comunicar-me que havia estat un dels seleccionats per la beca.

Trabajar en el grupo de Biotecnología Ambiental de la UB también me ha llevado a conocer a no solo compañeros de trabajo que me han ayudado en diferentes aspectos de mi investigación, si no a amigos los cuales podrán contar conmigo en un futuro. Gracias a todos vosotros por hacer más ameno el trabajo en un laboratorio cuyo olor ya dejó de sentirse con el tiempo: Sergi (Maestro Jedi), Maycoll (MacGyver), Irene (Cinturón negro de Karate, ojo!), Miriam, Núria (Mongui), Silvia (Corki), Carolina (Que te parece!), Albert (Man), Ruth, Jordi, Roger, Hilda, Paula, Eric, Guillermo, Adriana, Marc y más gente que seguramente este olvidando. Además del laboratorio, la sala, también tiene que dejar una huella profunda en los agradecimientos por la cantidad de horas trabajadas y los momentos de ocio (Comidas, cafés, presentaciones "locas" de tesis…) que tan necesarios fueron en esos momentos cuando sientes que tu cabeza va estallar con tanto metano acumulado en el cerebro. La sala no hubiera sido el lugar que es, sin vuestra presencia: Anto , Mireia, Angel, Núria, Sergi, Miriam, Renato, Oscar, Ana Justo, Anna May, Violette, Silvia, Xavi, Bruno, Raül (¿?)…
No obstante, otros muchos compañeros no se encontraban ni en el laboratorio ni en la sala y merecen también aparecer en esta sección como María Ángeles (Gracias por los papeles sobre todo!), Rodrigo (Que viva Jaén!), Roger y Nardi (Havieu d'anar junts si o si), Jordi Hug, Ricard, Bryshila, Esther, Blaia y todos los profesores del departamento de Ingeniería Química. Estoy muy contento de haber pasado 9 años y medio en la UB para la Licenciatura, Master y Doctorado.

Part of this thesis was also done at the University of Michigan. I want to thank Professor Lutgarde Raskin "Lut" for giving me the opportunity to work with her and her research group. You have really enlarged my passion for research and you have always shown how much you care about the research and about your students. I'm glad to meet such an amazing professor and person. Thank you also to the members of the research group that have received me with a huge kindness: Pedro, Shilva, Caroline, Nadine, Anton, Heather, Andrea, Chia Chen, Fei, Raghav, Tara, Becky, Ben, Nancy, Krista, Lauren, Jeseth, Kelly, Nigel, Sarah, Sean, Jimmy, Tom, Rick, Samayyah, Yinyin, Christian…
It would not be fear to no mention the members from the ICC Coop were I have lived in Ann Arbor: Mercedes, Nandu, Nishan, Vijeta, Aysh, Douglas, Djurdja, Ho-zhen, Aaron,….With you guys, I could not feel lonely even though I was 12 hours away from my friends and family.

Gracias también a todos esos locos que me habéis acompañado tanto en los momentos buenos como malos que a veces da la investigación. Por vosotros sí merece la pena trabajar para hacer un mundo más sostenible: Mis amigos de Andorra (Marc, Pichi, Toni, Esther, José, Carla, Nunu, Sandra, Laura, Lara, Alexia), los Aribau 127 (Iñaki, Flori, Hugo, Marcos, Marilyne y Marina), los Alcoholic Dreamers (Sergio, mi primo, Marian, Elena, Silvia e Iván) Javi Caballero, Laura Evangelio (Contigo empezó esto!), Mireia,…No dejéis de perseguir vuestros sueños y nunca dejéis de hacer lo que os gusta, que estamos en un país libre. Pero eso sí, que no os vean!
Otro gran apoyo ha sido sin duda mi familia. Gracias a mis padres por todos los buenos valores que me han enseñado, en especial, el trabajo y el respeto. Os quiero mucho y os echo mucho de menos.

Por ultimo quería no solo darle las gracias, si no también pedirle perdón a una persona en especial. Perdón por haber sido yo el ladrón de nuestro tiempo poniendo en repetidas ocasiones los experimentos por delante a la relación. Pero gracias por haber aguantado esto y los kilómetros de mar y tierra que en su momento nos bloquearon. Gracias por haberme apoyado como la que más en los malos momentos, por empujar junto a mí el pesado carro de mis sueños y sobre todo, por quererme. Te quiero mucho Isabel.

Muchas gracias a todos!

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Abstract

The energy demand increase and the generation of wastes is being the major problem regarding the next generation sustainability. Both problems can be corrected through the implementation of anaerobic digestion, a waste treatment technology able to produce electricity, heat and a fertilizer. The anaerobic co-digestion between two wastes with complementary characteristics has been widely studied to improve the methane production in anaerobic digesters. However, to increase the methane production from lignocelulosics substrates is still one of the main challenges of anaerobic digestion. Lignocelulosic components are a tridimensional structure between lignin, hemicellulose and cellulose, which bonds are extremely difficult to degrade by conventional anaerobic bacteria. Besides, those components can be found in a wide range of substrates such as municipal solid wastes, agro-wastes and energy crops.

In the following thesis, the increase of the economic viability of anaerobic digestion plants treating lignocelulosic materials has been studied.

Initially, the transitory state while the co-substrate was changed in the anaerobic co-digestion between sewage sludge and fruit waste was studied. The stability of the reactors was not drastically affected when the co-substrate was changed, but, the use of a co-substrate with a high concentration of fibers did not improve the methane production too much. Secondly, in order to consider the valorization of lignocellulosic components through the production of by-products, the effect of these components on the municipal solid wastes anaerobic digestion performance was evaluated. When the paper waste was removed, the biodegradability of the feedstock increased allowing the specific methane production to increase. Nevertheless, the digester was more fragile against instabilities and the digestate quality decreased if short retention times are applied. Next, low-temperature and ultrasounds pretreatments, strategies that have not been used too much for the degradation of lignocellulosic components, were studied to increase the methane production during the anaerobic co-digestion of barley waste and pig manure. Low-temperature and ultrasound pretreatment increased the methane production in a 27 and 12% respectively but only the first one had a positive energy balance. Finally, rumen, a waste from the slaughterhouse industry was used as inoculum and as co-substrate to bring hydrolytic bacteria able to improve the degradation of Napier grass. The results showed that, when rumen is used as inoculum it need to be mixed with an inoculum with high buffer capacity and a co-substrate with alkalinity

need to be used to avoid long start-up periods. The methane production only increased at the beginning and in a long-term, the microbial community was governed by the substrate and not by the rumen. However, rumen did not increase the methane production when it was used as a co-substrate because the digester conditions were not optimal for the activity of hydrolytic bacteria.

All the experiments were carried out in the laboratory and the conclusions are considered a progress for the energy production through the use of lignocellulosic substrates.

1. Introduction

- Mata-Alvarez, J., Dosta, J., Romero-Güiza, M.S., Fonoll, X., Peces, M., Astals, S., 2014. **A critical review on anaerobic co-digestion achievements between 2010 and 2013.** Renew. Sustain. Energy Rev. 36, 412–427.
- Shrestha, S., Fonoll, X., Raskin, L., Khanal, S.K. **Bioengineering strategies for enhanced hydrolysis of lignocellulosic biomass during anaerobic digestion.** In preparation

1.1 Energy demand and waste generation related problems

1.1.1 World population and energy consumption growth

World population grew rapidly by 14% from 2000 to 2011, surpassing 7 billion, and by the year 2050 the population is projected to reach over nine billion (Bedoussac et al., 2015). The percentage of populations living in urban areas is estimated to increase from 50% to 70% in 2050 and hence more households will be built (Ramaswami et al., 2012). Since this infrastructure is the first energy consumer (Fig. 1.1) in cities the energy demand is expecting to grow from 13.6 billion tons of oil equivalent (toe) to 44.6 billion toe (Bilgen, 2014).

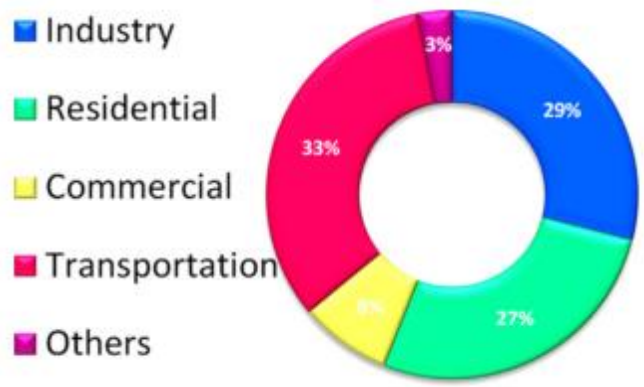


Figure 1.1 Sectorial shares of global energy consumption in cities (Nejat et al., 2015)

Energy is essential for the economic and social development of the new incoming generations in all countries but it also will be a grand environmental challenge (F. Li et al., 2014). Climate change, acid precipitation, stratospheric ozone depletion…are some of the environmental impact that comes from fossil energy which is the most common way to satisfy the actual energy demand (Fig. 1.2).

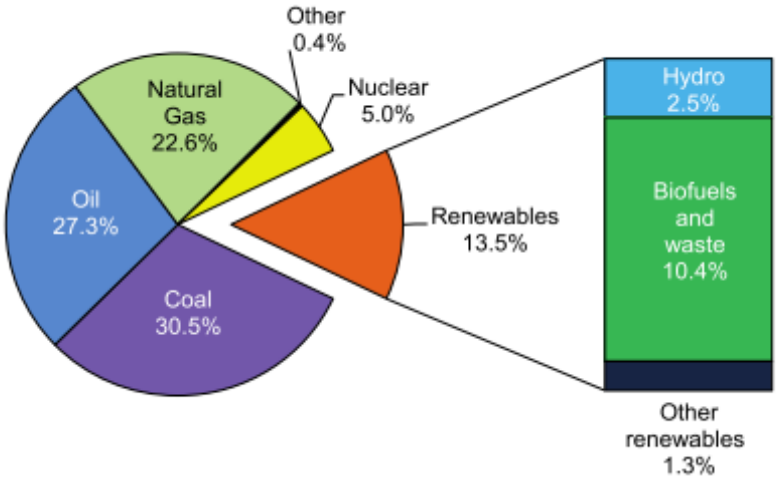


Figure 1.2 2013 fuel shares in world total primary energy supply (IEA, 2015)

Reducing GHG emissions from energy consumption requires stronger policy initiatives that are currently being discussed by policy makers. The countries successful at reducing their GHG emissions have employed restrictive and efficient policies, promoted the installation of renewable energy generation, shifted their energy mixes from high-emission fuels (coal and oil) to cleaner natural gas and electricity and imposed or incentivized higher energy standards for appliances. However, the a high contribution in GHG emissions is coming from the developing countries, such as China, India and Iran where there is a lack in efficient policy (Azhar Khan et al., 2014). China, Iran and India are among the 10 leading emitters with an increase of the CO2 emissions in the last twenty years around 25%, 245%, and 84% (Nejat et al., 2015).

1.1.2 Global waste generation

Economic growth is also bringing the problem of waste generation. Almost 1.3 billion of tones of municipal solid waste (MSW) were generated in 2010 by 161 of the world's countries where almost 50% of these wastes generated were organic (Fig 1.3). By 2025 the amount of wastes is expected to increase and it is predicted that the annual generation will be almost 2.2 billions of tones in 2025 (Table 1.1) (Hoornweg and Bhada-Tata, 2012; Yang et al., 2015).

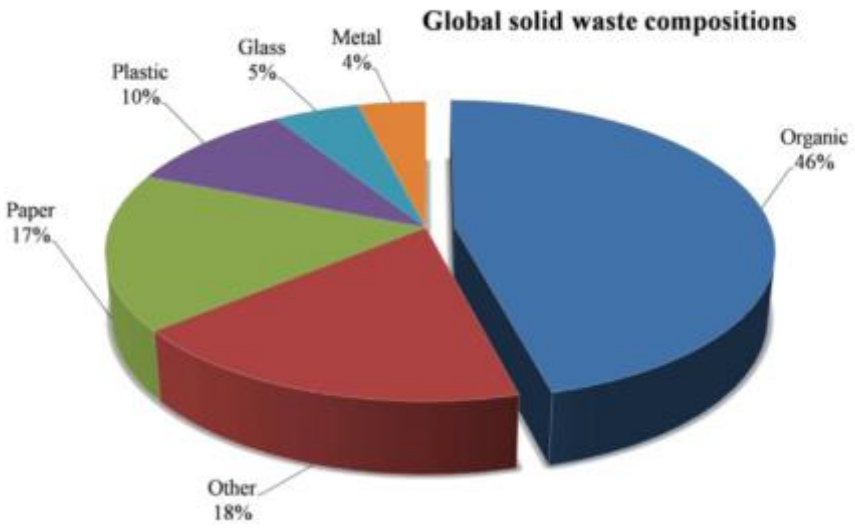


Figure 1.3 Global solid waste compositions (Yang et al., 2015).

Table 1.1 Waste generation in the world and its projection for 2025 (Ross and Rogoff, 2012)

Region	Current available data			Projections for 2025			
	Total urban population (millions)	Urban waste generation		Projected populations		Projected urban wastes	
		Per capita (kg/capita/day)	Total (Tons/ day)	Total populations (millions)	Urban population (Million)	Per capita (kg/capita/day)	Total (Tons/day)
AFR	260	0.65	169,119	1152	518	0.85	441,840
East Asia	777	0.95	738,958	2124	1229	1.5	1,865,379
Eastern & Central Asia	227	1.1	254,389	339	239	1.5	354,810
Latin America	399	1.1	437,545	681	466	1.6	728,392
Middle East North Africa	162	1.1	173,545	379	257	1.4	369,320
OECD	729	2.2	1,566,286	1031	842	2.1	1,742,417
South Asia	426	0.45	192,410	1938	734	0.7	567,545
Total	2980	1.2	3,532,252	7644	4285	1.4	6,069,703

Landfilling, which is the most common management in the US and in developing countries, can favor various ecological problems such as soil, surface and groundwater pollution from the leachate as well as uncontrolled methane emissions; a potent GHG. A bad control of landfills, which is frequent in developing countries, can even generate vectors for infectious diseases.

World population growth has also affect the agriculture sector which is more and more intensive. Agricultural residues (forestry residues, wastes from crops such as rice husks, cotton stalks or maize straw, manure from livestock, fruit wastes from the industry, pesticides…) generated primarily in rural areas, are amounting to 140 billion tones globally (UNEP, 2011)**.** These wastes also contribute to GHG emissions (CO2 and CH4) and contain high concentration of human pathogens, nutrients, heavy metals, veterinary pharmaceuticals and natural excreted hormones (Manyi-Loh et al., 2013). Different countries are facing the waste generation problem by the incentive of different management technologies and the implementation policies. For example, the EU has passed different laws focusing in on the waste management. One of them, the 2008/98/EC directive presents the waste hierarchy (prevention, reuse, recycling, other forms of recovery, and disposal of waste in landfills) which must be encouraged by member states to ensure the best environmental outcome.

1.1.3 Wastes as a resource. Renewables biotechnologies as one of the solutions

More than the 80% of the world energy demand is supplied with fossil resources which are limited. At the current consumption rates, the supply of petroleum, natural gas, and coal will only be able to last for another 45, 60, and 120 years, respectively (Guo et al., 2015). The nuclear energy source is also being considered as one of the alternate but because of its hazardous issues, relatively higher expenses and technological monopolies, it is not approachable for most of the countries of the world (Nayyar et al., 2014). Nevertheless, renewable energy will account for 80% of new generation in OECD (Fig. 1.4) countries and the European directives mention that wastes should not be seen as a burden anymore and be recovered to conserve natural resources (Eurostat, 2015). In fact, the 1999/31/EC directive, which has to prevent or reduce negative effects on the environment such as GHG emissions or groundwater pollution, is limiting the amount of organic wastes that can be dumped in landfill.

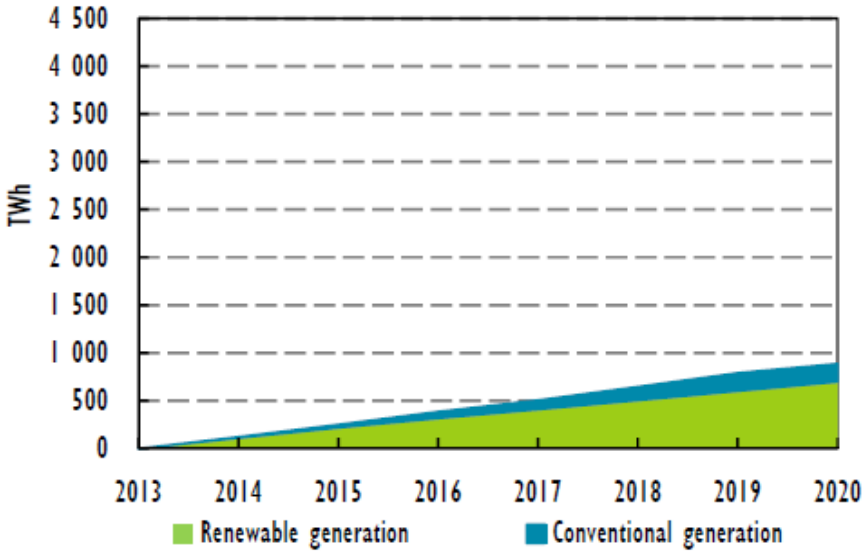


Figure 1.4 New energy production in OECD countries (Eurostat, 2015)

Due to the world energy demand, the lack of resources, the energy recovery from organic wastes through biotechnology processes could be one of the options to reach the sustainability for next generations. The ability of treating different kinds of wastes makes anaerobic digestion (AD) one of the best biotechnology candidates to produce energy from all the organic wastes generated worldwide (Mata-Alvarez et al., 2000).

1.2 Anaerobic digestion

AD has been worldwide implemented to treat different organic wastes streams (sewage sludge (SS), organic fraction of municipal solid wastes (OFMSW) and agricultural wastes) since it avoids volatile organic compound emissions, stabilizes organic matter, produces an effluent with good fertilizing qualities and, overall, recovers energy through biogas: a mixture of CH_4 and CO_2. With a heating value ranging from 21300 to 23400 kJ m^{-3} (as function of the percentage of CH_4), biogas is mostly used to produce electricity and heat through a cogeneration unit (Speece, 2008).

1.2.1 AD metabolic steps

The conversion of organic matter into biogas is carried out by a consortium of microorganisms through a series of metabolic stages: Hydrolysis, Acidogenesis, Acetogenesis and Methanogenesis) (Figure 1.5).

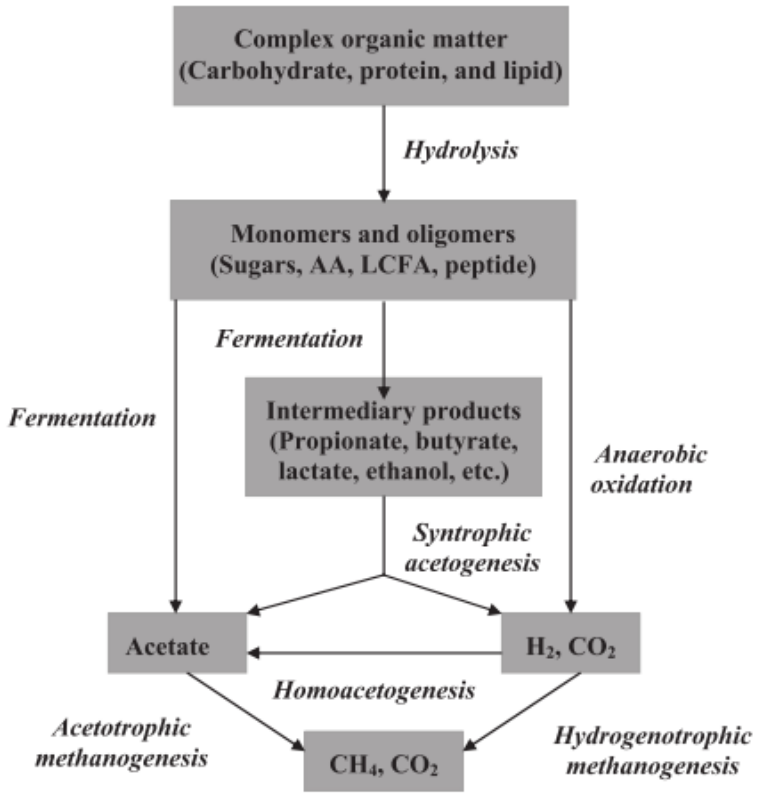


Figure 1.5 Scheme of the anaerobic degradation pathway (Surendra et al., 2014)

1.2.1.1 Hydrolysis

Hydrolysis step includes non-biological and extra-cellular biological processes mediating the breakdown and the solubilization of complex organic matter to soluble compounds (Batstone et al., 2002). In this step, the organic matter clusters are disintegrated into macromolecules (i.e. carbohydrates, proteins and lipids) and then, those macromolecules are hydrolyzed to soluble compounds. Specifically, the extracellular enzymes (cellulases, proteases and lipases) excreted by the fermentative bacteria solubilize carbohydrates, proteins and lipids to mono- and disaccharides (sugars), alcohols, amino acids and long chain fatty acids (LCFA) among others. Specifically, it is well established that the conversion of lignocellulosic materials (lignin, hemicellulose and cellulose) into CH_4 is limited by hydrolysis, the first step of the AD (Noike et al., 1985). The solubilization rate is affected by several parameters such as particle size, pH, temperature, biomass concentration or the intrinsic substrate characteristics (Veeken and Hamelers, 1999).

1.2.1.2 Acidogenesis

Acidogenesis, also known as fermentation, is carried out by a large group of facultative fermentative bacteria. In this stage, the fastest of the AD process, the soluble compounds obtained from the disintegration and hydrolysis step are able to be transported inside the bacteria and then converted to volatile fatty acids (i.e. acetate, propionate, butyrate, valerate), lactic acid, ethanol, pyruvate, ammonia, hydrogen sulphide, hydrogen and carbon dioxide. It should be noted that the acidogenesis of sugars and amino acids is carried out without an electron acceptor or donor, whereas LCFA are oxidized using hydrogen ions as electron acceptors (Batstone et al., 2002).

The main product of all acidogenesis reactions is acetate; however, the accumulation of hydrogen and/or acetate in the digester medium can promote the formation and accumulation of more reduced compounds such as propionate and butyrate.

1.2.1.3 Acetogenesis

Acetogenesis results in the conversion of organic acids into acetate and other simple products such as hydrogen and carbon dioxide, and it is characteristic of syntrophic relationships. For example, the degradation of saturated fatty acids and propionate occurs due to the syntrophic relationship between proton-reducing acetogens and methanogens. It is well known that acetogenesis reactions are only thermodynamically possible when the hydrogen concentration in the digester medium is low. Consequently, acetogens rely on the consumption of hydrogen, formate, and acetate by methanogens (Batstone et al., 2002).

1.2.1.4 Methanogenesis

The last stage of the AD process is carried out by methanogenic archaea, which convert the end products of the previous reactions into biogas. The majority of the methane (~70%) is generated by the aceticlastic methanogens, which split the two carbons of the acetate; one is reduced to methane and the other is oxidized to carbon dioxide ($CH3COOH \rightarrow CH4 + CO2$). Two different genera of aceticlastic methanogens, mutually exclusive, dominate as function of the ammonia and VFA concentration in the digester medium. Methanosaeta, characterized by its filaments, dominate when the volatile fatty acid and the ammonia concentration are low whereas Methanosarcina, characterized by its clumps, dominate when the volatile fatty acids and the ammonia

concentration are high (Karakashev et al., 2006). Minor methane production (~30%) is produced by hydrogenotrophic bacteria, which used hydrogen as electron donor and carbon dioxide as electron acceptor to produce methane ($4\,H2 + CO2 \rightarrow CH4 + 2\,H2O$). Finally, even been negligible, methyl groups can also be converted to methane ($CH3OH + H2 \rightarrow CH4 + H2O$).

1.2.2 AD in the world

Thousands of years ago in Assyrian bathhouses biogas used to be produced from organic matter degradation for heating water in Assyrian bathhouses and the first-recorded AD plant was constructed in 1859 in Bombay. A.M. Buswell started to study AD as a science in the 1930s to select best anaerobic bacteria and digestion conditions for promoting methane production (Bond and Templeton, 2011; Guo et al., 2015). It is estimated that worldwide 47–95 TW h of electricity were generated from biogas in 2012. Europe is the leader regarding the implementation of AD for energy production. In 2013 there were over 14,000 operational AD plants producing around 0.15 TWh of biogas which was converted in 23TWh (EurObserv'ER, 2014). The U.S. started to install manure-based digester systems on livestock farms to produce biogas in late 1970s, with financial incentives from the federal government. Biogas from the farm digesters provided sufficient heat to the farms and generated 541 million kWh of electricity in 2011 (Guo et al., 2015). Recently, the EPA launched AgSTAR, a program to promote AD in farms for livestock wastes. Even though the U.S. has 247 anaerobic digesters using livestock wastes, the plants are only economically attractive only for large dairy farms (more than 500 cows) (Klavon et al., 2013). AD is also a promising technology for developing countries since enormous volumes of organic waste remain underutilized. In developing countries, MSW is largely dominated by organic matter which accounts for over 55% of the total MSW and agriculture comprises a major fraction of the national economy leading to high amounts of wastes such as manures or crop residues. Among this reason, AD can also bring social, environmental and health benefits. In 1950s, China built 3.5 million family-sized, low-cost anaerobic digesters in the rural area to provide biogas for cooking and lighting (Figure 1.6). In 2012, the total number increased to 45 million, of which roughly 65% are in operation. India has more than 4.5 million small-scale anaerobic digesters to produce biogas from manures. There is a trend in these two countries toward using larger, more sophisticated digestion systems with improved biogas productivity and digester cleansing convenience.

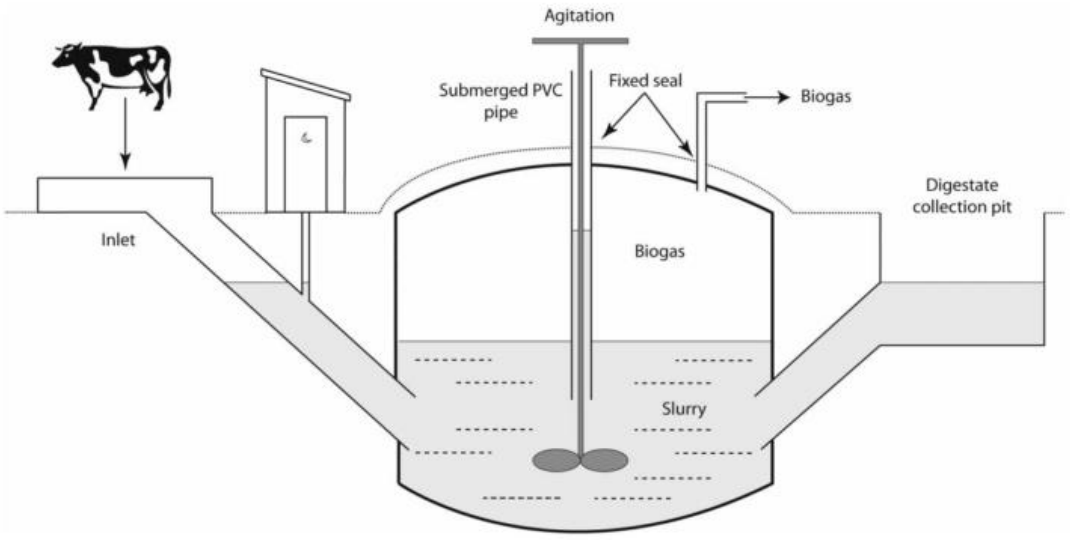


Figure 1.6 AD digester type in China (Surendra et al., 2013)

1.2.3 Anaerobic co-digestion: Increasing biogas production

AD of single substrates (mono-digestion) presents some drawbacks linked to substrate properties. For instance, (i) SS is characterized by low organic loads, (ii) animal manures have low organic loads and high N concentrations, that may inhibit methanogens, (iii) the organic fraction of municipal solid waste (OFMSW) has improper materials as well as a relatively high concentration of heavy metals, (iv) crops and agro-industrial wastes are seasonal substrates, which might lack N, and (v) slaughterhouse wastes (SHW) include risks associated with the high concentration of N and/or LCFA, both potential inhibitors of the methanogenic activity. Most of these problems can be solved by the addition of a co-substrate in what has been recently called anaerobic co-digestion (AcoD).

The interest in AcoD, the simultaneous AD of two or more substrates, have increase during the last years (Figure 1.7) because it is a feasible option to overcome the drawbacks of mono-digestion and to improve the economic viability of AD plants due to higher methane production.

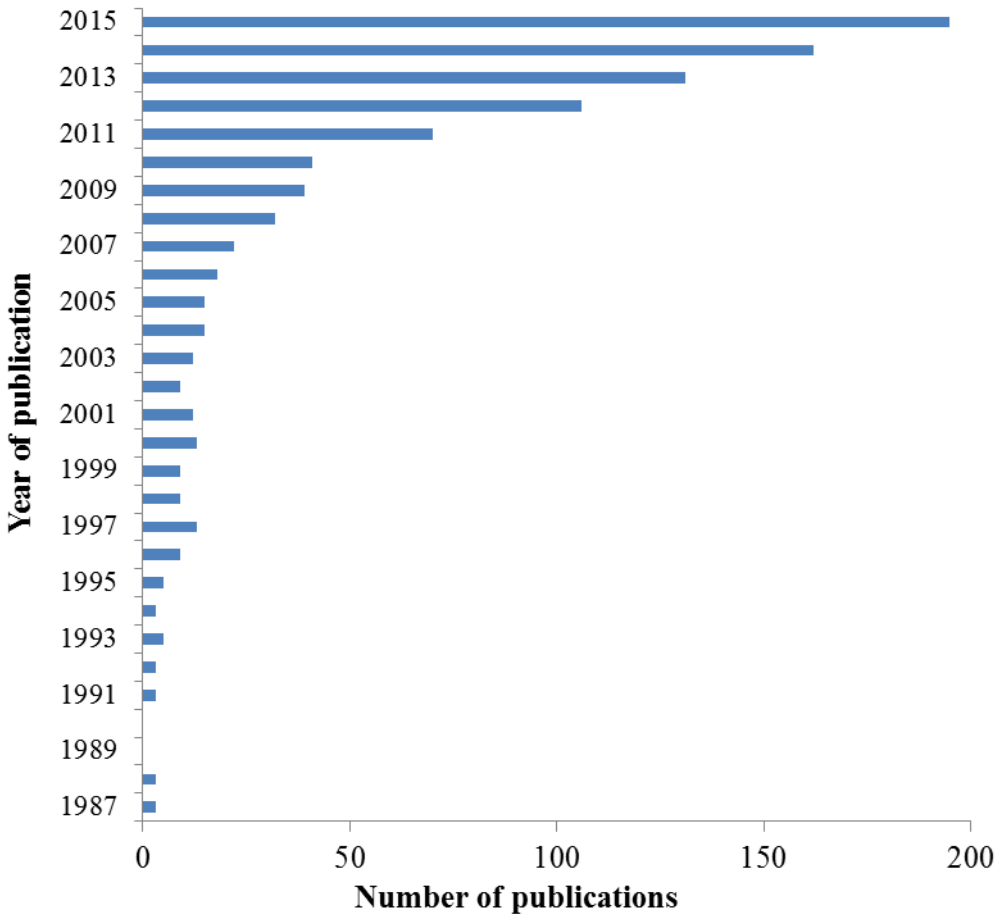


Figure 1.7 Evolution of number of papers published with the words co-digestion or co-digestion in its title

Initially, because of the research perspective, AcoD focused on mixing substrates which favor positive interactions, i.e. macro- and micronutrient equilibrium, moisture balance and/or dilute inhibitory or toxic compounds (Mata-Alvarez et al., 2011). Under these circumstances, synergisms may be achieved, that means, co- digestion is producing more methane than the addition of the methane produced in both single digestions. Moreover, the digestion of wastes produced in the same facility (lignocellulosic/agro wastes and animal manure) is more economically attractive than having a different waste-treatment technology for each of them (Alatriste-Mondragón et al., 2006). Actually, the transport cost of the co-substrate from the generation point to the AD plant is the first selection criteria. Despite this fact, it is still important to choose the best co-substrate and blend ratio with the aim of favoring synergisms, dilute harmful compounds, optimize methane production and not disrupt digestate quality.

1.3 Lignocellulosic compounds, the AD challenge

1.3.1 The recalcitrance of lignocellulosic biomass

Recalcitrant compounds such as lignin, hemicellulose and cellulose are present in a wide range of AD substrates such as agro-industrial, energy crops, and MSW (Fig. 1.8) (Azman et al., 2015; Baba et al., 2013; Yuan et al., 2014). Since microorganisms do not degrade these compounds efficiently, the methane yield of those substrates does not exceed 60% of the theoretical value in practice. The lack of efficient methods to overcome the refractory property of these biomass is one of the bottlenecks for their widespread utilization as a feedstock for AD. Specifically, it is well established that the conversion of lignocellulosic materials into CH_4 is limited by hydrolysis, the first step of the AD (Noike et al., 1985). Furthermore, the degradation products of hydrolysis and acidogenesis act as a substrate for other groups of bacteria and archaea and determine the rate and performance of the subsequent steps, i.e., acetogenesis and methanogenesis. Various high-throughput molecular techniques have been developed and applied for the comprehensive analysis of microbial communities in anaerobic digesters digesting lignocellulosic feedstocks. Such information can be applied to improve the efficiency and stability of AD operation for enhanced biogas production as well as to discover novel microorganisms and metabolic pathways important to AD.

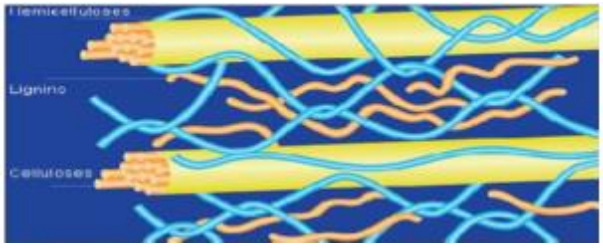


Figure 1.8 The position of lignin within lignocellulosic matrix (Abdullah et al., 2013)

Research on AD of lignocellulosic biomass has accelerated greatly during the last decade and a number of reviews have been published on this subject matter, focusing on process microbiology in general (Tsavkelova and Netrusov, 2012), the challenges during digestion process (Sawatdeenarunat et al., 2015), the types and role of different hydrolytic bacteria involved (Azman et al., 2015).

Lignocellulose forms the primary building block of plant cell wall, with the major constituents being cellulose (most abundant), hemicellulose, and lignin (Jørgensen et al., 2007; Martínez et al., 2005). In addition to these, non-structural carbohydrates (glucose,

fructose, sucrose…) proteins, lipids, and pectins are also present in varying amounts. The specific composition of lignocellulosic biomass, however, depends on plant species, age, and stage of growth.

Cellulose is a homo-polysaccharide of β-1,4-linked D-glucose units (Pérez et al., 2002). In most cases, cellulose fibers are embedded in a matrix of other structural biopolymers, primarily hemicelluloses and lignin. Hemicellulose is a complex heterogeneous polysaccharide, either linear or branched, and is composed of polymers of pentoses (D-xylose, L-arabinose), hexoses (D-glucose, D-galactose, D-mannose), D-glucuronic acid, 4-O-methyl-d-glucuronic acid or combination of these (Pérez et al., 2002). It serves to link the lignin and the cellulose fibers. Hemicellulose restricts access to cellulose cores by coating them, and its removal reduces the amount of cellulase required to convert cellulose into smaller units such as glucose (Himmel et al., 2007). The hydrolysis of both of the polysaccharides is coupled together, and degradation of either one of these two components in isolation is not efficient (Zverlov et al., 2010). Lignin is a large complex molecule formed by monomers of three different phenylpropane units: p-coumaryl, coniferyl, and sinapyl alcohol linked by aryl ether or C–C bonds in a three-dimensional structure (Martínez et al., 2005; Pérez et al., 2002; Zeng et al., 2014). It acts as glue that binds the different components in the lignocellulosic biomass together. It gives structural support to plants as well as contributes in increasing impermeability and resistance against microbial or enzymatic treatment. Besides being a physical barrier, the negative effects of lignin include non-specific adsorption of hydrolytic enzymes to "sticky" lignin, interference with, and non-productive binding of cellulolytic enzymes to lignin-carbohydrates complexes, and toxicity of lignin derivatives to microorganisms (Agbor et al., 2011).

The recalcitrance of lignocellulosic feedstocks can be attributed to various natural factors such as the epidermal tissue of the plant body, particularly the cuticle and epicuticular waxes, the arrangement and density of the vascular bundles, amount of sclerenchymatous tissue, cross-linking of cellulose with hemicellulose and lignin, crystallinity of cellulose, diverse architecture of cell wall, degree of lignification, and the inhibitors that are naturally present in cell walls or are produced during conversion processes (Himmel et al., 2007; Zeng et al., 2014). In plants, the inner face of the parenchymatous secondary walls is non-lignified whereas the scelrenchymatous secondary walls are lignified. The lignin content increases during the transition from the vegetative to the reproductive growth phase, which is mainly due to lignification of

parenchymatous secondary walls. This indicates that for higher bioenergy yield, it is better to collect plant biomass before the transition phase as lignin is difficult to degrade.

The ether and C–C linkages present in lignin are not susceptible to hydrolytic attack which makes it highly resistant to breakdown (Bugg et al., 2011). Cleavage of linkages between lignin units, aromatic rings of lignin monomers, and the bonds (benzylether, benzylester, phenylglyside, and acetal type) between lignin and hemicellulose can all release lignin from the polysaccharide (Zeng et al., 2014). Lignin content determines the extent of degradation of cellulose and hemicellulose and is negatively related to CH_4 yield during AD of lignocellulosic biomass (Brown et al., 2012; Y. Li et al., 2013; Liew et al., 2012; Surendra and Khanal, 2015). This indicates that lignin is one of the key factors controlling the AD of lignocellulosic biomass.

1.3.2 Strategies to further degrade lignocellulosic compounds

So far, a variety of strategies such as physical and chemical pretreatment of feedstock, use of an inoculum rich in cellulolytic/hemicellulolytic microorganisms, and co-digestion have been practiced to address this problem.

1.3.2.1 Pretreatment strategies

Table 1.2 Pretreatment strategies applied on lignocellulosic biomass

Substrate	Pretreatment	SMP (L_{CH4} gVS^{-1})	SMP improvement (%)	Cellulose removal (%)	Hemicellulose removal (%)	Lignin removal (%)	Reactor configuration	Reference
Olive husks Olive mill wastewater Dairy wastewater	Ultrasound (383kJ TS^{-1})	127	9				Batch	(Gianico et al., 2013)
Olive husks Olive mill wastewater Dairy wastewater	Thermal (503kJ TS^{1})	90	-23				Batch	(Gianico et al., 2013)
Barley waste	Thermal (120 °C)	338	41	No affected	No affected	No affected	Batch	(Menardo et al., 2012)
Wheat straw	Cut to 0.2 cm	334	84	No affected	No affected	No affected	Batch	(Menardo et al., 2012)
Barley waste*	0.3 gNaOH TS^{-1}	222	909				Batch	(Neves et al., 2006)

Corn Stover	0.05 gNaOH TS^{-1}	195	40				Solid state AD	(Y. Li et al., 2014)
Maize	Ensilage	357	1				Batch	(Kreuger et al., 2011)
Hemp	Ensilage	272	-10					(Kreuger et al., 2011)
Beets	Ensilage	405	-9					(Kreuger et al., 2011)
Maize	Ensilage (Additives)	420 (TS)	12				Batch	(Vervaeren et al., 2010)
Albizia chips	*Ceriporiopsis subvermispora*	124	265	10.5	15.0	24.0	Solid state AD	(Ge et al., 2015)
Corn Stover	*Ceriporiopsis subvermispora*			4	19	28	Solid state AD	(Wan and Li, 2011)
Switchgrass	*Ceriporiopsis subvermispora*			2	15	27	Solid state AD	(Wan and Li, 2011)
Wheat straw	*Ceriporiopsis subvermispora*			2	5	3	Solid state AD	(Wan and Li, 2011)
Soybean stalk	*Ceriporiopsis subvermispora*			0	3	0	Solid state AD	(Wan and Li, 2011)

Hardwood	*Ceriporiopsis subvermispora*			4	18	18	Solid state AD	(Wan and Li, 2011)
Corn Stover silage	*Phanerochaete chrysosporium*	265	23	20	32	23	Solid state AD	(Liu et al., 2014)
Olive mill wastewater	*Phanerochaete chrysosporium*	340	127				Anaerobic filter pilot plant	(Dhouib et al., 2006)
Sisal leaf decortications residue	CCHT-1 and *Trichoderma reesei*	292	101	-21	-127	16	Solid state AD	(Muthangya et al., 2009)
Cassava residues	Microbial Consortium	260	97				Batch	(Zhang et al., 2011)
Lignocellulose fraction of municipal solid wastes	Microbial Consortium	221	126				Batch	(Yuan et al., 2014)
Napier grass	Microbial Consortium	278	50	19	33	30	Batch	(Wen et al., 2015)
Sugar beet pulp	Enzymes	117 (COD)	20				CSTR	(Ziemiński et al., 2012)

Spent hops	Enzymes	72 (COD)	12				CSTR	(Ziemiński et al., 2012)
Dried sweet sorghum	Enzymes	274	15				Batch	(Matsakas et al., 2014)
Ensiled Sorghum forage	Enzymes	304	15	20	0	0	Batch	(Rollini et al., 2014)
Corn Stover	Liquid fraction of digestate	276	66	5	20	21	Batch	(Hu et al., 2015)

* Co-digested with sewage sludge

The removal of Cellulose, hemicellulose and lignin was performed during the pretreatment

TS = Total solids COD = Chemical oxygen demand SMP = Specific methane potential CSTR = Continuous stirred tank reactor

Pretreatment can greatly enhance the digestibility of lignocellulosic biomass by reducing the cellulose crystallinity, increasing the porosity of the biomass, and cellulose, hemicellulose and lignin removal and solubilization (Sun and Cheng, 2002). The pretreatment method should have i) a low capital and operational cost ii) should be effective on a wide range and loading of lignocellulosic material iii) should avoid the degradations of the solubilized products and iv) should produce no or little lignin degradation products that inhibit fermentative microorganism's growth or the action of hydrolytic enzymes, (Agbor et al., 2011; Zheng et al., 2014).Pretreatment methods such as physical (mechanical, thermal), physic-chemical, chemical (acid/alkaline hydrolysis) and biological (mediated by microbes or enzymes) methods have been widely studied for lignocellulosic biomass prior to AD (Gianico et al., 2013; Hendriks and Zeeman, 2009; Menardo et al., 2012; Neves et al., 2006; Zheng et al., 2014). Table 1.2 summarizes the effect of some of the pretreatment methods on the methane yield from different lignocellulosic substrates. Methane yield from rice straw could increase from 0.06 L_{CH4} gVS^{-1} to 0.13 L_{CH4} gVS^{-1} when Chandra et al. (2012) used a hydrothermal pretreatment followed by the addition of 5% of NaOH. You et al. (2014) improved the kinetics and the methane yield of mixture of swine manure and corn stover by pre-treating it at 35ºC with 6% of NaOH. The improve in methane yield was from 0.28 L_{CH4} gVS^{-1} to 0.35 L_{CH4} gVS^{-1}. However Risberg et al. (2013) did not presented very high methane yields (0.13 – 0.21 L_{CH4} gVS^{-1}) when cattle manure was co-digested with steam-exploded straw.

Biological methods are also an attractive option. Unlike other pretreatment methods they don’t require energy input or generate a variety of toxic contaminants (phenolic compounds and furfurals) that can affect the fermentation processes (Frigon et al., 2012; Wu and He, 2013). These methods usually involve the use of biological agents such as fungi or other microbial consortium or specific enzymes which could increase the methane yield between a 50% and 126% (Table 1.2). To get higher yield increments other strategies need to be implemented such as the integration of more than one pretreatments: Alkaline-enzymatic (Rollini et al., 2014), acid-alkaline-enzymatic (Gomez-Tovar et al., 2012) or thermal-enzymatic (Kabir et al., 2013)) or the addition of a co-substrate (Ziemiński and Kowalska-Wentel, 2015). The increases in the methane yield discussed above are quite similar and sometimes higher compared to the performance obtained with a physical or chemical pretreatment. Nevertheless, the main drawback of biological pretreatments could be the cost. The application of cultured

fungi, enzymes or microbial consortium can be very difficult for full scale AD plants since strict controlled conditions are needed for their growth.

Almost all the pretreatment studies are performed in batch essays and sometimes better results are obtained in continuous experiments since in long stationary conditions the microbial community is adapted to the substrate conditions instead of being adapted to the inoculum conditions (Table 1.2).

1.3.2.2 Anaerobic co-digestion

Table 1.3 AcoD experiments performed on lignocellulosic biomass

Substrate	Mixture	Reactor configuration	SMP (L_{CH4} $kgVS^{-1}$)	Improvement (%)	Pretreatment	Reference
Switchgrass:Dairy manure	1:1 (TS)	Batch	155	18	no	(Zheng et al., 2015)
Corn stover:Dairy manure	1:4 (TS)	CSTR	325 (TS)	-	no	(Z. Yue et al., 2013)
Various crops:Slaughterhouse waste	1:1 (ww)	CSTR	380	-	No	(Pagés-Díaz et al., 2015)
Various crops:Mixed Manure	1:1 (ww)	Batch	432	7	No	(Pagés-Díaz et al., 2014)
Various crops:Municipal solid waste	1:1 (ww)	Batch	470	-2	No	(Pagés-Díaz et al., 2014)
Cassava dregs:Pig Manure	6:4 (VS)	Two-phase	353	21	No	(Ren et al., 2014)
Oil seed radish:Cow and poultry manure	1:1 (VS)	CSTR	348	-	No	(Molinuevo-Salces et al., 2014)

Vegetable waste:Swine manure	3:1 (TS)	Batch	244	67	No	(Molinuevo-Salces et al., 2013)
Vegetable waste:Poultry litter	3:1 (TS)	Batch	223	32	No	(Molinuevo-Salces et al., 2013)
Salix:Cow manure:Fish	2:2:1 (VS)	CSTR	191	-	Steam explosion	(Estevez et al., 2014)
Quinoa:Llama manure	1:1 (VS)	CSTR	104	32	No	(Alvarez and Lidén, 2008)
Macrophytes:Llama manure	1:1 (VS)	CSTR	107	-24	No	(Alvarez and Lidén, 2008)
Grass silage:Dairy slurry	4:1 (VS)	CSTR	366	-12	Silage	(Wall et al., 2014)
Rice straw:Sewage sludge	1:1 (VS)	Batch	140	17	No	(Zhao et al., 2014)
Corn straw:Taihu blue algae	35:65 (VS)	CSTR	160	-	No	(Zhong et al., 2013)
Cassava Pulp:Pig manure	1:1 (VS)	Two-phase	126	-	No	(Panichnumsin et al., 2010)

Maize sillage:Cattle manure	1:1.6 (ww)	Batch	382	-	No	(Ziganshin et al., 2013)
Distiler grains:Cattle manure	1:14.8 (ww)	Batch	335	-	No	(Ziganshin et al., 2013)
Mize straw:Cattle Manure	1:6.7	Batch	220	-	No	(Ziganshin et al., 2013)

Due to their high Carbon to nitrogen ratio (C/N) (Sawatdeenarunat et al., 2015), lignocellulosic substrates have been used in co-digestion with other nitrogen-rich substrates such as animal manure to provide nutrients and buffering capacity to the AD system, and to increase the methane yield (Estevez et al., 2014; Jiménez et al., 2014; Molinuevo-Salces et al., 2015; Nakakihara et al., 2014; Pagés-Díaz et al., 2015, 2014). These AcoD studies have reported high SMP around 0.40 L_{CH4} gVS^{-1} while in some the SMP was pretty low (around 0.15 L_{CH4} gVS^{-1}) (Table 1.3). In fact, only a few of studies have focused on enhancing the degradation of lignocellulosic components and hence increase the hydrolysis rate step which, for most of the substrates, represents the bottleneck of the process rate (Lin et al., 2014; Nakakihara et al., 2014; Parameswaran and Rittmann, 2012; Yang et al., 2013; Zhao et al., 2014). Ruminant's anaerobic stomach is considered as the best model to improve biogas production from lignocellulosic biomass due to the presence of both fungi and methanogens co-cultures (Cheng et al., 2009; Youssef et al., 2013). The use of rumen content (RC) as a co-substrate, a waste generated in slaughterhouse, can be a great opportunity to introduce the microbes and/or enzymes necessary to break down the lignocellulosic components into the system. In this vein, co-digestion could be a very good strategy to improve hydrolysis since it allows the continuous addition of these beneficial microbial populations into the system and hence increase the methane production.

1.3.2.3 Inoculation strategies

The seed sludge used for inoculation of digesters should be selected such as to avoid a slow start-up and a prolonged acclimation period and thus making the digestion process more stable and efficient. Inoculum should be reused from one digester to another over a long time. This tends to select for the microorganisms capable of degrading diverse substrates and thus imitate the selection which occurs in animal guts (Godon et al., 2013). It is also very important to have an inoculum with diverse microbial consortia and high activity on the substrate to be digested (Keating et al., 2013; Quintero et al., 2012; Saady and Massé, 2013). Moreover, flexible microbial community (one with high level of dynamics together with a high bacterial diversity) is better suited to tolerate substrate overloading (VFA accumulation) than one with a stable community and can be correlated to higher process stability (De Vrieze et al., 2013). Most commonly animal manure or digested sludge from wastewater treatment plant or anaerobic digester in

operation is used as an inoculum during AD (Gu et al., 2014; Li et al., 2010, 2013a; Yue et al., 2012). Besides providing microbial consortia, inoculum such as digested manure, or liquid effluents from anaerobic digesters can also provide nitrogen, and other micro and macro nutrients to balance the C/N ratio during lignocellulose digestion, facilitate enzymatic activity or provide alkalinity to prevent acidification (Gu et al., 2014; Xu et al., 2013) without the addition of chemicals. Since enzymes are required for hydrolysis, enzyme activity of the inoculum can be assessed to ensure proper selection of inoculum. Gu et al.(2014) related the higher cellulose and hemicellulose degradation rate and higher specific methane production with the higher cellulase and xylanase activities and higher micronutrients contained in the inoculums (digested dairy manure) used. The enzyme activity increased after digestion which might have been caused by the microbial growth and adaptations of the inoculums in hydrolyzing the lignocellulose substrate. This further strengthens the need to re-use the inoculum from one digester to another. Xu et al.(2013) also attributed higher methane yield from corn stover to the presence of greater population of cellulolytic and xylanolytic bacteria present in the inoculum (0.24 L_{CH4} gVS^{-1} with dairy waste effluent). Hydrolysis can be enhanced by using an inoculum already acclimated to degradation of lignocellulosic biomass or having cellulolytic activity, such as rumen microorganisms that has both characteristics (Quintero et al., 2012; Tsavkelova et al., 2012a; Z.-B. Yue et al., 2013). Faster hydrolysis rates were observed in experiments with rumen inocula than with leachate from municipal solid waste and decreased with decreasing biomass concentrations of each inoculum type (Jensen et al., 2009; O'Sullivan et al., 2008). Rumen microorganisms solubilize cellulose faster than microbial communities from landfills or anaerobic digesters. However, due to the high capacity of rumen microorganisms to metabolize lignocellulose substrate into soluble compound, VFA accumulation can affect the stability of the digester. So, inoculum from different sources can be mixed together to promote synergistic action of mixed microbial population.

2. Objectives and thesis structure

2.1 Motivation and objectives

As stayed before the waste legislation in Europe is promoting the implementation of anaerobic digestion. In Europe over 14,000 are operational AD plants producing around 13.4 Mtoe of biogas. Although the advantage over others treatment and the great diversity of biogas applications, AD is presenting a reduction of the number of projects during the recent years, because of the difficult economic situation and the restrictive legislation in some countries about using energy crops as substrate. Increasing the economic viability of plants for the treatment of wastes or for the production of energy second generation biomass is necessary to ensure the application of this biotechnology in the future. Therefore, economical inputs through the generation of methane, compost and/or by-products must be considered. Recently, the use of AcoD to increase the OLR and to equilibrate the C/N ratio has been a widely applied strategy to increase the volume of methane produced.

Lignocellulosic components are present in a wide range of substrates like agricultural wastes, energy crops and MSW. The presence of those recalcitrant compounds in substrates is hindering its degradation and hence, less methane is obtained. A lot of interest about the degradation of those compounds using different strategies is presented in the scientific literature.

These considerations are the motivation of the present thesis, which deals with the study of strategies to improve the economic viability of AD plants considering the presence of recalcitrance compounds on the substrates used (MSW, agricultural wastes and second generation biomass). To reach this general objective, the following specific goals were proposed:

- To use agricultural wastes in AD plants treating sewage sludge with non-used capacity to improve their economic viability.

- To identify the effects on AD when lignocellulosic components from MSW are separated for the production of byproducts with high valorization.

- To enhance the production of methane using ultrasounds and low-temperature pretreatment on agricultural wastes.

- To evaluate separately the implementation of ultrasounds and low-temperature pretreatment considering the overproduction of methane.

- To improve lignocellulose degradation using an inoculum with potential hydrolytic bacteria.

- To study the microbial community involved in the degradation of lingo-cellulosic compounds when rumen is used.

- To use AcoD as a novel strategy to degrade lignocellulose and degrade lignocellulose. The co-substrate used was rumen, a waste harboring hydrolytic microbial populations.

2.2 Thesis structure

Chapter 1: Introduction

This chapter provides a general introduction regarding the main concepts included in this thesis. An overview is given about: The waste and energy situation of the world, the anaerobic digestion as a biotechnology for waste treatment and energy production, the characterization of the lignocellulose and the studied strategies to improve methane generation from lignocellulosic substrates.

Chapter 2: Objectives and thesis structure

This chapter summarizes the objectives and the thesis structure.

Chapter 3: Materials and methods

In this chapter, the biological reactors (discontinuous and semi-continuous) and the analytical and molecular methods used to perform the experimentation are detailed.

Chapter 4: Anaerobic co-digestion of sewage sludge and fruit wastes: Evaluation of the transitory states when the co-substrate is changed

To improve the methane production from the AD of SS, agricultural wastes from the fruit industry were used as a co-substrate. Due to the seasonality in agricultural industries, the effect of changing the co-substrate on AD performance was evaluated.

Chapter 5: Effect of waste paper suppression on OFMSW anaerobic digestion: Biogas and digestate evaluation

In this section the removal of lignocellulosic components from MSW to produce by-products was considered and the effects of this action on the AD performance and the digestate quality were studied.

Chapter 6: Anaerobic co-digestion of Agro-wastes under high ammonia concentrations: Low temperature and ultrasounds pretreatment application on barley waste

To improve the methane production during the AcoD of pig manure and barley spent grain; two pretreatments were used on barley waste. The pretreatments used were ultrasounds and low-temperature and the effect on the methane production was assessed.

Chapter 7: Anaerobic digestion of lignocellulosic substrates: Inoculation with rumen, a natural ecosystem harboring hydrolytic bacteria

A microbial community adapted to the degradation of lignocellulosic compounds was used as inoculum in the AcoD of Napier grass and cow manure to improve the degradability of recalcitrant components.

Chapter 8: Anaerobic digestion of lignocellulosic substrates with cow manure and rumen as potential co-substrates

As a novel strategy, rumen was used as a co-substrate to continuously bring a microbial community adapted to the degradation of lignocellulosic.

3. Materials and methods

3.1 Analytical methods

Most of the analytical methods were performed following the Standard Methods for the Examination of Water and Wastewater (APHA et al., 2012), however, different procedures were used according the laboratory.

3.1.1 University of Barcelona

- The Total solids (TS) and volatile solids (VS) were determined following the guidelines given by the standard methods 2540G, where VFA losses during the solids determination were taken into account and then combined to give a final TS and VS value
- Total (TA) and partial (PA) alkalinity were determined by a titration method at pH 4.3 and at 5.75 respectively. The intermediate alkalinity (IA) was determined by the difference between TA and PA (Ripley et al., 1986).
- Individual VFAs (acetate, propionate, iso-butyrate, n-butyrate, iso-valerate and n-valerate) were analyzed by a HP 5890-Serie II gas chromatograph equipped with a capillary column (NukolTM) and a flame ionization detector. Specifically, the chromatograph oven temperature program was as follows: hold 1.5 min at 85 °C; ramp to 120 °C at 15 °C min^{-1}; ramp to 145 °C at 10 °C min^{-1}; ramp to 175 °C at 20 °C min^{-1}, hold 2 min. Injector and detector temperature was set to 280 °C and 300 °C respectively, 33 mL min^{-1} of Helium at 5 psi was used as carrier gas.
- The biogas composition was determined with a Shimadzu GC-2010+ gas chromatograph equipped with a thermal conductivity detector and a Carboxen column. The chromatograph oven temperature program was as follows: hold 360 s at 40 °C; ramp to 230 °C at 0.42 °C s^{-1}, hold 120 s. Injector and detector temperature was set to 200 and 230 °C, respectively. Helium with a fix linear velocity of 0.29 m s^{-1} was used as carrier gas. The biogas and methane productions are reported at standard temperature and pressure conditions (i.e. 0 ºC and 1 bar).
- The concentration of ammonium (NH_4^+) was analyzed by the use of an 863 Advanced Compact Metrohm ionic chromatograph using Metrosep columns.
- The 5-day biochemical oxygen demand (BOD_5) was determined, with a WTW Oxitop® measuring system, following the 5210D Standard Methods procedure.

- The residual methane potential of the digestate was analysed by determining the methane released after 40 incubation days. Specifically, 200 mL of digestate were added to a 265 mL serum bottle. All bottles were flushed with N_2, sealed with a rubber stopper and placed in a 35 °C water bath. Methane production was calculated from the headspace pressure increase (vacuometer Ebro – VAM 320) and methane content, and expressed at standard temperature and pressure conditions (i.e. 0 ºC and 1 bar).

3.1.2 University of Michigan

- The TS, VS, TA, PA and IA were determined as at the University of Barcelona.
- Ammonia was analyzed using the phenate method.
- For the inoculum strategy discussed in chapter 7 VFA (formate, acetate, propionate, butyrate, and valerate) were determined with an ion chromatograph (ICS-1600, Dionex, Sunnyvale, CA) equipped with a conductivity detector, auto-sampler, and reagent free eluent generator to produce a KOH gradient. Eluent was passed through a Dionex AS-11HC column at 60°C at a flow rate of 0.30 mL min^{-1}. For the AcoD strategy discussed in chapter 8 VFAs (acetate, propionate, iso-butyrate, n-butyrate, iso-valerate, n-valerate, iso-hexanoate, n-hexanoate and heptanoate) were analyzed by a HP 5890-Serie II gas chromatograph equipped with a capillary column (NukolTM) and a flame ionization detector.
- Biogas methane content was measured with a gas chromatograph (Gow-Mac, Bethlehem, PA) coupled with a thermal conductivity detector (TCD).

3.2 Pretreatments

3.2.1 Ultrasounds

The specific energy (E_s) applied for ultrasound pretreatment (USP) was 5000 kJ $kgST^{-1}$ and the exposition time was calculated according to equation 1.

$$E_s = \frac{P \cdot t}{m \cdot TS} \qquad \text{Eq. 1}$$

Where P is the supplied power, t is the exposition time, m is the mass of the substrate used and TS is its TS concentration (gTS kg^{-1}). The samples were sonicated in a HD2070 Sonopuls Ultrasonic Homogenizer equipped with a MS 73 titanium microtip probe and working with an operating frequency of 20 kHz and a supplied power of 70

W. The ultrasonic probe was submerged until half-height of the sample. Temperature was not controlled during the USP.

3.2.2 Low-temperature pretreatment

The sample was heated in an oven for 24h at 60ºC inside of an air tight bottle flushed with N2.

3.3 Microbial analysis

Biomass samples collected in chapter 7 and 8 were pelletized by centrifugation at 7,000 x g for 10 min at 4°C, decanted, weighted and immediately stored at -80°C. DNA extraction from pelletized biomass was performed by three 2-min bead beating steps (Mini-Beadbeater-96, BioSpec Products, Bartlesville, OK) with 0.1 mm diameter silicon beads in lysis buffer, proteinase K digestion, and automated extraction using the Maxwell 16 Blood LEV kit according to manufacturer's instruction (Promega, Madison, WI). DNA quality and quantity were assessed via spectrophotometry (Nanodrop 1000, Thermo Fisher Scientific, Wilmington, DE) and Qubit 2.0 Flurometer (Invitrogen, Life Technologies) for samples obtained. Universal primers targeting the V4 region of the 16S rRNA of bacteria (Bact-338F/Bact-909R) and archaea (Arch-340F/Arch-915) (Caporaso et al., 2010) were used for PCR amplification. The quality of the extraction was assessed only in some samples from the same extraction run by PCR. PCR reactions were 20 μL and included primers at 500 nM, 10 μL 2x Accuprime buffer 11 (Invitrogen, Carlsbad, CA), 0.15 μL Accuprime TAQ, 0.5 ng template, and nuclease-free water. Thermocycling conditions consisted of an initial 2 min denaturation at 95°C, followed by 30 cycles of denaturing at 95°C for 20 s, annealing at 55°C for 15 s, and extension at 72°C for 5 min, followed by a final extension at 72°C for 5 min. Multiplexed amplicons were sequenced by the Host Microbiome Initiative via Illumina MiSeq using the MiSeq Reagent Kit V4 and sequences were processed with MOTHUR (Kozich et al., 2013) following the SchlossMiSeq SOP. Sequences were classified using the Ribosomal Database Project (Maidak et al., 1997) and further analyzed for operational taxonomic unit (OTU)-based clustering (average neighbor algorithm at 3% cutoff).

3.4 Experimental devices

Two types of anaerobic assays have been carried out: (i) discontinuous assays or biomethane potential (BMP) tests, and (ii) semi-continuous assays in laboratory stirred tank reactors (CSTR).

3.4.1 Biomethane potential test

The BMP test was done following the procedure defined by the German Standard Procedure VDI-4630 (2006) and by Angelidaki et al. (2009). The tests were carried out in serum bottles (250 mL) filled in with the corresponding inoculum and substrate considering that the VSsubstrate-to-VSinoculum ratio was 0.5. The blank assay, only filled with inoculum, was used to determine the background effect of the inoculum. In the UB deionized water was used to adjust the same effective volume for all the bottles. In order to deplete the residual biodegradable organic matter the inoculum was degasified at 37 ºC during 5 days. Before starting the experiment, all the bottles were flushed with nitrogen for one minute. The bottles were closed with PTFE/Butyl septums, which were fixed by an aluminum crimp cap. In the UB, the digesters were placed in a water bath set at mesophilic conditions (37±1 ºC) and mixed twice a day. In the UM the bottles were places in a shaking incubator heated at (37±1 ºC).

The biogas production during the running test was measured, after discarding the Overpressure generated during the first hour, by using a vacumeter. At each sample event, the methane content of the biogas accumulated in the bottle headspace was analyzed. The methane production in the course of time was obtained by multiplying the biogas production, once subtracted the vapor pressure and converted at standard temperature and pressure conditions (i.e. converted to 0 ºC and 1 atm), by the percentage of methane in the biogas. All tests and blanks were carried out in triplicate, and all error bars indicate 95% confidence in the average of the triplicate.

Figure 3.1 BMP bottle and vacuometer

3.4.2 Semi-continuous stirred tank reactor

3.4.2.1 University of Barcelona

Different semi-continuous stirred tank reactors with different volumes were used. The biogas production was measured and recorded with an on-line biogas measuring device (Ritter MGC-1). Biogas production was converted to standard temperature and pressure conditions (0 °C, 1 atm). The operational temperature (37 ºC) was ensured by circulating water from a heated water bath (HUBER 118A-E) through a jacket surrounding the reactor. The digester medium was continuously stirred at 60 rpm. The digesters were manually fed and purged once a day, and the mixtures were daily prepared before the feeding in order to avoid uncontrolled degradation.

Figure 3.2 Laboratory semi-continuous stirred tank reactors used in UB

3.4.2.2 University of Michigan

Semi-continuous stirred tank reactors (2L) were operated at mesophilic conditions (37ºC). A shaking water bath was used when the reactors were continuously mixed, otherwise, they were mixed manually once per day. The biogas collected in Tedlar gas bags was measured by a gas meter daily.

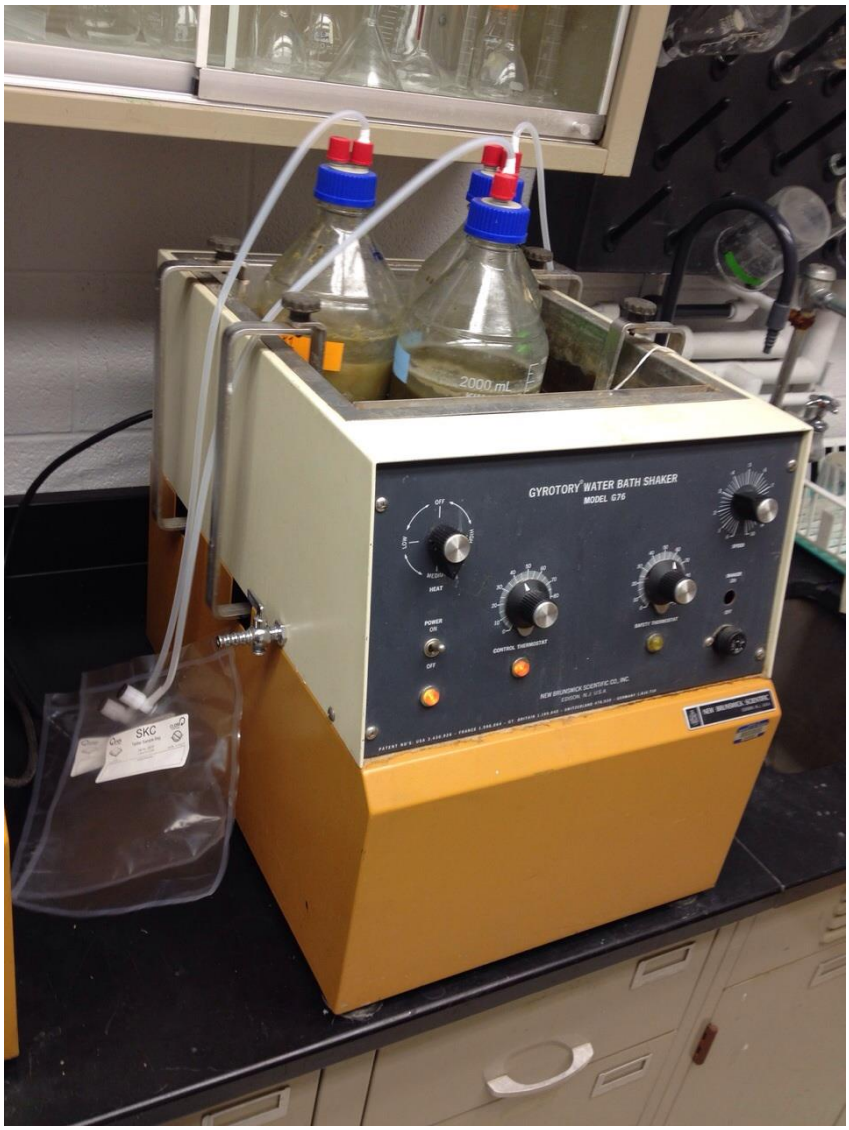


Figure 3.3 Laboratory semi-continuous stirred tank reactors used in UM

4. Anaerobic co-digestion of sewage sludge and fruit wastes: Evaluation of the transitory states when the co-substrate is changed

Abstract

Some existing anaerobic digesters treating sewage sludge have a non-used capacity. The use of this extra capacity by introducing additional wastes to conduct the co-digestion could enhance biogas production and plant economic feasibility. Fruit wastes from the food industry could be proper co-substrates due to their high biodegradability, but the harvesting seasons require the use of different kind of fruits causing many transitory conditions throughout the year. Two lab-scale semi-continuous anaerobic digesters treating sewage sludge were operated, one as a reference reactor and the other one as a co digester. The transitory state was evaluated when fruit waste supply was started, when the co-substrate was changed (peach, banana and apple waste) and when fruit waste supply was stopped. In the transition from mono- to co-digestion, volatile fatty acids concentration rose from 0.07 to 1.70 g L^{-1} due to the OLR increase, but this situation was recovered in only 5 days. The introduction of different kind of fruit wastes resulted in an alteration of alkalinity, without affecting volatile fatty acids concentration, and in an increase of methane production between 110% and 180% depending on the characteristics of the co-substrate.

Finally, when co-digestion was stopped, the parameters converged, at different rates, to the values recorded in the reference digester. It could be concluded that the change of one co-substrate by another one of the same type did not lead to system instability.

This chapter was presented as poster communication in:

Anaerobic co-digestion: focusing on the transitory state when the co-substrate is changed. 13th World Congress on Anaerobic Digestion, Santiago de Compostela, Spain, June 2013

As an oral communication in:

Sewage sludge and fruit wastes anaerobic co-digestion: Evaluation of the process. 2nd IWA Specialized International Conference - Ecotechnologies for Wastewater Treatment - ecoSTP2014, Verona, Italy, June 2014.

And then published as:

Fonoll, X., Astals, S., Dosta, J., Mata-alvarez, J., 2015. **Anaerobic co-digestion of sewage sludge and fruit wastes : Evaluation of the transitory states when the co-substrate is changed**. Chem. Eng. J. 262, 1268–1274.

4.1.1 Introduction

The food and agricultural industry is, with a billing of 18,000 million of euros per year, one of the most important sectors in Catalonia (NE Spain). As result of the productive activities, this sector produces more than one million tons of wastes per year (wet-basis) (Llena i Cortina, 2010). Among them, the fruit processing industry generates large amounts of wastes derived mainly from the washing and extraction processes (Ministerio de medio ambiente, 2006). Fruit wastes (FW) are characterized by high pollution loads and high concentrations of easily biodegradable organic matter (Angelidaki et al., 2003). In the same region, 600 thousand tons of SS, by-product of the physical, chemical and biological wastewater treatment, are annually produced by 340 municipal wastewater treatment plants (WWTP) (S. Astals et al., 2012; Obis et al., 2008). In this respect, the EU landfill directive has gradually restricted the disposal of organic waste in landfills and promoted the development and implementation of other management options (Stroot et al., 2001).

AcoD is a feasible option to overcome the drawbacks of only digesting SS or FW and to improve the economic viability of AD plants because of the higher biogas production (Esposito et al., 2012; Mata-Alvarez et al., 2011, 2000). In AcoD, it is important to choose the best co-substrate and blend ratio in order to promote positive interactions, dilute inhibitory and/or toxic compounds, optimize methane production and preserve digestate stability (Astals et al., 2011). Due to the high amounts of easily biodegradable organic matter, FW are ideal co-substrates for SS, substrate which is characterized by relatively low carbon-to-nitrogen ratios and high buffer capacity (Mata-Alvarez et al., 2011). Moreover, operational data have indicated some non-used capacity in SS anaerobic digesters, sometimes up to 30% (Di Maria et al., 2014; Montusiewicz and Lebiocka, 2011; Pagés-Díaz et al., 2014). Therefore, it would be profitable to use these extra capacities by introducing additional substrates to conduct the co-digestion in the existing anaerobic systems. Nevertheless, the seasonality of the fruit processing industry (fruit harvesting seasons last from 1 to 3 months) makes difficult to operate a co-digester under the same conditions during a long period of time, because waste supply can be frequently changed or stopped.

AcoD between SS and FW, either alone or together with vegetable waste (FVW), has already been investigated. However, most studies have focused on the effect of the co-

substrate ratio and the organic loading rate (OLR) on digester performance and biogas yield. Gómez et al. (2006), co-digested primary sludge and FVW, observing some fluctuations in the specific gas production (0.3 – 0.6 B_{iogas} g^{-1} VS) when changing the mixing conditions and the OLR. Di Maria et al. (2014) obtained good specific methane productions (0.25 L_{CH4} g^{-1} VS) when co-digesting sewage sludge and fruit wastes at a short hydraulic retention time (10 days). However, digestate stability was severely affected, likely due to the presence of easy biodegradable organic matter. In the case of SS and pear residues AcoD, Arhoun et al. (2013) evaluated the influence of two feeding strategies: discontinuous (once per day) and pseudo-continuous (liquid and pulp fed followed different patterns). Although the biogas yield remained constant when the OLR was changed (about 0.44 B_{iogas} g^{-1} VS), the pseudo-continuous scheme allowed to achieve higher OLR than the discontinuous one (10.5 and 6.0 g VS L^{-1} day^{-1}, respectively). Besides, a full-scale study was carried out the WWTP of Prince George (Canada) (Park et al., 2011). The co-digestion between SS and FVW led to an 8 – 17% increase of the biogas production but to a worse digestate quality, due to the presence of impurities in it. However, even though a lot of AcoD papers have been published during the last years (Mata-Alvarez et al., 2014a, 2011), no reference focused on the transitory state of the digester when the co-substrate was changed.

As a result of the high amount of VFA produced during the FVW anaerobic digestion, it is very important to monitor the process stability, i.e. VFA and/or alkalinity (Bouallagui et al., 2005; Montañés et al., 2014). On the one hand, VFA behavior provides information about the performance of the intermediate AD steps, where propionic acid is presented as a key parameter to be followed when analyzing AD stability (Blume et al., 2010; Nielsen et al., 2007; Peces et al., 2013a; Wang et al., 2009). On the other hand, alkalinity is the capacity of the digester medium to neutralize the VFA generated during the process and therefore to mitigate pH changes. According to Mata-Alvarez (Mata-Alvarez, 2002), to assure stable conditions the digester should have TA above 1.5 g $CaCO_3$ L^{-1}. Nonetheless, for AcoD some authors had reported digester instability at higher alkalinity values (S Astals et al., 2012a; Hassib Bouallagui et al., 2009; Habiba et al., 2009; Heo et al., 2004). Consequently, it is better to evaluate the AD stability through the volatile fatty acids-to-total alkalinity ratio (VFA/TA ratio). The critical values for the VFA/TA ratio are: VFA/TA $\leq$ 0.40 stable digester, 0.40 < VFA/TA <

0.80 some instability signs, and VFA/TA ≥ 0.80 significant instability (Callaghan et al., 2002).

The aim of the present study was to investigate the transitory state during AcoD when the co-substrate is changed as well as when the co-substrate supply is stopped. Specifically, the AcoD between SS and three different FW, i.e. peach waste, banana waste and apple waste, were evaluated.

4.2 Materials and methods

4.2.1 Substrates and inoculum origin

In the present study SS was used as a main substrate, whereas two different types of peach waste (PW1 and PW2), banana waste (BW) and apple waste (AW) were used as co-substrates. PW1 and PW2 were obtained from a fruit processing industry located in Lleida (Spain). PW1 was discarded peach generated during the step of fruit selection, while PW2 was fruit residue from juice extraction, consisting mainly of fibers. BW and AW were obtained from a grocery and then grinded in order to simulate the real wastes. The SS, obtained from a municipal WWTP of Barcelona metropolitan area (Spain), was a mixture of primary sludge (60% in wet-basis) and waste activated sludge (40% in wet-basis) diluted to reach a solid concentration of 30 g TS L^{-1}. After collection, all samples were stored at 4 ºC until its utilization. The inoculum was obtained from a stable lab-scale mesophilic digester treating SS at a hydraulic retention time of 20 days (S. Astals et al., 2012).

4.2.2 Lab-scale digesters

Two identical 2.5 L semi-continuous stirred tank reactors (R1 and R2), with a working volume of 1.5 L, were operated during 280 days at mesophilic conditions (37 ºC). The operational temperature was ensured by circulating water from a heated water bath through a jacket surrounding the reactor. The hydraulic retention time of both digesters was set at 20 days during the whole study. The reactors were purged and then fed once a day. The biogas composition of the digesters headspace was analyzed, three times per week.

The performance of R1 and R2 was carried as follows (see Table 4.1). Initially (stage I), both digesters were only fed with SS until both systems showed similar operational conditions (i.e. biogas production, pH and alkalinity). Then (stage II), R1 started to co-

digest SS and PW1, while R2 was kept as a reference digester. Ten days after the beginning of stage II, PW2 was supplied instead of PW1. Later on, the co-substrate was change for BW (stage III) and then for AW (stage IV). Finally (stage V), the co-digestion was stopped and R1 was only fed with SS. The organic loading rate (OLR) of R1 during the AcoD was fixed at 3.0 g VS L_R^{-1} day^{-1}, therefore the mixtures between SS and FW were done in order to obtain 60 g VS kg^{-1}. The reference digester (R2) had an average OLR of 1.2 g VS L^{-1} day^{-1}.

The characteristics of R1 and R2 feedstock are summarized in Table 4.2. It has to be noted that several SS batches were used throughout the experimental period.

Table 4.1 Performance of co-digestion digester (R1) during the different stages

Stages	Stage I	Stage II	Stage III	Stage IV	Stage V
Period of days	0-60	61-130	131-189	190-240	241-280
Feedstock	SS	SS + PW	SS + BW	SS + AW	SS
SS/FW mixture (ww/ww)	100/0	87/13	79/21	70/30	100/0

Table 4.2 Feedstock characteristics

		Stage I	Stage II			Stage III		Stage IV		Stage V
	Units	SS	SS + PW1	SS + PW2	SS	SS + BW	SS	SS + AW	SS	SS
TS	g L^{-1}	31.6 ± 1.1	66.8 ± 8.2	64.0 ± 2.5	31.1 ± 1.8	66.7 ± 2.2	30.9 ± 1.2	58.4 ± 2.0	29.9 ± 1.1	30.1 ± 1.7
VS	g L^{-1}	24.0 ± 0.9	61.1 ± 9.5	57.8 ± 1.7	23.7 ± 1.4	60.5 ± 2.1	25.1 ± 0.1	54.6 ± 0.8	26.1 ± 0.7	23.6 ± 2.0
PA	g $CaCO_3$ L^{-1}	0.4 ± 0.1	-	-	0.3 ± 0.1	-	0.1 ± 0.0	-	0.1 ± 0.0	0.2 ± 0.1
TA	g $CaCO_3$ L^{-1}	1.2 ± 0.2	1.0 ± 0.1	1.8 ± 0.5	2.0 ± 0.5	0.9 ± 0.2	1.7 ± 0.2	1.2 ± 0.2	1.5 ± 0.1	1.9 ± 0.5
pH	-	6.4 ± 0.2	4.9 ± 0.1	5.4 ± 0.1	6.4 ± 0.1	5.3 ± 0.1	6.0 ± 0.1	5.4 ± 0.2	6.0 ± 0.1	6.2 ± 0.1
VFA	g L^{-1}	1.4 ± 0.3	2.8 ± 0.3	2.0 ± 0.4	1.9 ± 0.1	2.1 ± 0.6	1.8 ± 0.6	2.2 ± 0.4	2.3 ± 0.3	2.1 ± 0.1

4.2.3 Analytical methods

TS and VS were determined following the guidelines given by the standard methods 2540G (APHA et al., 2012), where VFA losses during the TS determination were taken into account and then combined to give a final TS and VS value (S Astals et al., 2012a; Peces et al., 2014). TA and PA were determined by a titration method at pH 4.3 and at 5.75, respectively and the IA by the difference between TA and PA (Gianico et al., 2013). Individual VFA were analyzed by a HP 5890-Serie II chromatograph equipped with a capillary column and flame ionization detector (S Astals et al., 2012a). The biogas composition was determined with a Shimadzu GC-2010+ gas chromatograph equipped with a thermal conductivity detector and a Carboxen® column (Romero-Güiza et al., 2014a). The biogas and methane productions are reported at standard temperature and pressure conditions (i.e. 0 ºC and 1 atm).

4.3 Results and discussion

4.3.1 From mono-digestion to co-digestion.

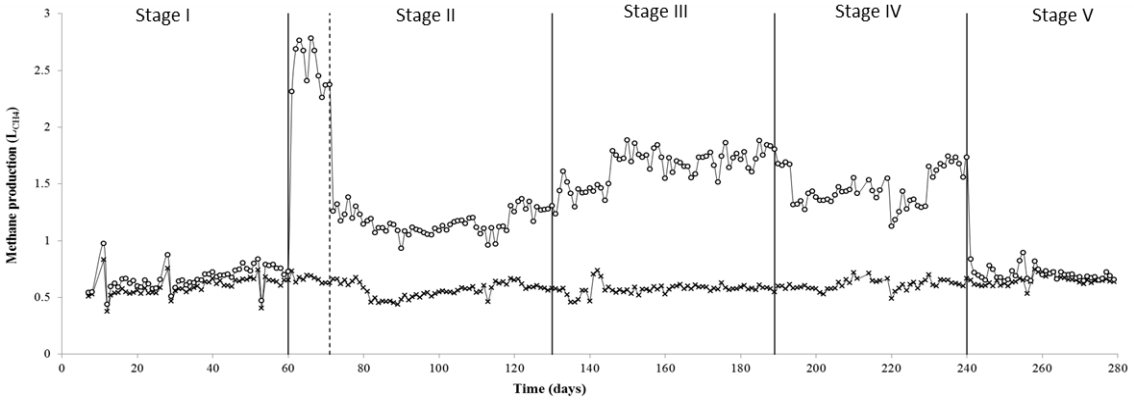


Figure 4.1 Methane production of R1 (○) and R2 (×); change of stages (▬▬).

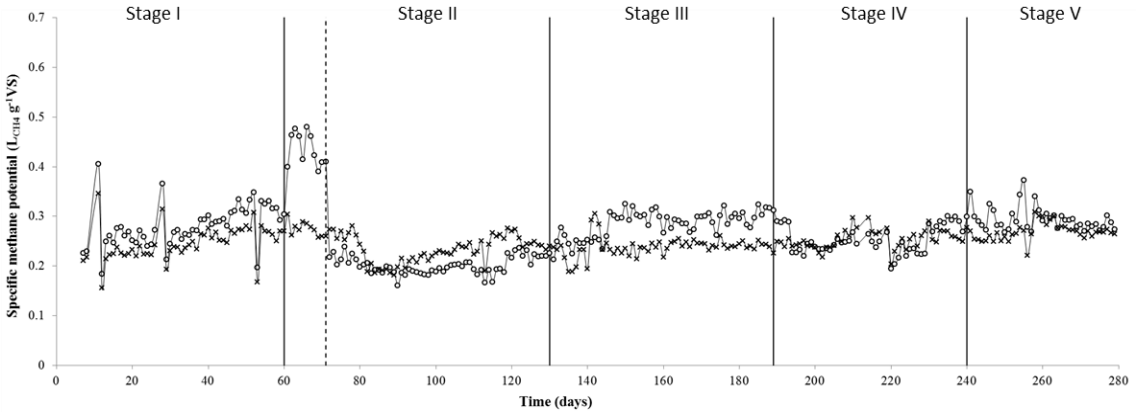


Figure 4.2 Specific methane production of R1 (○) and R2 (×); change of stages (▬▬).

In the figures each stage is divided by a black solid bar, while the black dotted bar represents the change from PW1 to PW2. At Stage I both digesters were fed only with SS until they achieved similar stationary conditions (pH 7.3 ± 0.1, TA 3.7 ± 0.2 g $CaCO_3$ L^{-1}, VFA 0.06 ± 0.01 g L^{-1} and 0.28 ± 0.04 L_{CH4} g^{-1} VS for R1 and 0.25 ± 0.03 L_{CH4} g^{-1} VS for R1). Later on, co-digestion started with the addition of PW1 in R1 feedstock (Stage II). The introduction of PW1 led to an increase of the OLR from 1.2 ± 0.1 to 2.9 ± 0.2 g VS L^{-1} day^{-1}. The increase of the OLR was reflected on VFA and alkalinity values during a short period of time (Fig. 4.3 and 4.4).

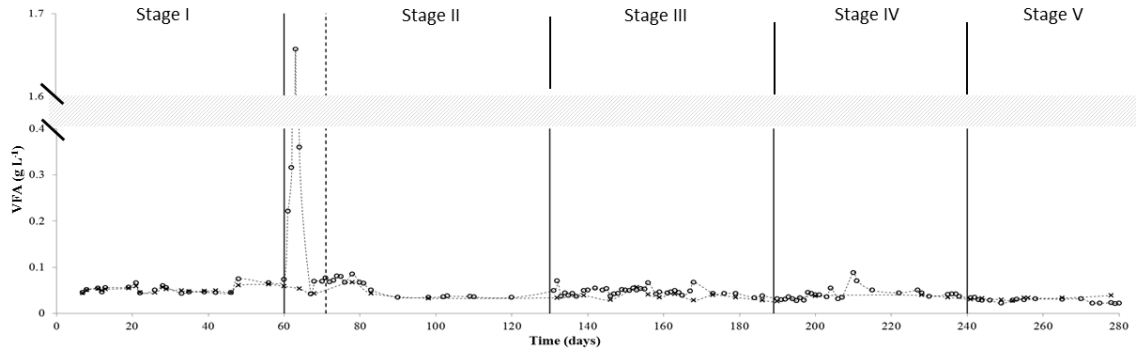


Figure 4.3 VFA from R1 (○) and R2 (×) effluent; change of stages (▬▬).

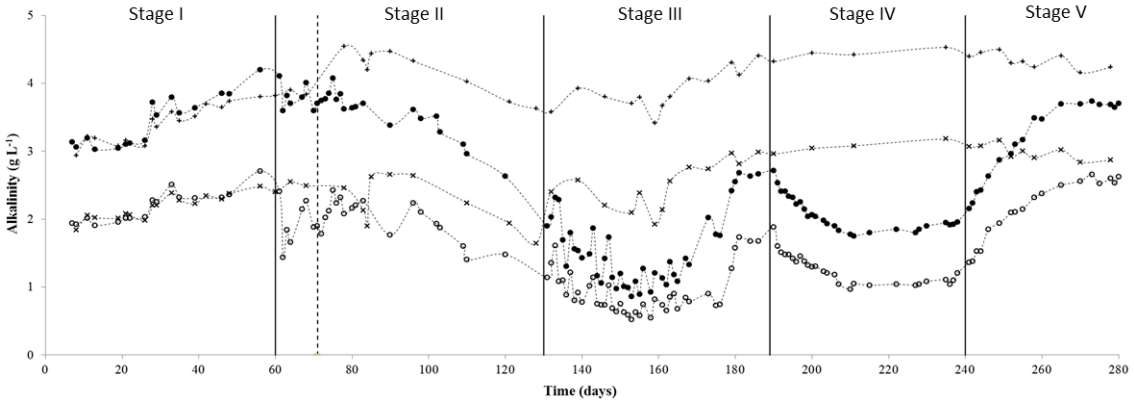


Figure 4.4 TA (●) and PA (○) of R1 effluent TA (+) and PA (×) of R2 effluent; change of stages (▬▬).

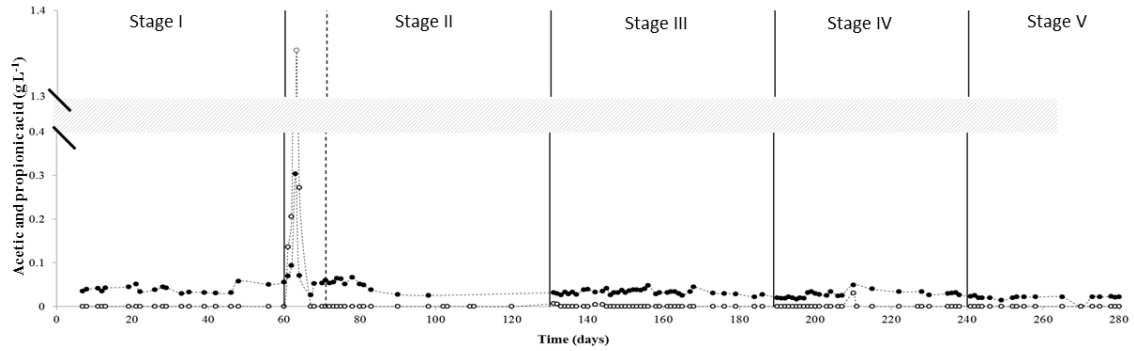


Figure 4.5 Acetic (●) and propionic (○) acid from R1 effluent; change of stages (▬▬)

On the one hand, the VFA concentration rose up from 0.07 to 1.70 g L^{-1} in only three days, being propionic acid (1.35 g L^{-1}) the main VFA (Fig. 4.5). In fact, only the levels of acetic and propionic acid increased, while the other VFA remained at the same level. Normally, in anaerobic digesters some intermediates, such as VFA, accumulate when the bacterial population is exposed to a sudden perturbation like a change in the OLR (Boe et al., 2010; Peces et al., 2013a; Peck et al., 1986). When the maximum concentration of VFA was achieved (3 days after the perturbation), acetic and propionic acid started to decrease at the same time during five days until constant values were achieved (0.04 g L^{-1} for acetic and below 0.01 g L^{-1} for propionic). While the propionic concentration peaked 1.35 g L^{-1}, which is within the critical limit reported in the literature (0.9 – 2.2 g L^{-1}), the prompt return of propionate to basal levels indicated that the system was not severely affected by the addition of PW1 as co-substrate (Blume et al., 2010; Nielsen et al., 2007; Wang et al., 2009).

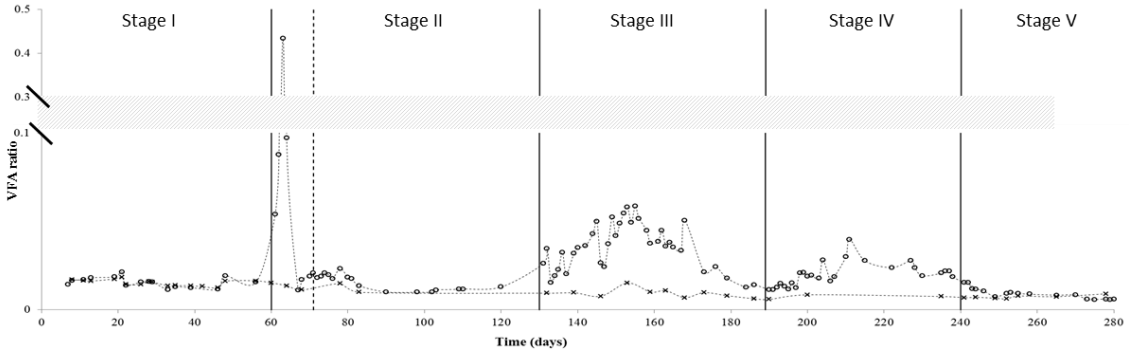


Figure 4.6 VFA/TA from R1 (○) and R2 (×) effluent; change of stages (▬▬).

The same conclusion can be obtained from the evolution of the VFA/TA ratio (Fig. 4.6). The ratio overcame the critical value for stable operation (VFA/TA > 0.4) three days after the co-substrate addition but returned to previous levels within two days (VFA/TA= 0.02). On the other hand, alkalinity values were also altered by the increase of the OLR. IA rose up from 1.5 to 2.2 g $CaCO_3$ L^{-1} as result of VFA accumulation; whereas PA decreased from 2.7 to 1.4 g $CaCO_3$ L^{-1} due to the neutralization of H^+ by the acid-base pairs (Fig. 4.4). However, as happened with the VFA concentration, the levels of these parameters returned to their previous levels 5 days after the perturbation (2.3 and 1.7 g $CaCO_3$ L^{-1} for PA and IA, respectively). It should be mentioned that the recorded stability levels were slightly better than those reported in the literature for stable AcoD operation between SS and FW (VFA: 500 - 550 mg L^{-1}; TA: 2.0 - 5.0 g $CaCO_3$ L^{-1}; VFA/TA 0.1 - 0.2) (H Bouallagui et al., 2009; Habiba et al., 2009).

At the 70th day PW1 was changed for PW2, a peach residue that presented a high quantity of fibers and seeds. As illustrated in all the figures, the change of the PW composition did not significantly disturb R1 stability (e.g. the VFA/TA ratio remained at the same level, p=0.11), but reduced the SMP from 0.45 to 0.22 L_{CH4} g^{-1} VS (p<0.01). Moreover, when using PW2 as co-substrate the SMP of R1 (0.20 ± 0.03 L_{CH4} g^{-1} VS) was significantly lower than the recorded in R2 (0.23 ± 0.03 L_{CH4} g^{-1} VS) (p<0.01), nonetheless, in absolute values the methane production of R1 was about 110% higher than in R2 (Fig. 4.1). At day 100, when the SS was changed the alkalinity fell down from 3.3 to 2.1 g CaCO3 L^{-1} while the methane production was not significantly influenced (p<0.01).

4.3.2 First co-substrate change: From peach waste to banana waste co-digestion

At day 131, PW2 was changed by BW which represented a change in the feedstock composition while keeping the same OLR. As happened before, when PW1 was changed by PW2, the amount of VFA was not considerably affected by the co-substrate change. Actually, the VFA concentration in R1 was nearly constant during all the third stage and similar than the R2 levels with an average of 0.04 ± 0.01 g L^{-1} ($p=0.30$) (Fig. 4.3). This behavior demonstrated that the biomass was not disturbed when only the co-substrate was changed. Acetic and propionic fluctuations at days 142 and 143 were related to digester operation rather than to AcoD.

Besides, the alkalinity in R1 presented an important change. TA decreased in R1 from 2.0 to about 1.1 g $CaCO_3$ L^{-1} (Fig. 4.4), likely due to the waste composition. In contrast to PW1 and PW2, BW was collected from the grocery and grinded in the laboratory so it had not suffered an industrial extraction process before it was fed to the digester. Therefore, the biodegradability of the BW was higher and its addition led to a decrease in the TA. Because of the higher biodegradability, the R1 SMP presented higher values (0.30 ± 0.02 L_{CH4} g^{-1} VS) than those achieved by the reference digester (0.24 ± 0.01 L_{CH4} g^{-1} VS) and during the co-digestion with PW2 (0.20 ± 0.03 L_{CH4} g^{-1} VS) ($p<0.01$ and $p<0.01$, respectively) (Fig. 4.2). It is worth noting that stable methane productions were reached just one week after the co-substrate change. Bolzonella et al. (2003) also observed the same behavior when the most biodegradable waste was added in a semi-dry anaerobic digester. Specifically, when the OLR of the reactor treating the most biodegradable waste was increased, the variation of the stability parameters was higher and the TA was lower than before even if the reactor achieved stable conditions. Finally, it can be seen that, at the end of the stage, the alkalinity rose up in both digesters because the alkalinity of the sewage sludge fed increased from 0.8 ± 0.2 to 1.5 ± 0.3 g $CaCO_3$ L^{-1}.

4.3.3 Second co-substrate change: From banana waste to apple waste co-digestion

Similar behavior than in the transitory state between II and III was observed when BW was changed by AW while keeping the OLR. Again, a change in the co-substrate did not induce a rise in the VFA concentration. However, TA decreased progressively in 20 days from 2.7 to 1.8 g $CaCO_3$ L^{-1} and afterwards it remained at this value until the end

of the stage (Fig. 4.4). As before, AW biodegradability was expected to be higher than PW biodegradability, since it was obtained from the grocery. Nonetheless, the current feedstock presented a lower alkalinity than the other mixtures inducing that change in alkalinity values ($p<0.01$).

During the co-digestion of AW the R1 SMP was identical to R2 ($p=0.49$), but lower than the values registered during BW co-digestion ($p<0.01$). That behavior suggested that AW was less biodegradable than BW. These results are in accordance to those published by Raposo et al. (2012) who reported less biodegradability for apple waste than for banana waste in AD batch tests. Even though, the biodegradability of the FW obtained from the industry depends on the fruit processing. However, such difference could also be related to the organic matter composition (COD-to-VS ratio) rather than to a lower waste biodegradability.

Table 4.3 Average of SMP and methane production obtained from R1 and R2

	Units	**Stage I**	**Stage II**	**Stage III**	**Stage IV**	**Stage V**
SMP R1	L_{CH4} g^{-1} VS	0.28 ± 0.04	0.20 ± 0.03	0.30 ± 0.02	0.26 ± 0.03	0.28 ± 0.03
SMP R2	L_{CH4} g^{-1} VS	0.25 ± 0.03	0.23 ± 0.03	0.24 ± 0.01	0.26 ± 0.02	0.27 ± 0.02
Methane production R1	L_{CH4} day^{-1}	0.67 ± 0.10	1.16 ± 0.10	1.72 ± 0.09	1.45 ± 0.2	0.69 ± 0.25
Methane production R2	L_{CH4} day^{-1}	0.6 ± 0.08	0.55 ± 0.06	0.58 ± 0.02	0.62 ± 0.05	0.66 ± 0.05

4.3.4 From co-digestion to mono-digestion

At the end of the study (Stage V), R1 was fed again only with SS and therefore the OLR of R1 was decreased to 1.2 ± 0.1 g VS L_R^{-1} day^{-1}. As expected, when the OLR was decreased, VFA did not present any change or fluctuation and remained at the same level (0.02 g L^{-1}) (Fig. 4.3). These results show that a decline in the OLR does not make any fast change in the stability parameters indicating that the digester was working under stable conditions. However, the levels of alkalinity presented a significant change due to the fed of only SS. TA increased in 25 days from 2.2 to 3.7 g $CaCO_3$ L^{-1}; however, even after 40 days TA values in R2 where lower than that the observed in R2 ($p<0.01$) (Fig. 4.4). As expected, co-digestion suppression resulted in a significant reduction of the methane production (from 1.1 to 0.5 L_{CH4} L_R^{-1} day^{-1}) because of the OLR decrease (Fig. 4.1).

4.4 Conclusions

To make the most of the non-capacity of waste water treatment plant Anaerobic digester, the Anaerobic co-digestion of sewage sludge with agro-industrial wastes was performed. Due to the seasonality of fruit wastes, the co-substrate was changed during the experiment and the reactor performance was evaluated during the transitory state. The main conclusions drawn from the study are summarized as follows:

- When the co-substrate was changed the process stability was not affected. Only volatile fatty acids concentration increased during a short period of time when Anaerobic co-digestion started due to the sudden increase in the organic loading rate.
- Due to the high biodegradability of some fruit wastes, the alkalinity decreased during Anaerobic co-digestion. To avoid the process failure, the sewage sludge used must have a good buffer capacity.
- The methane production could be very low if the co-substrate contains a high amount of fibers. A strategy to improve the hydrolysis of those co-substrates needs to be studied to avoid negative economical balances.

5. Effect of waste paper suppression on organic fraction of municipal solid waste anaerobic digestion: Biogas and digestate evaluation

Abstract

In mechanical biological treatment plants municipal solid waste is separated and/or treated to obtain different products like metals, refuse derived fuel, compost and methane. Waste Paper can be used in anaerobic digestion to generate methane but it can be used to produce other byproducts which, depending on the economic scenario, they can be more economically valuable than methane.

In the following study, the effect of removing half or all the waste paper on the methane production, digester performance and digestate quality was evaluated.

Since waste paper contains recalcitrant compounds such as lignin, its removal increased the feedstock biodegradability and hence increasing the SMP in a 27% when all the paper is removed and in a 17% when only half of it is removed. Nevertheless the digester performance and the digestate quality were negatively affected due to the increase in biodegradability. The digester treating a feedstock with waste paper needed fewer amounts of days to recover from an instability period. Besides, more residence time will be needed to obtain all the methane from the feedstock and therefore to get a digestate with a good quality.

This chapter was presented as poster communication in:

MBT plant revamping: Does waste paper can be separated prior OFMSW anaerobic digestion? *IWWATV - Industrial Water & Wastewater Valorization & Treatment,* Athens, Greece, 21-23rd May 2015.

As an oral communication in:

Effect of paper fraction on the mesophilic anaerobic digestion of OFMSW. Biogas and digestate evaluation. 9th International Conference ORBIT 2014. New Challenges, New Responses in the 21st Century, Gödöllő, Hungary, June 2014.

And then in preparation for publication as:

Fonoll, X., Astals, S., Dosta, J., Mata-Álvarez, J., 2015. **Effect of waste paper suppression on OFMSW anaerobic digestion: Biogas and digestate evaluation.**

5.1 Introduction

Even if wastes have been always presented as a burden for the society, nowadays, MSW are presented as new resource for the generation of energy (biogas production or incineration) and other materials such as compost, refuse derived fuel (RDF) or bioethanol (Pandyaswargo et al., 2012).

For the OFMSW, several life cycle analysis studies have claimed that mechanical-biological treatment (MBT) with AD have a better environmental performance compared to other waste management options (Abeliotis et al., 2012; Beylot et al., 2015; Pires et al., 2011). These plants are constituted by different mechanical processing steps which prepare MSW for the following biological stages. Those stages are designed according to MSW characteristics and its collection type. Usually, the plant outputs after the treatment of MSW are recyclable (mostly metals) and compostable materials, RDF, biogas and a fraction of residuals. Sometimes, the mechanical steps are not well designed and non-organic residuals materials can be introduced in the digester leading to a plant malfunction or even failure due to pipe/equipment wearing and dossing material accumulation in the digester. These problems are especially noticeable in MBT plants treating mixed or residual waste. Some studies to solve them have recently been published in the literature. One of these studies (Romero-Güiza et al., 2014b) was based on the changing of the old pulper system by new sorting system mainly composed of an optical sorter. The studied plant (Sant Adrià del Besós, Barcelona; 186000 tons per year of residual waste) got an energy efficiency index of 2.2 kWhproduced/kWhconsumed thanks to an improvement on its digester feedstock. With such improvement new scenarios of MSW recycling are opened.

The characterization of the MSW can change according to different countries, however, paper and cardboard are the second main compound in MSW (15%-30% in wet basis) (Bolzonella et al., 2006; Montejo et al., 2010; Saint-Joly et al., 2000). Due to the good performance achieved with the optical sorter , this kind of technologies can be more implemented in the new MBT plants and the separation of new recyclable materials like waste paper (WP) could be possible. Waste papers are recently presented as a perfect candidate for the production of bioethanol because of many reasons: (1) they are abundant in MSW, (2) they contain high levels of carbohydrates necessary for the production of bio-ethanol and (3) they are easy to digestate when compared to other lignocellulosic feedstock. The demand for bioethanol in the EU is expected to rise to

28.5 billion liters by 2020 and some studies have proved that bioethanol produced from waste papers is cheaper than petrol production (Wang et al., 2013, 2012a). However, the effect of WP removal on the AD process needs to be studied to evaluate how the digester performance and digestate quality can be affected.

Even though there are many AD studies regarding the effect of paper on biogas production, they do not pay attention on the digestate condition which is also another important economic input for the plant. Those studies concluded that, despite achieving an increase in the SMP due to an increase in the feedstock biodegradability, the removal of WP will also decrease the absolute methane production being prejudicial for the viability of the MBT plant. According to the substrate type, when digesters present low SMP the digestate quality can be affected due to a high remained methane potential on it (Lehtomäki et al., 2007; Wrap, 2010). Recalcitrant AD compounds such as lignin present in WP increase the respirometry index of digestate which means that the compost process will require longer operation times increasing the operational costs (Bsi, 2011; Montejo et al., 2010; Ponsá et al., 2008). Besides, the viability of the plant does not need to be negatively affected by the suppression of WP from the feedstock since an economic input can be obtained from the bioethanol produced or a co-substrate such as sewage sludge or fats, oils and greases (FOG) from waste water treatment plants (WWTP) can be used to improve the absolute methane production (Mata-Alvarez et al., 2014b). These kinds of co-substrates are good candidates since WWTP are usually located close to MBT plants.

In the present study, the methane production, the stability of the process and the digestate quality were evaluated when WP was removed from the OFMSW.

5.2 Materials and methods

5.2.1 Substrate and inoculum collection

The samples used for the substrate preparation were obtained from a MBT plant in Sant Adrià del Besós (Barcelona). Residual waste (organic matter remaining after bio-waste collection from a non-source sorted collection) (RW) was collected just before the digester feeding. One sample of MSW was obtained before being processed by the optical sorter and it was divided in three categories: Bio-waste which represented a 38% (only organic matter) (BioW), WP (23%) and others (38%) (This last fraction was discarded). The BioW and WP samples were characterized (Table 5.1) in order to make sure that all the BioW and WP batches used were prepared with the same

characterization. Water process was obtained also just after the centrifugation of the AD digestate.

The inoculum used was obtained from a lab-scale digester treating similar OFMWS at a HRT of 18 days (Fonoll et al., n.d.). All the samples were stored at 4ºC until their use.

Table 5.1 Characterization in % of BioW and WP (ww)

	BioW		WP
Vegetable	36	Cellulose	49
Fruits	43	Cardboard	22
Meat	13	Office paper	8
Carbohydrates	8	Newspaper	21

5.2.2 Reactor configuration and feedstock preparation

Two 5 L lab-scale semi-continuous stirred tank reactors with a working volume of 3.5 L were used at mesophilic conditions (35ºC). The operational temperature was ensured by circulating water from a heated water bath through a jacket surrounding the reactor. To aim an optimum waste treatment scenario (High flows of waste treated and low digester volume), the hydraulic retention time (HRT) of both digesters was set at 15 days. The biogas production was recorded every day and its composition was evaluated from the headspace once per week.

The study was divided in 4 stages (Table 5.2). During the first stage both reactors were fed with RW which is normally fed in the plant to evaluate the biogas production and the digestate stability that the plant usually has and to compare the results with stage II, where BioW was used. This comparison will evaluate the performance achieved after the revamping strategy. In order to evaluate the same parameters when the paper is half or not separated at all, during the third stage R2 was fed with a mixture of 85% of BioW and 15% of WP. In the fourth stage, R2 was fed with a mixture of 70% of BioW and 30% of paper, as if the paper was not removed. In the plant, the feedstock contains a 60% of pure organic matter a 25% of WP and 5% of inert matter. For this study the substrate contained 30% of WP because the mixtures were prepared without using inert materials to avoid operational problems. During the last two stages R1 was kept as a reference reactor and it was fed with the same BioW without changing any characteristic. Besides, to really evaluate the impact of paper in the digester performance both reactors were operated at same OLR (2.9 gVS L_R^{-1} day^{-1}) even though

the OLR varied due to the intrinsically heterogeneity of MSW.

Before using RW, the substrate was screened using a sieve with a pore diameter of 1 cm. The fraction with a particle size below 1cm was grinded before used. In the MBT plant the feedstock is first grinded and diluted with water process obtained after the digestate centrifugation (Romero-Guiza et al. 2014). This action was performed on the feedstock used in stages II, III and IV. The same water process was used to dilute the BioW and WP used before grinding them. The substrate was diluted to have a TS% equal to 5, the same used in the MBT plant.

Table 5.2 Performance of both reactors during the whole study

Stages	Stage I		Stage II		Stage III		Stage IV	
Reactor	R1	R2	R1	R2	R1	R2	R1	R2
Period of days	0-40		40-80		80-123		123-210	
Feedstock	RW	RW	BioW	BioW	BioW	BioW + WP	BioW	BioW + WP
mixture (ww/ww)	100/0	100/0	100/0	100/0	100/0	85/15	100/0	70/30

5.2.3 Analytical methods

Most of the analytical methods were performed following the Standard Methods for the Examination of Water and Wastewater (APHA et al., 2012). TS and VS were determined following the guidelines given by the standard methods 2540G, where VFA losses during the solids determination were taken into account and then combined to give a final TS and VS value (Peces et al., 2014). TA and PA alkalinity were determined by a titration method at pH 4.3 and at 5.75, respectively and the IA was determined by the difference between TA and PA (Ripley et al., 1986). Individual VFAs (acetate, propionate, iso-butyrate, n-butyrate, iso-valerate and n-valerate) were analyzed by a HP 5890-Serie II gas chromatograph equipped with a capillary column (NukolTM) and a flame ionization detector. The biogas composition was determined with a Shimadzu GC-2010+ gas chromatograph equipped with a thermal conductivity detector and a Carboxen column. The biogas and methane productions are reported at standard temperature and pressure conditions (i.e. 0ºC and 1 bar). The concentration of ammonium was analyzed by the use of an 863 Advanced Compact Metrohm ionic chromatograph using Metrosep columns.

The digestate stability was assessed by BOD_5 (aerobic) and residual methane test (anaerobic) of both digesters effluents. The aerobic respiration rate was selected by many authors as the most suitable parameter to assess aerobic biological activity and hence digestate stability (S Astals et al., 2012a; Ponsá et al., 2008; Trzcinski and Stuckey, 2011). The BOD_5 was done following the 5210D standard method procedure (APHA et al., 2012). A post-methane production test was done on the digestates by disposing them in close vessels at mesophilic conditions. Pressure and methane measurements were taken during 40 days to assess its methane production.

5.3 Results and discussion

Table 5.3 Averaged values of the performance of both reactors during the last 15 days of each stage

	Units	**Stage I**		**Stage II**		**Stage III**		**Stage IV**	
		R1	R2	R1	R2	R1	R2	R1	R2
SMP	L_{CH4} g^{-1} VS	0.30 ± 0.03	0.34 ± 0.03	0.41 ± 0.06	0.41 ± 0.04	0.43 ± 0.06	0.36 ± 0.03	0.47 ± 0.05	0.34 ± 0.04
Methane production	L_{CH4} day^{-1}	3.3 ± 0.3	3.6 ± 0.4	4.2 ± 0.7	4.2 ± 0.5	4.5 ± 0.7	3.8 ± 0.4	4.3 ± 0.4	3.1 ± 0.2
SMP	L_{CH4} g^{-1} VSremoved	0.10 ± 0.01	0.12 ± 0.01	0.13 ± 0.01	0.13 ± 0.01	0.12 ± 0.01	0.11 ± 0.01	0.16 ± 0.01	0.12 ± 0.01
VS removal	%	66 ± 3	66 ± 2	74 ± 2	74 ± 2	79 ± 2	77 ± 2	68 ± 2	67 ± 2
pH	-	7.7 ± 0.1	7.8 ± 0.07	7.8 ± 0.1	7.8 ± 0.1	7.7 ± 0.0	7.6 ± 0.1	7.8 ± 0.1	7.8 ± 0.1
PA	gCaCO3 L^{-1}	7.3 ± 0.4	7.2 ± 0.1	5.2 ± 0.4	5.2 ± 0.4	4.8 ± 0.1	4.6 ± 0.1	6.3 ± 0.1	6.5 ± 0.2
VFA	mg L^{-1}	162 ± 44	180 ± 53	239 ± 85	239 ± 85	64 ± 14	66 ± 18	973 ± 155	474 ± 105
HAc	mg L^{-1}	150 ± 40	167 ± 46	202 ± 85	202 ± 85	42 ± 7	45 ± 10	920 ± 157	439 ± 108
HPr	mg L^{-1}	6 ± 4	7 ± 5	24 ± 15	24 ± 15	10 ± 3	10 ± 3	18 ± 3	12 ± 2

VFA/TA	-	0.02 ± 0.00	0.02 ± 0.01	0.08 ± 0.07	0.08 ± 0.07	0.01 ± 0.00	0.02 ± 0.00	0.15 ± 0.02	0.07 ± 0.02
NH3	mg L^{-1}	105 ± 30	135 ± 27	109 ± 28	109 ± 28	73 ± 7	62 ± 11	153 ± 44	173 ± 37

5.3.1 Effect of paper fraction on process stability

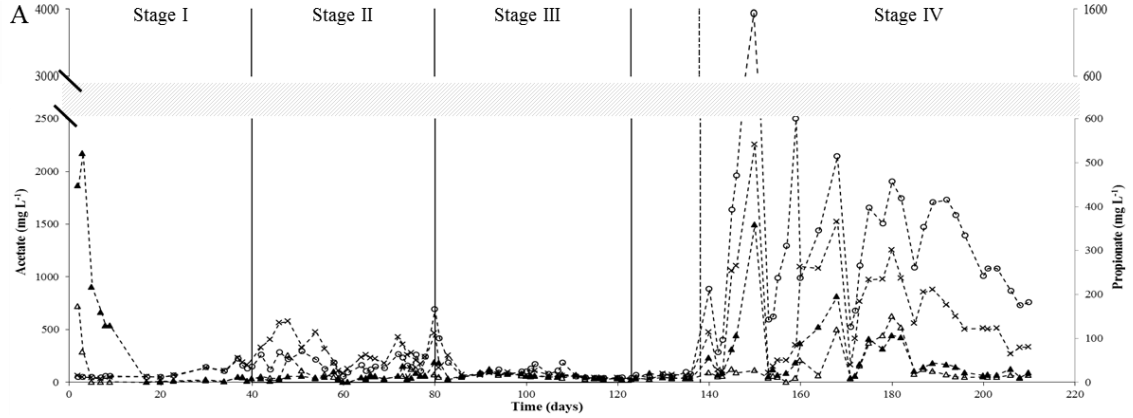


Figure 5.1 Acetic acid in R1 (○) and R2 (×) and propionic acid in R1 (▲) and R2 (Δ); change of stages (▬▬), Process water acidification (▬ ▬)

In Figure 5.1 all the stability parameters (VFA, Acetic and propionic acid and PA) of both digesters are presented. The vertical black lines show the separation between each stage. The start-up was done without any instability problem. At the beginning propionate levels were not the same in both digesters (Fig. 5.1). In R2 propionate levels were decreasing until they could not be detected in the 5th day. However, in R1 propionate levels were higher (500 mg L^{-1}) and took around two weeks to decrease until being not detectable. 2.5 HRT passed already being all the parameters equal and constant between both reactors (VFA=150 mg L^{-1}, PA=7600 mg$CaCO_3$ L^{-1}, VS removal = 65%, NH_3=100 mg L^{-1}, pH= 7.6 and VFA/TA=0.01; see Table 5.3). Then, at day 40, stage II started.

In this stage, only BioW was fed in both reactors without the presence of paper or any inorganic material. As is usual for AD (Fonoll et al., 2015) when the new feedstock was added the parameters changed. The PA decreased from 7500 to 5500 mg$CaCO_3$ L^{-1} in ten days because the feedstock biodegradability was higher than before (Table 5.3). Therefore, VFA were produced in high amounts and more alkalinity was needed. The VFA increased in R2 from 150 mg L^{-1} to 600 mg L^{-1} in 8 days, but, after reaching this concentration the levels could decrease to 300 mg L^{-1} in 10 days (Fig. 5.1). After those changes PA and VFA levels remained constant in both reactors as well as the other stability parameters during the whole stage (Table 5.3). Actually, those changes did not affect the stability of both systems. The VFA/TA (0.06) ratio was under its limit (0.4) (Callaghan et al., 2002), the ammonia was around 100 mg L^{-1} and the pH was constant around 7.7 (Table 5.3).

At the 80th day R2 started to be fed with a mixture of 85% of BioW and a 15% of WP. The stability was similar in both reactors since the parameters did not change too much (PA=4500 mg$CaCO_3$ L^{-1}, NH_3= 100 mg L^{-1}, pH= 7.6 and VFA/TA=0.02). Because of the decrease in the OLR (2.2 gVS Lr^{-1} day^{-1}), the VFA levels (especially acetate) started to be lower than 100 mg L^{-1} again in both reactors (Fig. 5.1).

At the 123th (Stage IV) day R2 started to be fed with a mixture of 70% of BioW and 30% of WP. During the first 15 days the stability was good in both reactors, any value changed except the PA which increased from 5500 mgCaCO3 L^{-1} to 6500 mgCaCO3 L^{-1} (Table 5.3). Unfortunately, the process water was acidified on the 138th day due to a technical problem with the fridge (dotted line) affecting the stability of the process. The VFA increased from 100 mg L^{-1} to 4500 mg L^{-1} for R1 and 2400 mg L^{-1} for R2 in 10 days (Fig. 5.1). All the VFA levels increased except caproic and heptanoic acid. The PA was not affected so the ratio VFA/TA, which normally refers to the stability in AD, only reached the value of 0.3, suggesting that the system was not working under unstable conditions. To recover the reactor performance the feeding was stopped during some days (152th, 153th, 162th, 163th, 165th, 169th and 170th) to degrade the remaining VFA and the process water was changed by a new batch. A new batch was used then in the day 150. Some of the acids (propionate, iso-valerate and iso-caproic) could decrease to their previous levels at the 185th day (35 days after the peak of acids). The other acids did not have time to decrease to their previous levels, however, only acetate presented high levels (750 and 350 mg L^{-1} for R1 and R2 respectively) while the other acids remained below 50 mg L^{-1}. This scenario suggested that the reactor became fragile against instability periods due to the WP suppression from the feedstock. As can be seen in Figure 5.1, R1 took more time to degrade acetate and its levels were 120% bigger than the acetate levels in R2 at the end of the experiment. Usually, when the WP is removed from the feedstock its biodegradability increase (S Astals et al., 2012b; Romero-Güiza et al., 2014b; Saint-Joly et al., 2000) and the methanogenesis rate is limited by the slow activity of acetoclastic methanogens, since their growing rate is several times slower than that observed for bacterial populations (Noike et al., 1985; Ferry James 1993). Therefore, when WP is removed from a system with a very low HRT, methanogenesis becomes the rate limiting step

5.3.2 Effect of paper fraction on methane production

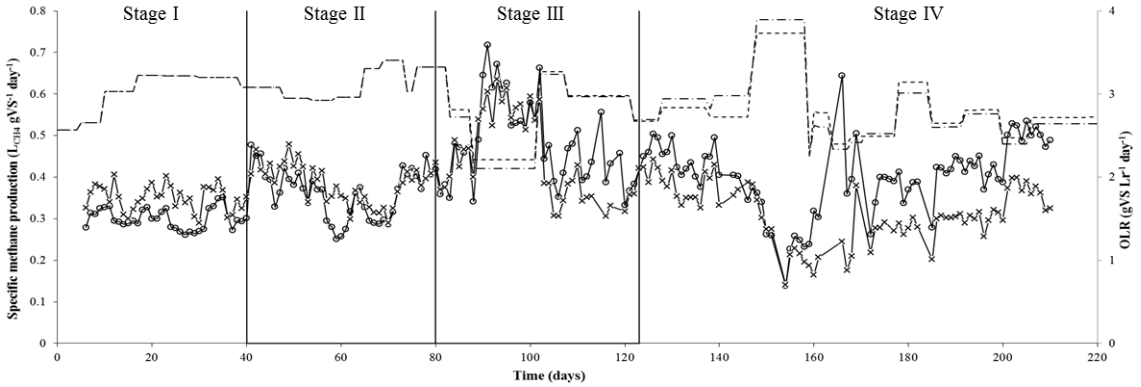


Figure 5.2 Specific methane production of R1 (○) and R2 (×); change of stage (——), OLR in R1 (— —) and R2 (— • —).

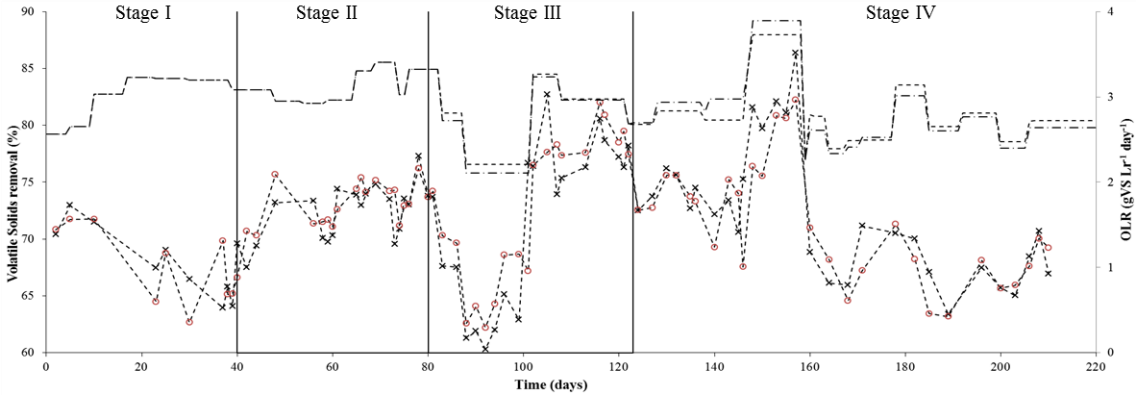


Figure 5.3 Volatile solids removal in R1 (○) and R2 (×); change of stage (——), OLR in R1 (— —) and R2 (— • —).

During the first stage the SMP was quite constant during the whole experiment even though it was a little bit higher in R2. For the last 15 days the averaged SMP in R1 was 0.30 ± 0.03 LCH_4 gVS^{-1} day^{-1} and in R2 0.35 ± 0.03 LCH_4 gVS^{-1} day^{-1} (Table 5.3). The VS removal decreased with time and got to the same value in both reactors (65%; p=0.94) (Fig. 5.3).

In the second stage the parameters regarding the biogas production and the VS removal presented some changes (Fig. 5.2 and 5.3). At the beginning of this stage the SMP (0.40 ± 0.03 LCH_4 gVS^{-1} day^{-1} and 0.42 ± 0.03 LCH_4 gVS^{-1} day^{-1} for R1 and R2 respectively) started to be higher than the SMP recorded in stage I (Fig. 5.2). However, the methane production decreased in both reactors from the 55^{th} day to the 70^{th} day (Table 5.3). A new batch was used on this period. Even if the OLR and the characterization was the

same (Table 5.1) a different kind of vegetable or fruit could be used and could have affected the biodegradability. In fact, MSW is normally affected by seasonality changes. After that period the SMP could achieve the same values than the beginning of the stage and be equal between both reactors (0.42 ± 0.05 LCH4 gVS^{-1} day^{-1}, p=0.9). Besides, the system presented an increase in the VS removal from 65% to 75% (Fig. 5.3). These improvements in the SMP and in the VS removal with respect to stage I, is certainly due to an increase of the feedstock biodegradability. Since the levels of acetate and VFA did not decrease (Fig. 5.1) we can assume that methanogenesis and the fermentation rate did not increase either. Therefore, the increase in VS removal and SMP is probably due to an increase of the hydrolysis rate. This makes sense because no paper was added in the feedstock and in stage I the feedstock used contained a 25% of paper (Romero-Güiza et al., 2014b). Therefore WP removal increased both the hydrolysis rate and the feedstock biodegradability (increasing by a 20% the SMP).

When the third stage started the VS removal decreased in both reactors and was not constant during that period because a new batch was used with a low VS concentration (Fig. 5.3). As mentioned before, MSW presented a high heterogeneity which normally leads to a variation in the feedstock characteristics. In 8 days the VS removal decreased from 75% to 60% but 10 days later it could recover in both reactors to its previous value, 75%. Because of that, when the OLR was very low the SMP was also higher when compared to the rest of the experiment, but once the OLR was normal again in the 102^{th} day, the SMP was around the previous values (Fig. 5.2). During the last 20 days the SMP decreased a bit in R2 (0.36 ± 0.03 LCH_4 gVS^{-1} day^{-1}) being lower than the SMP in R1 (0.43 ± 0.06 LCH_4 gVS^{-1} day^{-1}, $p<0.05$). The decrease in the SMP and equality between both reactors in terms of VS removal suggests that the presence of 15% of paper in the feedstock affects its biodegradability decreasing the SMP by a 17% (Table 5.3).

Due to the acidification of the process water in the fourth stage, the SMP started to decrease at the 148^{th} day. The archaea were not performing correctly due to the acetate build up (Fig. 5.1) and the SMP did not stop to decrease during the next 20 days from 0.43 LCH_4 gVS^{-1} day^{-1} until achieve 0.15 LCH_4 gVS^{-1} day^{-1} (Fig. 5.2). When a new batch was used and the reactor could recover its stability the SMP could increase again. However, because of the paper addition up to 30% the SMP in R2 was 0.34 ± 0.04 LCH_4 gVS^{-1} day^{-1}, 27% lower than R1 (Table 5.3). Besides, due to the use of the new batch the VS removal decreased and was 65% (Fig. 5.3).

By the removal of WP the SMP obtained was very high. The values were very similar than some studies where pretreatments were applied with a high success. For example, by the use of a wet oxidation pretreatment Lissens et al. (2004) increased the SMP of food waste up to 0.57 $LCH_4\ gVS^{-1}\ day^{-1}$. Shahriari et al. (2013) obtained a SMP of 0.50 $LCH_4\ gVS^{-1}\ day^{-1}$ using thermal microwave pre-treatment in a single stage AD and working at an HRT of 15 days. Those increments are not very high compared to the production obtained in this study by just removing paper and those pre-treatments are usually expensive (Cesaro and Belgiorno, 2014). Previous studies suggested that WP can affect the overall methane production. This could be solved by the introduction of other wastes as co-substrates. For example, Martín-González et al. (2011) could increase the absolute methane potential from 1.52 $L_{CH4}\ L_R^{-1}\ day^{-1}$ to 2.45 $L_{CH4}\ L_R^{-1}\ day^{-1}$ (a 60% of improvement) using FOG from a wastewater treatment plant. Cuetos et al. (2008) used as a co-substrate SHW and the methane production increased from 0.95 $L_{CH4}\ L_R^{-1}\ day^{-1}$ to 1.85 $L_{CH4}\ L_R^{-1}\ day^{-1}$ (95% of improvement). The co-substrates used by the authors can be easily found in the city near the MBT plants.

5.3.3 Digestate stability

Table 5.4 Results from the BOD5 and the post-methane test done in R1and R2.

	BOD_5 (mg $O_2\ gVS^{-1}$)		Post-methane (mg $O_2\ gVS^{-1}$)	
	R1	R2	R1	R2
Stage I	238 ± 84	213 ± 108	478 ± 40	488 ± 40
Stage II	264 ± 16	260 ± 20	595 ± 34	617 ± 43
Stage III	281 ± 6	255 ± 17	669 ± 34	584 ± 20
Stage IV	267 ± 17	191 ± 18	561 ± 37	466 ± 11

The results of BOD_5 and the post methane test are both methods, presented in Table 5.4, could lead to the same conclusions regarding digestate stability. As expected, in stage I and stage II the digestate from both reactors had the same stability but, it decreased when both reactors were fed with BioW instead of RW. As BioW was a mixture of only organic matter without any recalcitrant compound such as paper or others (Table 5.1), and both reactors were fed under the same OLR, it was expected to the digestate from stage II to be more stable. Even if the SMP was higher than before, due to the low HRT in the system not all the methane potential could be recovered during the process, and

therefore, all the potential remained in the digestate. When Trzcinski and Stuckey (2011) evaluated the effects of different parameters such as SRT or OLR on the digestate stability, they had similar behavior in the digestate. The digestate coming from a very biodegradable feedstock which presented high VS removal had worse stability than other substrates less biodegradable and working under the same SRT.

WP contains high levels of lignin which cannot be degraded in anaerobic conditions and due to cross-linking with cellulose, its degradation is physically impossible for enzymes (Tsavkelova and Netrusov, 2012; Tsavkelova et al., 2012a). For that reason lignin and cellulose can be present in the digestate affecting the degradation rate and making the time length of the process longer (Donovan et al., 2010; Montejo et al., 2010). However, in this study, after the digestion of BioW and WP together, the digestate obtained presented better stability than the digestate that comes from the digestion of BioW only. As was suggested before, this difference between R1 and R2 digestates can be explained by the low HRT used and the high biodegradability of the feedstock. Thus, when WP is removed from the feedstock the digestate stability is affected and longer time processes would be needed to obtain compost with the same quality. To avoid this problem, if WP is separated, the HRT of the plant should not be so low to get all the methane potential from the high quality feedstock. This fact would also increase the absolute methane production.

The BOD_5 test has its limitations since they tend to preferentially decompose the readily degradable component and thus may not indicate potential long-term biodegradability. In fact, anaerobic test last longer enough to decompose the non-readily degradable matter. In fact the milligrams of O_2 per grams of VS that represents the results obtained with the post-methanation test are higher than the values obtained with the BOD_5 (data not shown). Besides, the residual methane potential of the digestate is an important factor because emissions of methane and odors are not desirable for a stable digestate and contributes to the climate change (Lehtomäki et al., 2007). Nevertheless, all the post-methanation values presented in the digestates were under the limit ($0.25\ L_{CH4}\ gVS^{-1}\ day^{-1}$) set by Wrap (2010) for market placement. An increase in the HRT will lead to a lower waste treatment capacity or to a higher concentration of metals in the digestate since less process water will be used (Trzcinski and Stuckey, 2011).

5.4 Conclusions

In this work, the reactor performance was evaluated when waste paper, a lignocellulosic substrate, was removed from the feedstock. The conclusions are summarized as follows:

- The waste paper separation from the municipal solid wastes increased the feedstock biodegradability.
- Half or total separation of waste paper can increase the specific methane and 27% respectively.
- Due to the feedstock biodegradability increase the reactor became fragile against instability periods. In case that such episode occurs, a digester without waster paper in the feedstock will take long time to recover.
- The digestate quality gets worse when waste paper is removed if low hydraulic retention times are applied.
- The waste paper removal will decrease the absolute methane production in the plant, so, the market of biogas and by-products obtained from waste paper should be studied before removing it. The use of a co-substrate can be also another solution.

6. Anaerobic co-digestion of agro-wastes under high ammonia concentrations: Low temperature and ultrasounds pretreatment application on barley waste

Abstract

Pig manure is a waste highly generated due to the population increase and the intensive livestock farming. The management of Pig manure through anaerobic digestion produces methane and compost, products very attractive for the agriculture. Due to the low C/N ratio, the methane production form pig manure only is very low and hence, anaerobic co-digestion with high C/N ratio substrates is almost mandatory. However, agricultural wastes such as barley spent grain present high concentration of recalcitrant compounds.

Two different pretreatment (Ultrasound and Low-Temperature) were applied on barley spent grain to degrade lignocellulosic compounds and the substrate was co-digested with pig manure in a semi-continuous stirred reactor. Ultrasound and low-temperature pretreatment could increase the methane production by 12% and 26% respectively but, after 37 days, the production decreased to the same level than the reference reactor where no pre-treatment was applied.

An energy balance was applied during the period where the methane production was higher. Only low-temperature pretreatment was suitable for application in a full scale scenario because the overheating can be reused to heat the digester. Besides, during the AcoD of pig manure and barley spent grain the levels of ammonia were extremely high suggesting that syntrophic ammonia oxidizing bacteria were active.

This chapter was presented as <u>poster communication</u> in:

Anaerobic co-digestion for agro-wastes: Ultrasounds pre-treatment to improve biogas production. 13th Mediterranean Congress of Chemical Engineering, Barcelona, Spain, September 2014.

And then in preparation for <u>publication</u> as:

- Fonoll, X., Roig, R., Dosta, J., Mata-Álvarez, J., 2015. **Anaerobic co-digestion of barley waste and pig manure under high ammonia concentrations: Low temperature and ultrasounds pretreatment application.**

6.1 Introduction

In 2010 this sector in Europe was composed by 12 million of farms, 172 million hectares of agricultural land and 25 million people involved in agriculture activities (EC, 2013a). It is strongly recognized that agricultural sector provide a huge potential for the provision of public good but it is also responsible for a large share of the pollution. Among all the wastes provided by agriculture the more pollutant usually are manures. These wastes contribute to GHG emissions and contain high concentration of human pathogens, nutrients, heavy metals, veterinary pharmaceuticals and natural excreted hormones (Manyi-Loh et al., 2013). Therefore member States will have to spend at least 30 % of their rural development funding from the EU budget on certain measures related to land management and the fight against climate change (EC, 2013b).

Treating those wastes through AD is very interesting from the point of view of agriculture since the effluent can be used as a fertilizer (Insam et al., 2015; Möller and Müller, 2012). However, animal wastes are characterized to have a very low methane yield and C/N ratio and high Total Kjeldahl Nitrogen concentrations which can lead to toxicity problems related to ammonia. Because of all these facts AD technology must be carefully monitored and making it economically suitable only for big farms or centralized units (Angelidaki and Ellegaard, 2003; S Astals et al., 2012a; Faulhaber et al., 2012; Swindal et al., 2010). Agro-industrial wastes like lignocellulosic materials, crop residues, vegetable oils or glycerin are presented as an attractive co-substrate for this kind of system due to their high C/N ratio (Mata-Alvarez et al., 2014c). With an economic input of 5100M€/year, Spanish brewery industry is crucial for the country economy. Brewers spent grain (BSG) represents the 85% of the residues generated in this industry and the best methods to obtain energy from it are thermochemical and biological processes (Gómez et al., 2010). Recently BSG has been used as a substrate for AD with excellent results and its characteristics are suitable the AcoD of Manure (Goberna et al., 2013; Menardo et al., 2012).

Despite having a suitable C/N ratio for the AcoD of PM, agro-industrial waste present the problem of having high concentration of lignocellulosic compounds (Azman et al., 2015). Lignin cannot be degraded in anaerobic conditions and some cellulose structures are really recalcitrant (Barakat et al., 2014). Moreover, the cross linking structure of lignin, hemicellulose and cellulose make very difficult the availability of hemicellulose and cellulose for enzymes (Tsavkelova and Netrusov, 2012; Tsavkelova et al., 2012b). All these facts make hydrolysis the rate limiting step and therefore not all the methane

potential is recovered (J. C. Frigon and Guiot, 2010; Noike et al., 1985).

Different kind of pre-treatment (Physical, chemical, biological or a combination of them) have been used for the degradation of lignocellulosic compounds and therefore, for the improve of the mixtures methane yield (Zheng et al., 2014). Methane yield from rice straw increased from 0.06 to 0.13 LCH_4 gVS^{-1} when Chandra et al. (2012) used a hydrothermal pretreatment followed by the addition of 5% of NaOH. Since each substrate have a different lignocellulosic structure, the same pretreatment can lead to different results. For example, *Ceriporiopsis subvermispora* was ineffective during the pretreatment of soybean (Wan and Li, 2011), but, the same kind of fungi (Ge et al., 2015) could increase the methane yield in a 265% of Albizia Chips.

Peces et al. (2015) used low-temperature and ultrasounds pretreatments (LTP and USP) on BSG in a batch experiment. After doing an economic analysis, the authors concluded that both pretreatments consumed more energy than the extra energy that could be obtained from them. Almost all of the pretreatment studies are performed in batch essays (Table 1.2) and sometimes better results are obtained in continuous experiments since in long stationary conditions biomass is adapted to the substrate.

The objective of the present study is to run the AcoD between PM and BSG in a semi-continuous experiment and evaluate the use of two different pretreatment (LTP and USP) on BSG to increase the SMP of the mixture.

6.2 Materials and methods

6.2.1 Substrates and inoculum

The substrate used for the experiment was a mixture of PM and BSG and the reactors were seeded with digested PM. The PM, a mixture of 95% (ww) of urine and feces and a 5% (ww) of food-industrial wastes, and the inoculum were both obtained from an AD centralized plant in Lleida, Spain. The BSG was obtained from a brewery in Barcelona after the saccharification process. The inoculum and PM were stored at 4ºC and BSG was stored in batch at -20ºC until their use. The substrates and inoculum characteristics can be seen in Table 6.1.

Table 6.1 Inoculum and substrates characteristics

		BSG	PM	Inoculum
TS	mg L^{-1}	208 ± 21	56 ± 5	31 ± 2
VS	mg L^{-1}	196 ± 22	43 ± 5	20 ± 2
pH	-	n.a.	7..8 ± 0.2	8.0 ± 0.1
PA	gCaCO3 L^{-1}	n.a.	2.10 ± 0.23	5.38 ± 1.0
TA	gCaCO3 L^{-1}	n.a.	6.16 ± 0.54	10.18 ± 2.2
VFA	g L^{-1}	n.a.	11.85 ± 1.87	0.19 ± 0.06
NH_3	mg L^{-1}	n.a.	204 ± 10	319 ± 25

n.a. Non analyzed

6.2.2 Experimental design

Three 5 L lab-scale semi-continuous stirred tank reactors were used for 170 days. The working volume was 3.5L and the digesters were heated at 35ºC by circulating hot water through a jacket surrounding the reactor. The biogas production was measured through a biogas counter (Ritter MGC-1) and its composition was analyzed once per week.

For all the reactors the OLR was set to 3.3 gVS L_R^{-1} day^{-1} during the first two weeks and after that period the OLR decreased to 2.5 gVS L_R^{-1} day^{-1} and remained constant for the whole experiment. Some variations could exist due to the heterogeneity of BSG. The HRT was 20 days during the first two weeks and after the HRT increased to 27 days. To set those parameters the reactors were fed with a mixture of PM (85%; ww) and BSG (15%; ww).

The experiment was divided in two periods. In the first period (start-up) all three reactors were fed with the same mixture of PM and BSG. At the 110^{th} day the second period started and R1 was kept as a reference reactor, R2 evaluated the effect of applying LTP on BSG and R3 evaluated the effect of applying USP on BSG. In a previous study (Peces et al., 2015) the effect of TS concentration on LTP and USP was evaluated. The highest methane production was obtained when the TS% of BSG was 10%. In order to obtain the highest methane production, the digestate was centrifuged and the supernatant was used to dilute the BSG in order to obtain a TS% of 10. The

supernatant was used instead of water because, in a practical scenario, it is more economically attractive.

6.2.3 Pretreatments

The parameters for the application of LTP and USP were set according to the highest methane yields obtained by (Peces et al., 2015) who studied the same pretreatments applied to the BSG obtained at the same time and the same brewery. Ultrasounds: The specific energy (E_s) applied for USP was 5000 kJ $kgTS^{-1}$ and the exposition time was calculated according to equation 1.

$$E_s = \frac{P \cdot t}{m \cdot TS} \qquad \text{Eq. 1}$$

Where P is the supplied power (0.07kW), t is the exposition time (s), m is the mass of diluted BSG (34g) used and TS is its TS concentration (gTS kg^{-1}). Diluted BSG samples were sonicated in a HD2070 Sonoplus Ultrasonic Homogenizer equipped with a MS 73 titanium microtip probe and working with an operating frequency of 20 kHz and a supplied power of 70 W. The ultrasonic probe was submerged until half-height of the sample. Temperature was not controlled during the ultrasound (US) pretreatment.

Low-Temperature: 34g of diluted BSG were heated in an oven for 24h at 60ºC inside of an air tight bottle flushed with N2.

6.2.4 Analytical methods

TS and VS solids were determined according to the guidelines given in APHA et al. (2012). TA and PA alkalinity were found out by a titration method at pH 4.3 and 5.75, respectively (Ripley et al., 1986). The VFAs and biogas composition were analyzed with a Shimadzu GC-2010+ gas chromatograph. For VFA determination the chromatograph was equipped with a capillary column and a flame ionization detector. The biogas composition was determined using a thermal conductivity detector and a Carboxen® column. The biogas and methane productions are reported at normal conditions (0 ºC and 1 atm). The concentration of ammonium was analyzed using an 863 Advanced Compact Metrohm ionic chromatograph equipped with a Metrosep columns. Nevertheless, due to technical problems with the device, NH_4^+ was analyzed using an ammonia probe (pH/mV CRISON MicropH 2002) between the 88^{th} and the 144^{th} day.

6.2.5 Energy balance

The implementation of USP and LTP was evaluated through an energy balance calculating the energy required by the pretreatment (Ei) and the energy that can be recovered through the improved methane production (Eo). The energy balance was applied in a hypothetical scenario where, in the LTP case, BSG is heated in a cylindrical tank at 60ºC for one day and, in the case of USP, BSG is treated using a commercial sonication system from Hielscher (48kW), quite implemented in waste water treatment plants (Cesaro and Belgiorno, 2014; Tyagi et al., 2014). The flow of BSG used for this hypothetical case was 1.0 tn day^{-1} In this study AcoD was run with a 15% (ww) of BSG, so, the mass flow of PM and supernatant were 6.6 and 1.1 tn $year^{-1}$ respectively. To evaluate the implementation of both pretreatments the energy required (Ei) and the energy that can be recovered by the pretreatment through the improved methane production The feasibility of the pretreatment was evaluated by the energy ratio=Eo/Ei, where Eo/Ei > 1 would indicate that the energy from the additional methane generated covers, at least, the pretreatment requirements (Passos et al., 2014)

The energy needed for LTP ($E_{i,LTP)}$ was calculated as follows:

$$E_{i,LTP} = (E_{i,LTP1} + E_{i,LTP2}) \quad \text{Eq. 2}$$

$$E_{i,LTP1} = w_b \cdot C_{pb} \cdot (T_{LTTP} - T_i) + w_s \cdot C_{ps} \cdot (T_{LTTP} - T_i) \quad \text{Eq. 3}$$

$$E_{i,LTP2} = A_{opt} \cdot U_i \cdot (T_{LTP} - T_i) \quad \text{Eq. 4}$$

Where: $E_{i,LTP}$ is the total energy needed for LTP, $E_{i,LT1}$ is the energy needed to heat BSG up to 60ºC and $E_{i,LTP2}$ represents the heat losses due to convection In equation 3 and 4 w_b and w_s are the mass flow of BSG (1.0 tn day^{-1}) and supernatant (1.1 tn day^{-1}) and C_{pb} and C_{ps} are the heat capacities of BSG and supernatant calculated according to Adl et al. (2012). A_{opt} is the tank surface, U is the heat transfer coefficient of the tank equal to 1.0 W/m^2 K (Adl et al., 2012), T_{LT} is the pretreatment temperature (60ºC) and T_i is the outside temperature (20ºC).

The tank to heat the diluted BSG was designed according to the following equations (Adl et al., 2012):

$$V = \frac{w_f}{\rho_f} \cdot 1.15 \quad \text{Eq. 5}$$

$$D_{opt} = \sqrt[3]{\frac{8V}{\pi}} \quad \text{Eq. 6}$$

$$A_{opt} = \frac{2\pi D_{opt}^{2}}{4} + \frac{4V}{D_{opt}} \quad \text{Eq. 7}$$

Where V represents the tank total volume, w_f is the mass flow of diluted BSG and ρ_f is its density of BSG mixed with the supernatant and measured in the laboratory (1.1 kg L^{-1}). The volume of the tank was a 15% overestimated. In Eq. 8 D_{opt} is the optimal diameter.

The heat recovered from LTP ($E_{o,LTP}$) to heat the digester was also calculated as an energy input according to Eq. 8:

$$E_{o,LTP} = w_f \cdot C_p \cdot (T_{LTTP} - T_D) \quad \text{Eq. 8}$$

Where T_D is the digester Temperature (37ºC).

The energy needed for USP (E_{iUSP}) is the power supplied by the commercial sonication system (48kW).

The energy obtained by the overproduction of methane (E_o) was calculated as follows:

$$E_o = \Delta P_{CH_4} \cdot \xi \cdot \varphi_{CHP} \quad \text{Eq. 9}$$

ΔP_{CH4} is the methane yield increase after the pretreatment (L CH_4 $kg_{feedstock}^{-1}$); ξ is the methane heating value (35.8 kJ L^{-1} CH_4); and φ_{CHP} is the efficiency of the CHP unit, 0.55 heat and 0.35 electricity generation (S. Astals et al., 2012).

Finally the ratio (Ei/Eo) was calculated to determine the pretreatments viability.

6.3 Results and discussion

Table 6.2 Averaged values of the performance of R1, R2 and R3

	Units	**Start-up**			**Under pretreatment**		
		R1	R2	R3	R1	R2	R3
SMP	L_{CH4} g^{-1} VS	0.32 ± 0.02	0.34 ± 0.03	0.32 ± 0.02	0.33 ± 0.02*	0.37 ± 0.02*	0.42 ± 0.03*
Methane production	L_{CH4} day^{-1}	2.86 ± 0.13	3.02 ± 0.15	2.90 ± 0.22	2.81 ± 0.15*	3.15 ± 0.21*	3.55 ± 0.22*
VS removal	%	56 ± 2	58 ± 3	60 ± 4	49 ± 1*	56 ± 2*	55 ± 1*
pH	-	8.3 ± 0.1	8.3 ± 0.1	8.3 ± 0.1	8.2 ± 0.1	8.2 ± 0.1	8.2 ± 0.1
PA	gCaCO3 L^{-1}	8.6 ± 0.9	10.7 ± 0.3	8.4 ± 0.6	8.1 ± 0.3	10.3 ± 0.2	8.0 ± 0.6
VFA	mg L^{-1}	118 ± 33	172 ± 76	160 ± 55	133 ± 53	155 ± 78	115 ± 77
HAc	mg L^{-1}	83 ± 8	73 ± 23	65 ± 5	90± 9	96 ± 32	77 ± 36
HPr	mg L^{-1}	3 ± 2	2 ± 2	1 ± 0	3 ± 1	3 ± 1	2 ± 1
VFA/TA	-	0.01 ± 0.00*	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.00	0.01 ± 0.01	0.01 ± 0.00
NH_3	g L^{-1}	0.7 ± 0.2	0.5 ± 0.1	0.6 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1

* These values were averaged between 112th and the 132nd day

6.3.1 Anaerobic co-digestion of pig manure and barley waste

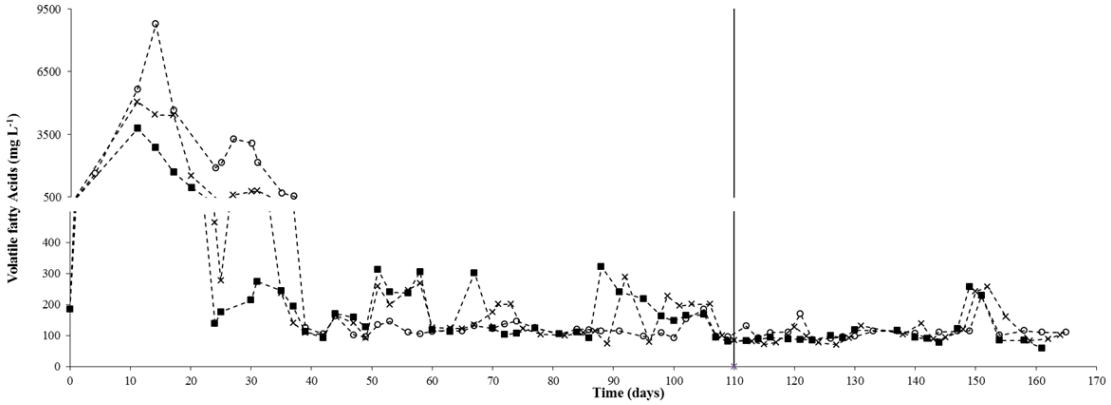


Figure 6.1 Volatile fatty acids levels in R1 (○), R2 (×) and R3 (■); change of stages (▬▬)

Table 6.2 shows the average and the standard deviation of all the parameters during the start-up and while pretreatments were applied. The reactors started with an OLR of 3.3 gVS L_R^{-1} day^{-1} and an HRT of 20 days. Despite that other AcoD studies could work under the same conditions without stability problems (Cornell et al., 2012; Giuliano et al., 2013; Wang et al., 2010), in this case the start-up was quite challenging. Even though the buffer capacity was very high (Table 6.2) the concentration of VFA in the digester did not stop to increase (Fig. 6.1).

As an example, R1 VFA continuously increased from 180 mg L^{-1} to 8700 mg L^{-1} in two weeks and therefore the ratio VFA/TA, which represents the stability of the system, was above its limit (VFA/TA=0.95>0.4) (Table 6.2). The reason for the VFA build up might be that the digester was working with a higher OLR than the digester which inoculum was used to seed the reactors.

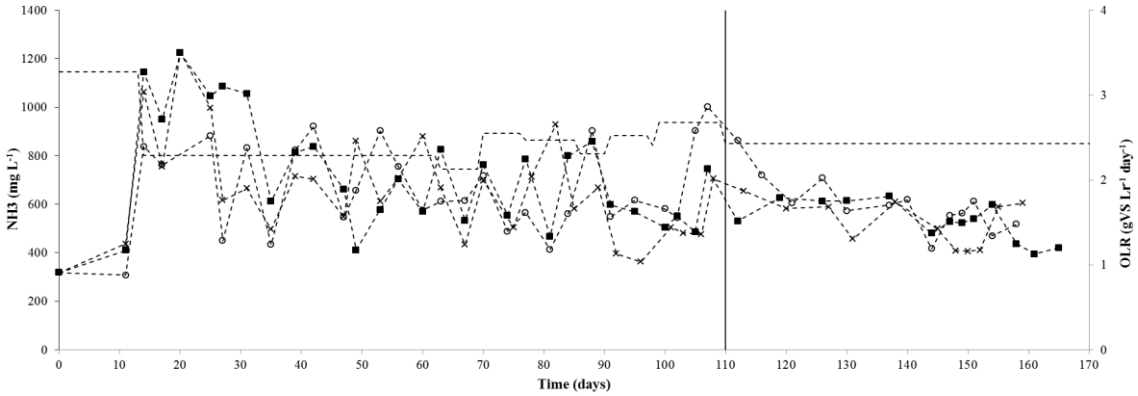


Figure 6.2 NH_3 levels in R1 (○), R2 (×) and R3 (■); change of stages (▬▬); OLR (▬ ▬)

As this inoculum have had already high ammonia concentrations (320 mg L^{-1}) (Table 6.1) the high OLR and short HRT used lead to an increase of the NH_3 in the system around 850 – 1150 mg L^{-1} (Fig. 6.2). Nitrogen is a key element in AD since it is necessary for microorganism growth. However, when manure is used as feedstock, the degradation of proteins can lead to high nitrogen levels in the form of NH_3 which can be very toxic for acetoclastic methanogens in particular (Batstone et al., 2002; Borja et al., 1996). When ammonia levels are above 200 mg L^{-1} it is considered as a toxicological agent for archaea leading to an increase in the VFA levels (Chen et al., 2008; Giuliano et al., 2013). Because of the VFA accumulation the methane production decreased at the 8th from 3.1 to 1.5 LCH4 day^{-1} (Fig. 6.3). To solve this situation the feeding was stopped on the 10th, 13th, 14th and 15th day and at the 16th day the OLR decreased from 3.3 to 2.5 gVS L_R^{-1} day^{-1} and the HRT increased from 20 to 27 days.

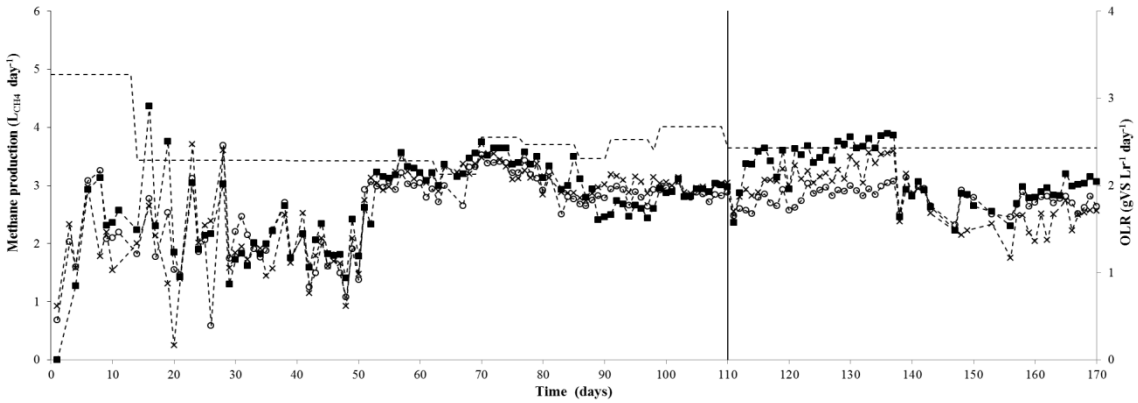


Figure 6.3 Methane production of R1 (○), R2 (×) and R3 (■); change of stages (——); OLR (— —)

After this change the overall VFA concentration decreased immediately in the three reactors (Fig. 6.1). At the 39th day the VFA levels were around 100 mg L^{-1} in all three reactors. Moreover, the methane production could increase and sometimes it achieved high values (4.4 LCH_4 day^{-1}) due to the degradation of the VFA accumulated (Fig. 6.1). The methane production presented many fluctuations due to the changes in the OLR and HRT but at the 30th day the production was constant and equal in R1, R2 and R3 until the 50th day (p=0.36).

At the 25th day a layer of foam appeared at the top of the liquid in R1, R2 and R3. In full scale plants foaming can cause problems such as blockages of gas mixing devices or the creation of dead zones due to the accumulation of solids on the top (Ganidi et al.,

2009). Since foam is a gas-liquid dispersion where gas account for more than 90%, foaming is also the responsible for methane losses (Kougias et al., 2013). Foaming in manure digesters is constant problem since this substrate contains high levels of proteins. Proteins films can surround gas bubbles creating foam layers and a methane losses, moreover, NH_4^+, which is one of the products after protein degradation, is also recognized as a stabilizer agent for foams (Boe et al., 2012). It was decided to use a different kind of stir blade with three wings to induce better rheology in the system and avoid foam formation. The blade was installed on the 49th day and the foam could disappear completely. Due to the new mixing system at the 52th day the methane production increased from 1.7 to 3.2 LCH4 day^{-1} (Fig. 6.3).

Despite these two problems from the 40th day all the parameters were mainly the same in the three reactors. However the methane production and the VS removal fluctuated quite a bit with time which can be normal when heterogeneous substrates such as BSG are used (Fig. 6.3 and 6.4).

It is important to highlight that the reactors performance was good (Methane production=2.86-3.02 LCH_4 day^{-1} and VFA= 118-172 g L^{-1}) even though the NH_3 and pH levels were very high (NH_3=0.5-0.7 g L^{-1} and pH=8.3) (Table 6.2). Nevertheless, other studies presented also similar conditions (Cornell et al., 2012; Giuliano et al., 2013; Karakashev et al., 2006; Schnürer and Nordberg, 2008; Werner et al., 2014). Usually syntrophic acetate oxidizers bacteria, which oxidize acetate into H_2 and CO_2, are the responsible for this kind of behavior (Shimada et al., 2011). It is possible that this kind of bacteria was already present in the full-scale digester where the inoculum was taken from. The full-scale plant also reported high concentrations of NH_3 during the operation. Therefore, the use of an inoculum with high levels of ammonia is recommended to avoid toxicity problems related to high levels of NH3.

Since the methane production was equal between the three reactors during that period of days (Fig. 6.3), LTP and USP started to be applied on BSG.

6.3.2 Ultrasound pretreatment

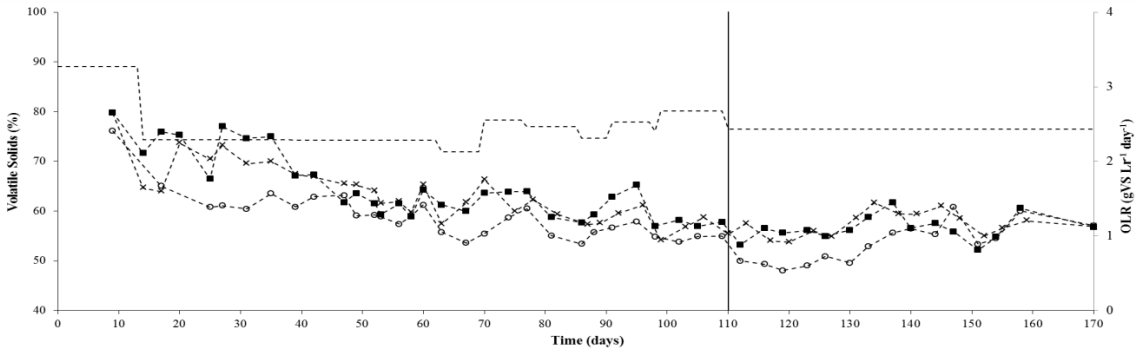


Figure 6.4 Volatile solids removal in R1 (○), R2 (×) and R3 (■); change of stages (▬▬); OLR (▬ ▬)

When USP was applied any stability parameter changed (VFA= 115 mg L^{-1}; PA= 10.3g CaCO3 L^{-1}; pH= 8.2; VFA/TA=0.01) (Table 6.2). The only parameter that changed was the methane production (Fig. 6.3). In R1 VS removal decreased to 49% because the BSG was diluted in all three feedstocks and OLR decreased as well. However, in R2 VS removal did not decrease and remained constant (56 ± 2%) being 14% higher than R1 (Fig. 6.4).An increase in the hydrolysis rate could be the reason of this increment. However, the methane production (3.1 LCH4 day^{-1}) was only a 12% higher than R1 (2.8 LCH4 day^{-1}) (Fig. 6.3). When USP is applied, particulate compounds are degraded by two mechanisms: A physical one (Cavitation) and a chemical one due to the formation of radicals in water. However, in other studies the methane production was very high after applying USP. In a Batch test, Fernández-Cegrí et al. (2012) could increase the methane yield of sunflower oil cake in a 54% after applying USP at 24,000 kJ kg TS^{-1}. When the USP effect was tested in a semi-continuous digester, the SMP obtained was also 47% higher compared to the SMP obtained from a non-pretreated substrate (De La Rubia et al., 2013).

Unfortunately, at the 138th day the methane production and the VS removal started to be equal than the reference reactor (2.8 LCH4 day^{-1} and 55%). In fact, 10 days later the methane production started to be even lower than R1 and R3. This behavior could not be explained since the stability parameters in R1 and R2 did not change during the second stage.

6.3.3 Low-temperature pretreatment

When the pre-treatment was applied all the parameters except the methane production remained constant and equal until the end of the experiment (VFA= 115 mg L^{-1}; PA=

8.0 g CaCO3 L^{-1}; pH= 8.2; VFA/TA=0.01) (Table 6.2). During the following 27 days the methane production of the system could increase up to 3.6 LCH_4 day^{-1} from 2.7 LCH^4 day^{-1} being a 26% higher than the reference reactor (2.8 LCH4 day^{-1}) (Fig. 6.3). In this case, also the VS removal was higher 14% higher than R1 suggesting that the hydrolysis rate was higher in R2 and R3 than R1 (Fig. 6.4).

LTP has been largely applied on sewage sludge with a lot of success (Appels et al., 2010a; Ferrer et al., 2008; Ruiz-Hernando et al., 2014). When the pretreatment is applied in short periods of time for such a substrate, biogas production increases due to the disruption of the cell walls chemical bonds which lead to the solubilization of many organic compounds. For example, Appels et al. (2010) got a methane production 10 fold higher than the control when a LTP at 90ºC was applied on sludge during 60 min. Besides, Skiadas et al. (2005) could reduce the pathogens load in the effluent after applying LTP on sludge at 70ºC. However the mechanism is different in the case of lignocellulosic substrates. In the study of Menardo et al. (2012) when LTP was applied on agricultural by products the levels of solubilized compounds did not increase and the fibers composition did not change either. However, biogas production from barley straw could be increased in a 40% as well. Apparently, instead of solubilizing recalcitrant compounds, LTP could enhance the biological activity of some hydrolytic bacteria or enzymes, break weak hydrogen bond linking cellulose and hemicellulose or induce a positive change on the microbial community in AD (González-Fernández et al., 2012; Nielsen et al., 2004). In a previous study were the same BSG and inoculums were used, the LTP gave worse performance at 80ºC than 60ºC (Peces et al., 2015). Such a conclusion discarded the hypothesis that the performance is mainly due to solubilization of recalcitrant compounds since solubility increases with temperature. It is important to highlight that the BSG used in this case was the same than the one used in Peces et al. (2015) study, collected after a brewery process. Brewery processes involve the action of enzymes such as α-amylases, β-amylases and β -glucanases which are inactivated at temperatures higher than 70ºC. In this study it can be also assumed that the higher methane production is mainly due to the re-activation of these enzymes during the pretreatment step.

According to the methane production, LTP seemed to perform better than USP. Even though the VS removal was the same in R2 and R3, the mechanisms to disrupt the particulate matter are different in USP and LTP. Therefore, the products released can have a different biodegradability according to the pretreatment used.

Unfortunately, at the 138th day the methane production and the VS removal started to be equal than the reference reactor (2.8 LCH4 day^{-1} and 55%). This behavior could not be explained since the stability parameters in R1 and R3 did not change during the second stage and usually no recalcitrant compounds are produced during LTP.

Nevertheless, Similar to R2, the methane production and the VS removal were equal to R1 at the 138th. No explanation could be obtained for that behavior since, as it happened with R3, any other parameter changed for this reactor.

6.3.4 Energy assessment

The calculations for the energy assessment were done using the methane production obtained between the 113th and the 137th when pretreatment showed a good performance.

In this case, E_{iUSP} was equal to 48.0 kW and the E_{oUSP}, the energy obtained by increasing methane production, was 6.5 kW leading to an energy ratio of 0.1. Therefore, the increment of methane obtained after USP is not enough to compensate the pretreatment energy expenses.

For LTP the tank volume would have a capacity of 10 m^3 and the optimal area would be 26 m^2. The energy spent for this pretreatment was 4 kW and the energy obtained from the heating recovery and the increment in methane production was 17 kW. The energy ratio was then 16.9 showing that LTP can be a suitable option to pretreat BSG.

6.4 Conclusions

In this work, the anaerobic mesophilic co-digestion of pig manure with bagasse spent grain was performed at laboratory scale. In order to break/degrade the bagasse lignocellulosic components, a low-temperature pretreatment and a ultrasound pretreatment were applied on it. The main conclusions extracted from the study are summarized as follows:

- When low-temperature and ultrasounds pretreatment were applied on bagasse, the methane production could increase in a 26 and 12% respectively.
- According the energy balance results, only the low-temperature pretreatment would be recommended for its application in a full scale system. The reasons for the positive energy balance were the increase in the methane production and the availability to recover the heat used.

- The methane production of reactors where the pretreatments were applied decreased in 30 days at the same level than the reference reactor. Any other parameter changed so no explanation could be obtained for this behavior.
- The use of an inoculum already adapted to high ammonia levels allows performing the Anaerobic co-digestion of pig manure under high ammonia levels without toxicity problems.

7. Anaerobic digestion of lignocellulosic substrates: Inoculation with rumen, a natural ecosystem harboring hydrolytic bacteria

Abstract

The refractory property of lignocellulosic components is one of the bottlenecks for their utilization in anaerobic digestion. Since the microbial community in the rumen of ruminants facilitates the degradation of lignocellulosic compounds in animal feed, the use of rumen content as an inoculum can be a potential strategy to enhance methane generation from lignocellulosic feedstocks. Three anaerobic bioreactors were operated to evaluate this strategy for the co-digestion of Napier grass and cow manure. R1 was inoculated with rumen content, R3 with a conventional anaerobic digestion inoculum, and R2 with a mixture of both inocula. During the first days of experiment, R2 presented the highest archaea/bacteria diversity, with the presence of microorganisms able to degrade lignocellulose compounds and VFA which resulted in a fast start-up. At the end of the experiment the three digesters presented the same stability, the same methane production (0.15 $LCH4\ gVS^{-1}\ day^{-1}$) and the same microbial community.

The start-up period should be closely monitored and the use of a co-substrate with high buffering capacity is highly recommended for the efficient digestion of Napier grass.

This chapter was presented as <u>poster communication</u> in:

- **Use of rumen content to enhance anaerobic digestion of lignocellulosic biomass.** 14th World Congress on Anaerobic Digestion, Viña del Mar, Chile, November 2015.
- **Anaerobic Digestion of Lignocellulosic Biomass Using Rumen Contents For Enhanced Biogas Production.** The Science and Engineering For a Biobased Industry and Economy. Ohio, United States of America, August 2015.

This chapter is also <u>presented</u> in:

- Shilva Shresta. **Use of rumen content to enhance anaerobic digestion of lignocellulosic biomass.** Master thesis (University of Hawaii)

And then in preparation for <u>publication</u> as:

- Shrestha, S., Fonoll, X., Mata-Alvarez, J., Khanal, S., Raskin, L. **Anaerobic digestion of lignocellulosic substrates: Inoculation with rumen, a natural ecosystem harboring hydrolytic bacteria.**

7.1 Introduction

Even though 3-4 times of the energy needed is stored in plants through photosynthesis, 80% of the energy world demand is covered by the use of fossil fuels which are non-renewable and generate a high amount of GHG (Guo et al., 2015). Many countries have different energy policies/targets regarding the use of renewable energies (Popp et al., 2014; Surendra et al., 2014; Waramit and Chaugool, 2014). The use of energy crops in AD to get energy is recognized as a suitable option to decrease the dependency on fuels such as natural gas and, at the same time, an effluent with high fertilizer qualities is generated (EurObserv'ER, 2010; Koçar and Civaş, 2013; Surendra et al., 2015). Europe is the best example regarding the implementation of AD for energy production. There are over 14,000 operational AD plants being energy crops the most used substrate (EurObserv'ER, 2014). AcoD with crops could be the solution for the implementation of digesters in small farms where not many tones of manure are produced (Klavon et al., 2013; Mata-Alvarez et al., 2014b). However, the use of crops as AD substrates became a dilemma. Energy crops are high water and fertilizer demanding, induces more greenhouse gas emissions than it prevents and decrease the land availability for food production increasing its prices (Stankus, 2014). Therefore, the new German law (EEG 2014) does not incentive anymore the use of crops for methane generation and the biogas market is suffering some difficulties because of that (EurObserv'ER, 2014).

In contrast, the so-called second generation biofuels (SGB) (Energy grasses or crops wastes) are more suitable for sustainable energy production (de Souza et al., 2014; Del Grosso et al., 2014; López-Bellido et al., 2014). SGB such as giant reed could be the key to re-activate the biogas market in Europe since their cultivation is suitable for marginal areas where the cultivation of food is not advantageous (Cappelli et al., 2015). Moreover, the use SGB in AD can be also very attractive for developing countries. The presence of SGB in the tropical and sub-tropical regions is very high and many studies suggest that the implementation of AD in developing countries can decrease their imported energy dependency and promote education, the creation of a research and development culture and the creation of new jobs opportunities (Lohri et al., 2013; Pandyaswargo et al., 2012; Surendra et al., 2013). As an example, Thailand is focusing its energy policy towards the use of NG in AD (Waramit and Chaugool, 2014). NG is perfect candidate among all the energy grasses since it presents a high biomass yield potential (87 tn yr^{-1} ha^{-1}), high lignocellulose content, low water and fertilizer demand, and positive environmental impact (Sawasdee and Pisutpaisal, 2014). Nonetheless, the

progress of this technology is being slow due to technical and financial issues.

SGB are rich in carbohydrates but their nutrient levels are low, so, AcoD with low C/N ratio such as manures is fully recommended (Ferrer et al., 2014; Mata-Alvarez et al., 2011). However due to the high concentration of the lignocellulosic compounds, hydrolysis is being the rate limiting step and therefore not all the methane potential is recovered (J. C. Frigon and Guiot, 2010; Noike et al., 1985). In nature, cows are perfect lignocellulosic degraders as they are usually fed with grass. The cow's stomach content harbors a complex and diverse microbial community of bacteria, archaea, fungi, and protozoa able to degrade the lignocellulosic biomass despite its recalcitrant nature (Sirohi et al., 2012). Rumen content (RC) is one of the wastes produced in slaughterhouse which is usually composted with other wastes. The integration of RC in the AcoD of NG and Cow manure (CM) could be a potential option to enhance the degradation of lignocellulosic compounds presented in NG (Z.-B. Yue et al., 2013). Nevertheless, not too many studies regarding the use of RC in AD have been published and none of them have focused on the microbial community.

In the present study RC was used as inoculum to enhance the methane production during the AcoD of NG and CM. Molecular tools were also performed to evaluate the changes in the microbial community structure during start-up and the role of RC microbial populations.

7.2 Materials and methods

7.2.1 Substrate and inoculum origin

Napier grass (4 months old) was harvested from Waimanalo Research Station (Waimanalo, HI, USA). The biomass samples were shredded using a cutting mill (Vincent Corporation, Tampa, FL, USA) and air dried to reduce the moisture content to less than 10%. It was then passed through a second laboratory cutting mill with a screen size of 6mm. 50% (ww) of CM, obtained from a dairy farm in Michigan State University (MSU), was blended with 50% (ww) of water to get a TS content around 6.5% but at the 81st day water was replaced by urine (CM:Urine=30:70; ww) also obtained from MSU farm. One of the inoculums used (MSU inoculum) was obtained from a full-scale AD plant at MSU treating a mixture of food waste and cow manure at mesophilic conditions. The second inoculum, Solid and liquid samples of RC, were obtained from a fistulated cow in MSU farm and stored at 4ºC during the transportation

(Hervás et al., 2005). Once in the lab both samples were mixed together to get a TS content of 12% and simulate better RC from slaughterhouses (Tritt and Schuchardt, 1992). NG, CM mixtures were stored at 4ºC and the inoculums were used immediately. The substrates and inoculum characteristics can be seen in Table7.1.

Table 7.1 Substrate and inoculum characteristics

		NG	CM + Water	CM + Urine	R1 inoculum	R2 Inoculum	R3 Inoculum
TS	mg L^{-1}	917 ± 21	69 ± 3	77 ± 6	43	32	34
VS	mg L^{-1}	775 ± 27	62 ± 3	56 ± 6	39	23	25
pH	-	n.a.	6.6 ± 0.7	8.7 ± 0.4	5.4	7.2	7.9
PA	gCaCO3 L^{-1}	n.a.	0.6 ± 0.3	20.1 ± 7.6	0.1	3.6	7.8
TA	gCaCO3 L^{-1}	n.a.	2.9 ± 0.4	27.0 ± 9.0	1.6	5.9	11.2
Acetate	g L^{-1}	n.a.	1.4 ± 0.4	3.2 ± 0.7	1.3	0.9	0.5
Propionate	g L^{-1}	n.a.	0.7 ± 0.1	0.6 ± 0.2	0.8	1.2	1.6
NH_4	g L^{-1}	n.a.	0. 1 ± 0.0	3.7± 1.0	0.1	1.0	1.8

n.a. Non analyzed

7.2.2 Experimental design

Three 2-L semi-continuous anaerobic bioreactors, R1, R2, and R3, each with a working volume of 1.3 L were operated at mesophilic conditions (37ºC). The reactors were continuously mixed using a shaking water bath and after the 31st day they were mixed manually once per day. During start-up, the reactors were fed once every two days (organic loading rate (OLR) = 0.75 gVS L^{-1} day^{-1}) for two weeks and then the reactors were fed every day (OLR = 1.5 $gVSL^{-1}day^{-1}$). All three reactors were fed with a mixture of NG and CM at a ratio of 30:70 (w:w; wet basis) to obtain a C/N mass ratio around 20. The C/N ratio was estimated by using characteristics for NG and CM reported in the literature. The mixture TS content started to be 3.5% for rheological reasons and later on (day 89) the concentration was increased up to 6.0%. R1 was inoculated with RC, R2 was inoculated with a 50:50 (w:w, wet basis) mixture of RC and AD biomass to provide a high concentration of methanogens and buffer capacity to RC and R3 was inoculated with the AD biomass only. Water was added to R1 and R2 inocula to have approximately the same total solid content for all three reactors (~4%). The biogas collected in Tedlar gas bags was measured by a gas meter daily. Some issues related with low pH and low alkalinities levels induced to some changes during the experiment:

- Initially, the pH in R1 and R2 was maintained by addition of $NaHCO_3$ or NH_4HCO_3.
- The OLR and HRT were changed during the experiment to check if a reduction in loading rate would stabilize the pH (Table 7.2).
- Systems under high mixing conditions can disrupt microbial flocs and increase the VFA levels. Therefore mixing was stopped to decrease the build-up of VFA.
- Different concentrations of NH_4^+ were added every day to the CM diluted with water to increase its nutrient content. 0.7, 1.0, 2.0 and 3.0 gNH_4^+ L^{-1} started to be added on the 38th, 48th and the 75th day respectively.
- On the 81st day urine was added to the CM at a ratio of 70:30 (w:w, wet basis) instead of water to increase its buffering capacity consistent with the need to treat different waste streams generated at a farm.

Table 7.2 Reactors design parameters

	R1			**R2**			**R3**		
Period of days	OLR ($gVS\ L^{-1}\ day^{-1}$)	HRT (days)	TS content (%)	OLR ($gVS\ L^{-1}\ day^{-1}$)	HRT (days)	TS content (%)	OLR ($gVS\ L^{-1}\ day^{-1}$)	HRT (days)	TS content (%)
$0 – 14^{th}$	0.75	20	3.6	0.75	20	3.6	0.75	20	3.6
$14^{th} – 22^{th}$	1.5	20	3.6	1.5	20	3.6	1.5	20	3.6
$22^{nd} – 89^{th}$	1.0	35	3.6	1.5	20	3.6	1.5	20	3.6
$89^{th} – 96^{th}$	2.0	20	4.8	2.0	20	4.8	2.0	20	4.8
$96^{th} – 143^{rd}$	3.0	20	6.8	3.0	20	6.8	3.0	20	6.8

7.2.3 Analytical methods

TS, VS, TA, PA, IA, NH_3, VFA and biogas composition were measured as stated in the section 3.1.2.

7.2.4 Microbial analysis

All the microbial analyses were performed as stayed in section 3.3.

7.3 Results and discussion

7.3.1 Reactor performance

The characteristics of feedstock and inocula are summarized in Table 7.1. CM presented very low levels of ammonium (0.1 ± 0.0 g L^{-1}) and pH (6.6 ± 0.7), which is not usual for this kind of waste (Astals et al., 2011; Molinuevo-Salces et al., 2015; Pagés-Díaz et al., 2014). Due to the low alkalinity and pH presented in R1 inoculum (PA= 0.1 ± 0.0 gCaCO3 L^{-1}; pH=5.4 ± 0.4), a combination of RC and MSU inoculum was used for R2, in addition to the RC alone (R1) and the MSU inoculum alone (R3). Therefore, R2 was expected to have lignocellulose degrading microorganisms originating from the RC and exhibit a more stable start-up. Propionate levels (1.6 ± 0.7 g L^{-1}) were three times higher than acetate levels (0.6 ± 0.0 g L^{-1}) in MSU inoculum suggesting that syntrophic bacteria might be inhibited.

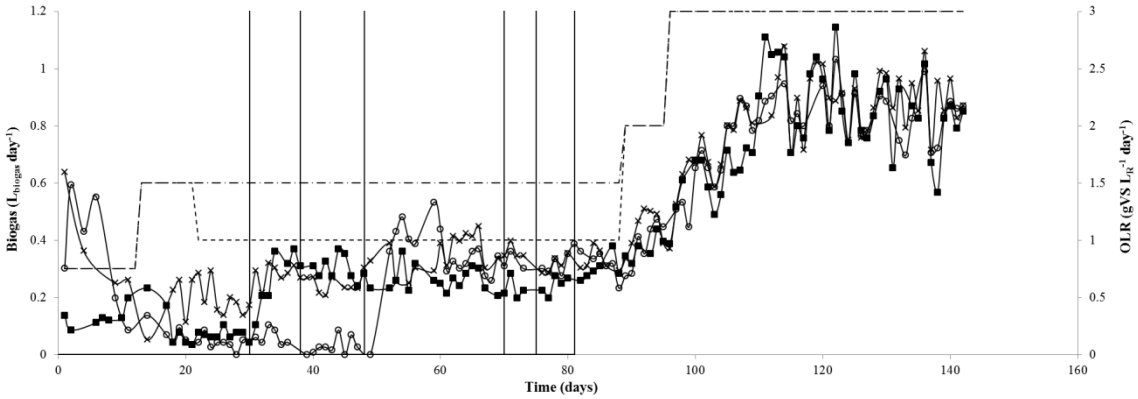


Figure 7.1 Biogas production in R1 (○), R2 (×) and R3 (■); change of stages (——), R1 OLR (— —) and R2 and R3 OLR (— • —)

The black bars in the figures represent the different changes performed in the system commented in section 7.2.2. During the first two weeks the three digesters worked

under low OLR (0.75 gVS L_R^{-1} day^{-1}) by feeding them every two days only. The first days of the start-up, the reactors inoculated with RC presented a higher hydrolytic activity compared to R3 because of the higher biogas production that R1 and R2 presented. Although, the biogas production could be overestimated in R1 due to the alkalinity added to increase the pH (Fig. 7.1). Actually, at the 1st day the biogas production in R2 (0.6 L day^{-1}) was higher than R1 (0.3 L day^{-1}) probably because some recalcitrant organic matter remained in MSU inoculum and could be degraded by the microorganisms contained in RC (Fig. 7.1). Quintero et al. (2012) obtained similar results in a study where the BMP of fique's bagasse was evaluated using different inocula. Although rumen fluid presented the highest hydrolytic activity, the bottle inoculated with rumen fluid and pig waste sludge presented the highest SMP (0.3 LCH4 gVS^{-1}).

After this episode of fast hydrolysis, the biogas production in R1 and R2 felt, being the SMP less than 0.10 LCH_4 gVS^{-1} day^{-1} (Fig. 7.2).

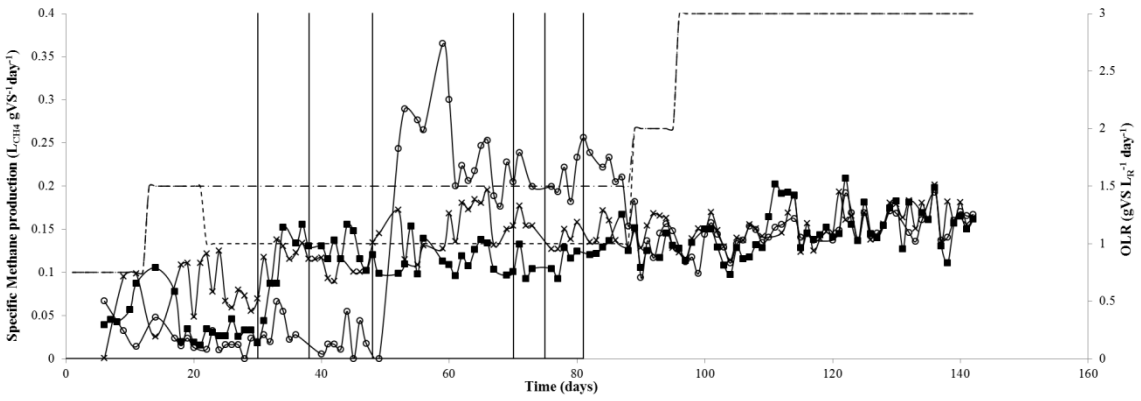


Figure 7.2 Specific methane production in R1 (○), R2 (×) and R3 (■); change of stages (——), R1 OLR (— • —) and R2 and R3 OLR (— —)

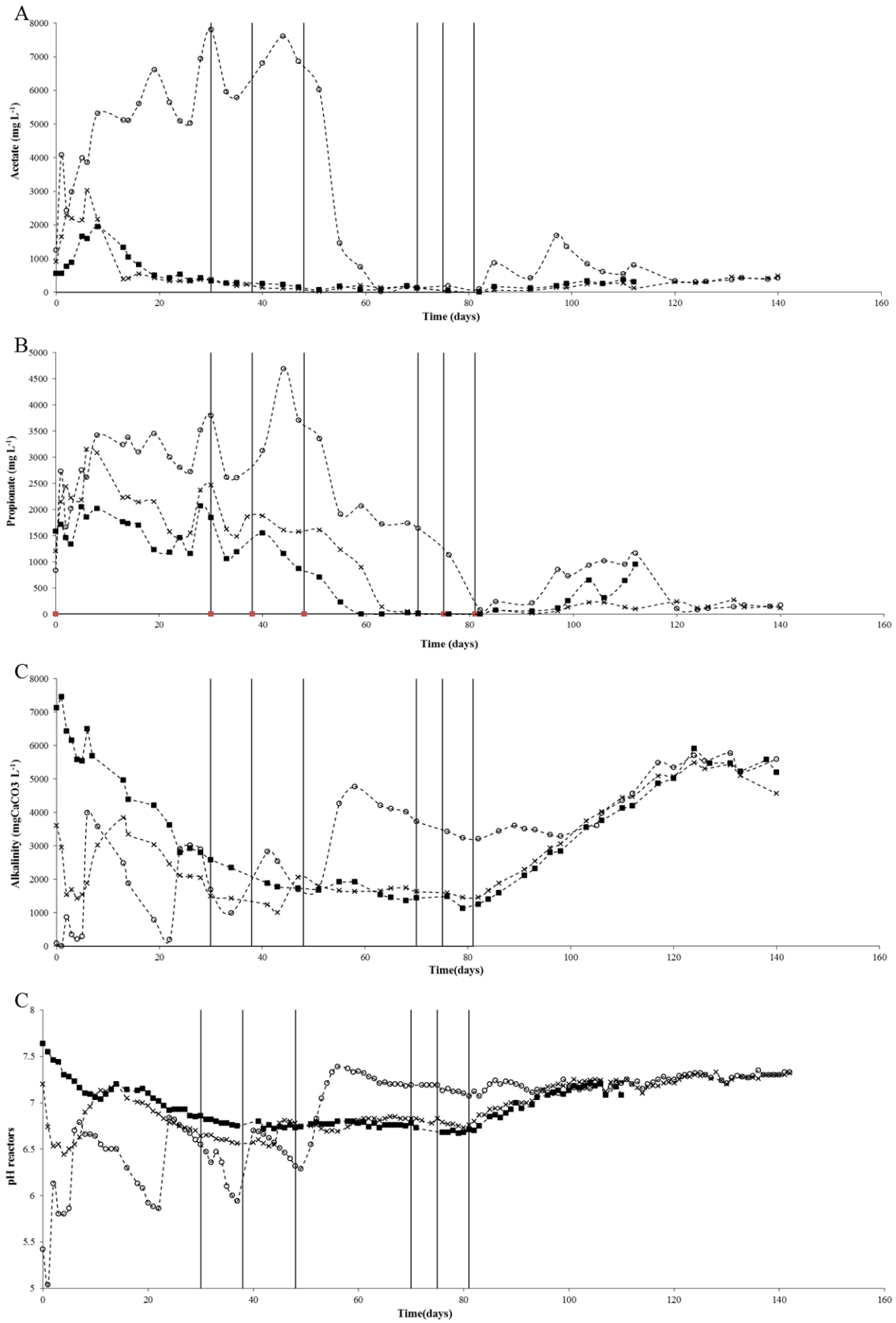


Figure 7.3 Acetate (A), propionate (B), PA (C) and pH (D) levels from R1 (○), R2 (×) and R3 (■);(■); change of stages (▬▬)

Figure 7.3 shows the levels of some stability parameters such as acetate, propionate, PA and pH. The low SMP presented in R1 could be explained by the stability parameters. Due to the extreme conditions that RC presented (Table 7.1), the VFA in R1 sharply increased in 8 days from 2.7 g L^{-1} to 10.0 g L^{-1}. Those levels did not decrease even though alkalinity in form of $NaHCO_3$ and NH_4HCO_3 was added to increase the pH up to 6.8 whenever the pH in the reactor was below 6.0 (Fig. 7.3a and b). Every time alkalinity was added in the system, the PA could increase between 3.0 and 4.0 gCaCO3 L^{-1}, but, after the addition, PA and pH started to decrease (Fig. 7.3c and d) and the VFA, especially acetate, remained high between 12.0 g L^{-1} and 14.0 g L^{-1}, demonstrating that archaea were probably inhibited (Fig. 7.2a and b). To some extent, R2 and R3 had a similar behavior. Since the beginning of the experiment VFA increased and the pH and PA decreased at a constant rate (fig. 7.3). VFA increased faster in R2 than R3 probably due to the imbalance between the hydrolytic and methanogenic bacteria. Due to the high hydrolytic activity at the beginning of the experiment in R2, VFA increased from 2.7 g L^{-1} to 7.8 g L^{-1} and the pH decreased from 7.2 to 6.5 in 6 days (Fig. 7.3). The fact that PA and pH decreased and VFA increased so fast at the experiment start in R1 and R2, even though the OLR was very low, is confirming the hypothesis that the microbial community contained in RC induced the system to a fast hydrolysis.

Since the pH was very low at the 7^{th} day 2 g of NH_4HCO_3 were added in R2 and the feeding was stopped in R3. Due to this change acetate levels could decrease and hence VFA decreased to 3.5 g L^{-1} (Fig 7.3a and b). Moreover, propionate did not decrease in those reactors, remaining constant all the time between 1.5 g L^{-1} and 2.0 g L^{-1} (Fig. 7.3b) and propionate/acetate (P/A) ratio was quite high around 6.4 and 3.6 for R2 and R3 respectively (Table 7.3). Hill et al. (1987) reported that a ratio higher than 1.4 is an indicator of process failure which could be associated to the low SMP registered in those reactors. This fact supported the theory that syntrophic bacteria were already inhibited in MSU inoculum. Even though R3 was inoculated completely with MSU inoculum, P/A ratio from R2 was higher than R3 probably because in R2 the pH decreased up to 6.4 at the 4^{th} day further inhibiting syntrophic populations (Fig. 7.3d).

In fact, all three digesters presented the same behavior, low SMP, high concentrations of VFA and a constant decrease of the pH, PA and NH_3 levels (Fig. 7.2). For example, the

SMP in R1 was critical around 0.02 ± 0.02 LCH_4 gVS^{-1} day^{-1} (Fig 7.2). Different actions were performed to solve this situation:

Day 30^{th}: Systems under high mixing conditions can disrupt microbial flocs. Interspecies hydrogen transfer is one important syntrophic interaction, where hydrogen is transferred directly between acetogenic and methanogenic microorganisms (producers and consumers of hydrogen) to keep the partial hydrogen pressure low, which would otherwise increase the concentration of VFA (Lindmark et al., 2014). Nevertheless, when mixing was stopped any change was noticed in the concentration of VFA during the next 10 days so another change was induced.

Day 40^{th}: As mentioned in section 1.3.2.2 manure has been widely used as a co-substrate to supplement the nutrients which are normally washed out in the mono-digestion of plant biomass (Bruni et al., 2010). Nges and Björnsson (2012) stood out the importance of nutrients when the mono-digestion of beets produced 383 LCH4 gVS^{-1} day^{-1} under an OLR of 4.5 gTS L_R^{-1} d^{-1} and a HRT of 40 days. Usually, plants using little or no manure were found to operate at an average HRT of 170 days.

In this study, the CM used had low levels of ammonium (0.1 ± 0.0 mg L^{-1}). To test if the no degradation of propionate was due to a lack of nutrients in the cow manure, different concentrations of NH_4Cl were added continuously in the feedstock to further evaluate the lack in macro-nutrients. When the concentration of NH_4^+ in the feedstock increased to 1.0 g L^{-1} VFA started to be degraded in all three reactors. In R1 acetate was degraded from 6.0 g L^{-1} to 0.1 g L^{-1} in 10 days and propionate was degraded from 3.3 g L^{-1} to 0.1 g L^{-1} in 30 days. In R2 and R3 propionate was degraded from 3.3 g L^{-1} to less than 0.1 g L^{-1} in 20 days. The degradation of the VFA could increase the SMP in R1 being around 0.22 ± 0.02 LCH_4 gVS^{-1} day^{-1}. However, the pH and PA remained very low (Except in R1 since more NH_4HCO_3 needed to be added before the VFA were degraded) and the SMP did not increase in R2 and R3. Actually, a parallel BMP test proved that nutrients were not lacking in the system. In this test the three inoculums were mixed together and NG was used as substrate. Macro- and micro-nutrients were added in some bottles while in other bottles no nutrients were added at all. The methane production remained the same and no difference was observed between the addition and the no addition of nutrients. Maybe, the degradation of VFA in all reactors was due to the stop of mixing, which consequences were not immediately, or to an adaptation of bacteria and archaea to the digester conditions since the pH could be maintained above 7.0.

Day 81st: Since CM did not presented a high buffer capacity (Table 7.1) and the reactors presented low pH and NH3 levels, urine, which usually has a high buffer capacity was mixed with CM. The mixing of urine with CM is a realistic scenario since both wastes are mixed together in the sewage. By adding urine the pH and PA increased in all three reactors to 7.2 and to 4500 $gCaCO3\ L^{-1}$ respectively.

Table 7.3 Process parameters during the start-up and at the end of the experiment

Parameter	Units	0 – 30th day			113th – 143rd day		
		R1	R2	R3	R1	R2	R3
SMP	$LCH_4\ gVS^{-1}\ day^{-1}$	0.02 ± 0.02	0.08 ± 0.03	0.04 ± 0.02	0.16 ± 0.02	0.16 ± 0.02	016 ± 0.02
VS removal	%	*	*	*	47 ± 2	48 ± 3	48 ± 2
pH	-	6.6 ± 0.8	6.8 ± 0.2	7.1 ± 0.2	7.3 ± 0.0	7.3 ± 0.1	7.6 ± 0.1
PA	$gCaCO3\ L^{-1}$	1.6 ± 1.4	2.4 ± 0.8	5.0 ± 1.6	5.5 ± 0.2	5.2 ± 0.3	5.3 ± 0.3
VFA	$g\ L^{-1}$	9.5 ± 2.9	4.1 ± 1.7	2.9 ± 0.8	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1
Acetate	$g\ L^{-1}$	4.8 ± 1.7	1.2 ± 0.9	0.9 ± 0.5	0.4 ± 0.0	0.4 ± 0.1	0.3 ± 0.0
Propionate	$g\ L^{-1}$	2.8 ± 0.8	2.2 ± 0.5	1.6 ± 0.3	0.1 ± 0.0	0.2 ± 0.1	0.2 ± 0.1
NH3	$mg\ L^{-1}$	6 ± 8	10 ± 7	29 ± 26	20 ± 2	20 ± 3	20 ± 3
VFA/TA	-	1.6 ± 1.0	0.8 ± 0.3	0.4 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
P/A	-	0.6 ± 0.1	3.6 ± 2.2	2.5 ± 1.3	0.4 ± 0.1	0.5 ± 0.2	0.7 ± 0.4

* VS removal was not reported during the start-up due to the high fluctuation

Since the conditions in all three reactors were stable, it was decided to set the OLR and the HRT to the target values (3.0 gVS L_R^{-1} day^{-1} and 20 days) so the TS% in the feedstock increased to 6.8% (Table 7.2). The stability parameters remained in good levels (Table 7.2) and the SMP was the same in all three reactors 0.15 ± 0.02 LCH_4 gVS^{-1} day^{-1}. The addition of RC to perform the start-up of NG and CM AcoD led to differences in the stability of the reactors, especially if RC was used alone as inoculum. The addition of a co-substrate with a high buffer capacity is extremely necessary when RC is used as inoculum to avoid VFA build-up that can affect the microbial community and hence, the SMP. Nevertheless, after long period of days (143 days) the effect of RC was no noticed anymore and all three reactors were having exactly the same performance. In fact, the inoculum seemed to have a stronger effect on the stability rather than the SMP. De Vrieze et al. (2015) studied the effect of inoculation on AD performance in a long-term experiment. The authors observed that, in a long-term, the microbial community of reactors receiving the same substrate is quite similar even though different inoculums were used. However, when high concentrations of ammonia were added, the reactors had a difference response according to the NH_3 concentrations that the inoculums presented. RC could increase the SMP only at the beginning while during the last days of the experiment the SMP was very low and equal between all three reactors ($p=0.68$). Actually, the SMP decreased when the HRT in R1 changed from 35 to 20 days suggesting that the hydrolysis rate was very low and that the system needed more time to degrade NG. When Z. Yue et al. (2013) co-digested manure with corn stover also obtained the highest SMP at the highest HRT. Cellulose reduction increased with the increase of HRT indicating that the longer the feed resided in the digester, the more cellulose was degraded and utilized by the microbes. Besides SMP, the VS removal was below 50% confirming that hydrolysis was not successfully performed towards the end of the experiment.

7.3.2 Bacteria populations

7.3.2.1 Inoculums and substrate

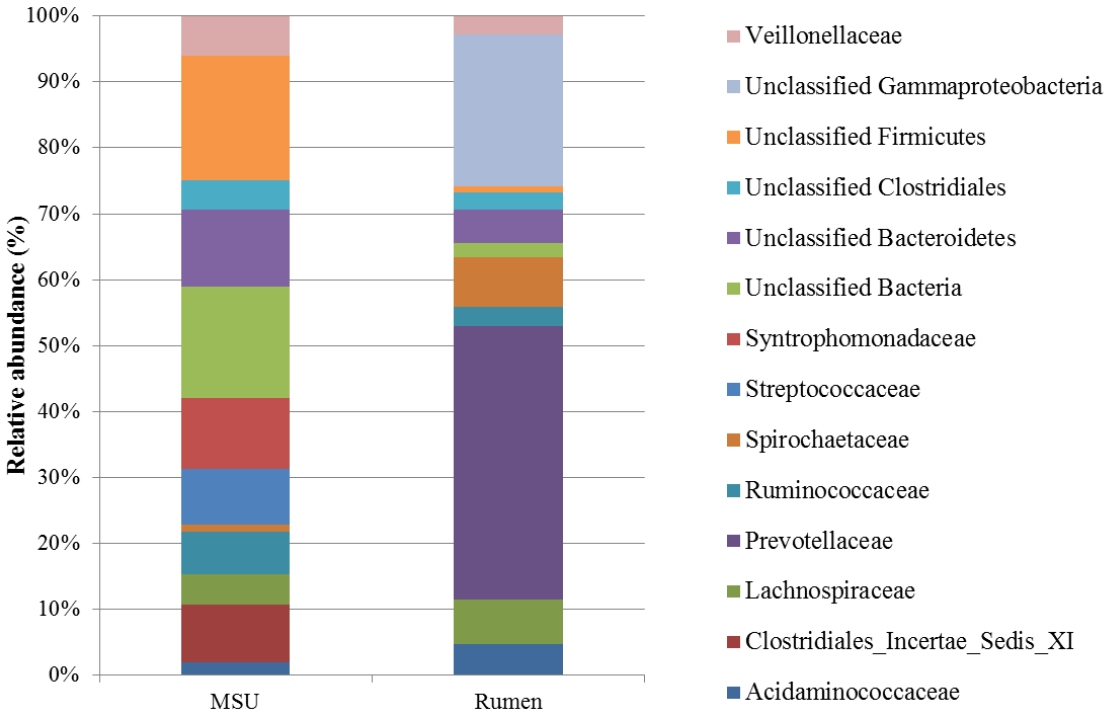


Figure 7.3 Bacterial community in inoculums

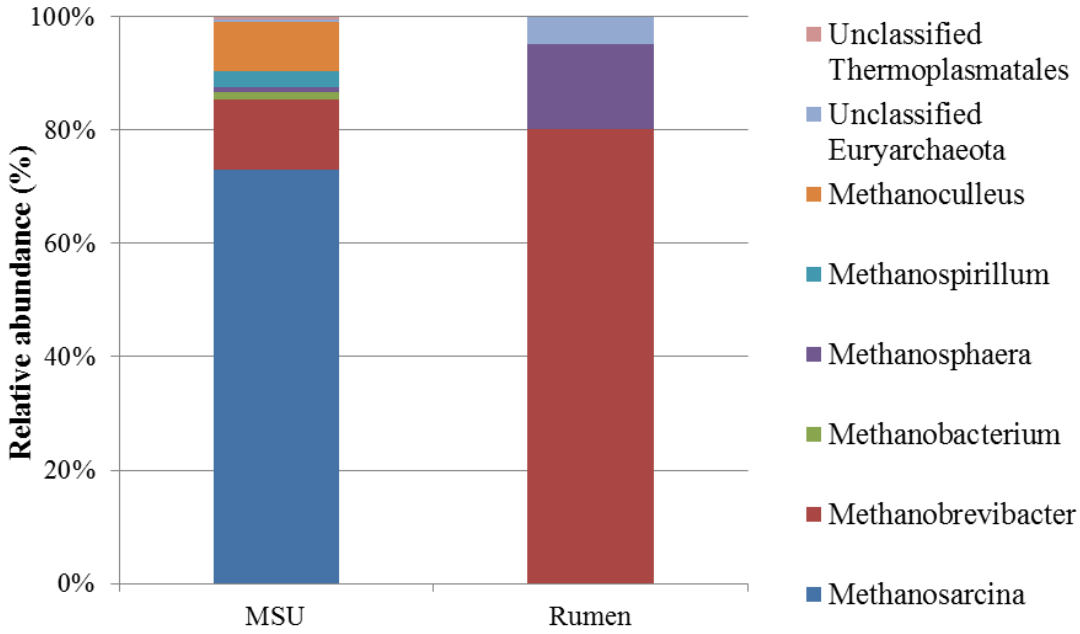


Figure 7.4 Archaeal community in inoculums

MSU and RC inocula were rich in *Bacteroidetes* and *Firmicutes.* Those phyla are rich in anaerobic bacteria able to degrade lignocellulose components, and to perform fermentation using hydrolysis products such as amino-acids, carbohydrates or long- and mid-chain fatty acids (Ren et al., 2014; Sun et al., 2013; Wang et al., 2014; Zheng et al.,

2015). In MSU and RC inoculum only 42% and 55% of the reads could be classified into families. Among the cultured bacteria, the most dominant families in MSU were *Syntrophomonadaceae*, a family of bacteria able to degrade butyric and propionic acid into acetate (Smith et al., 2015), *Clostridiales*, a genus of bacteria that usually dominates in AD which species have high cellulolytic activity (Azman et al., 2015) and *Streptococcaceae*. Moreover, RC microbial community was dominated by *Prevotellaceae* family and *Gammaproteobacteria* order. Those populations are typical in rumen but their activity is not usually involved in the degradation of lignocellulosic components (Piao et al., 2014). However, there is the high presence of unclassified *Bacteroidetes* and *Firmicutes* that might have high hydrolytic activity.

Regarding the archaeal community, MSU and RC inoculum were clearly dominated by acetoclastic methanogens and hydrogenotrophic methanogens respectively. In fact, the acetoclastic community in MSU was governed by the genus *Methanosarcina*, which can convert acetate into CO_2 and CH_4 under high concentrations of Acetate (Peces et al., 2013b). Differently to MSU inoculum, RC methanogenic community is just composed of hydrogenotrophic archaea. The Shannon diversity index was also very different among the two inoculums being higher in MSU inoculum than RC for bacteria and archaea (Table 7.4) .In this vein, the microbial information could predict the behavior of R1. According to this analysis, MSU seemed to be the best candidate for NG AD since its microbial community was dominated by families with hydrolytic, syntrophic and acetoclastic activity. RC presented a less even bacteria and methanogenic community. In fact, the unstable period presented by R1 at the beginning was certainly due to the archaea community which was only dominated by hydrogenotrophic populations. However, it could be possible that, among the unclassified *Bacteroidetes* and *Firmicutes* some populations with a high hydrolytic activity were present. Therefore, the integration of RC to lignocellulosic AD systems could be interesting, although, the mixing with an stable inoculum such as MSU inoculum is a highly recommended strategy.

Table 7.4 Shannon index for the bacterial and archaeal community in inoculums and CM at different days

Shannon Index	MSU	RC	CM 7	CM 21	CM 34	CM 56	CM 75	CM 89	CM 105	CM 140
Bacteria	2.50	1.09	2.02	2.40	2.20	1.92	2.26	2.53	1.14	1.85
Archaea	0.95	0.61	0.44	0.33	0.36	0.38	0.30	0.52	0.53	0.67

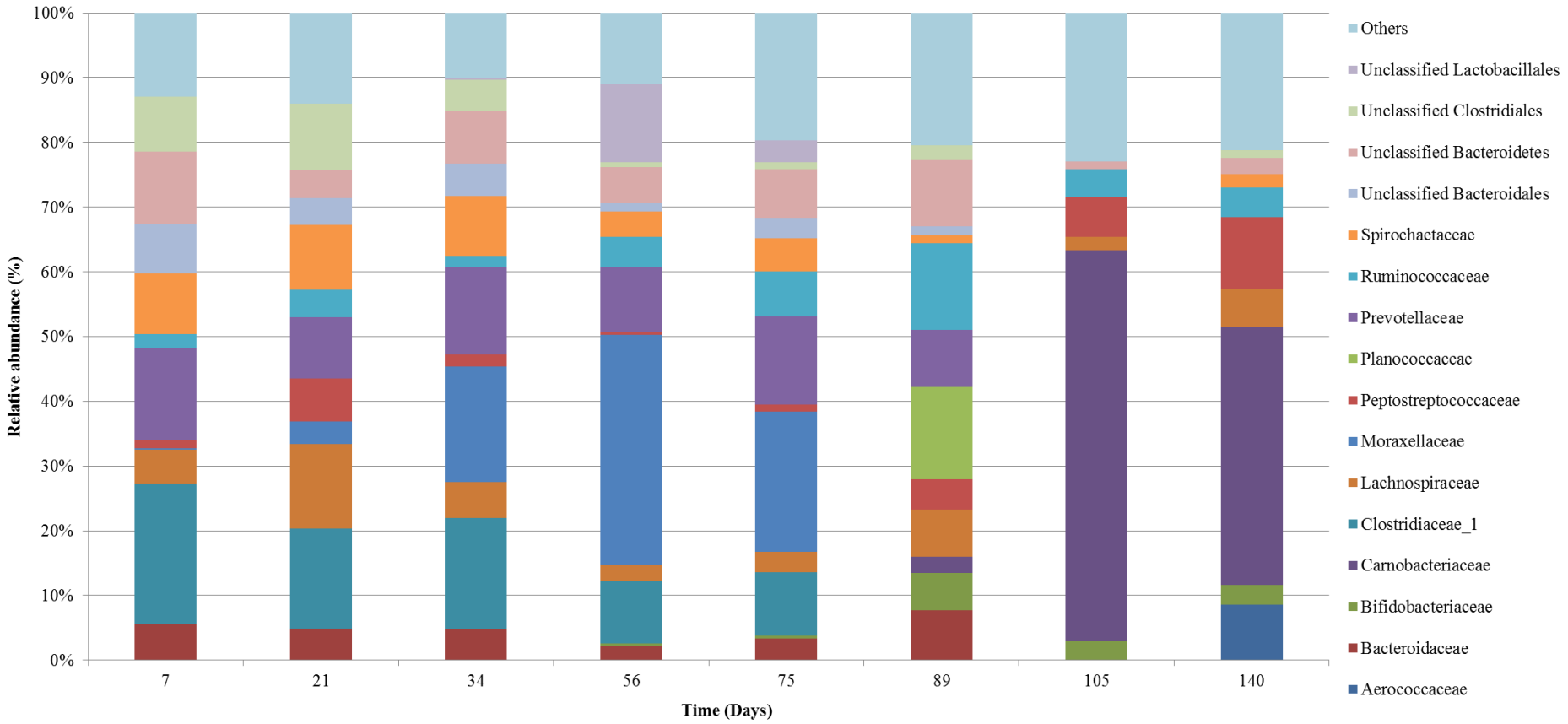


Figure 7.5 Bacterial community in CM

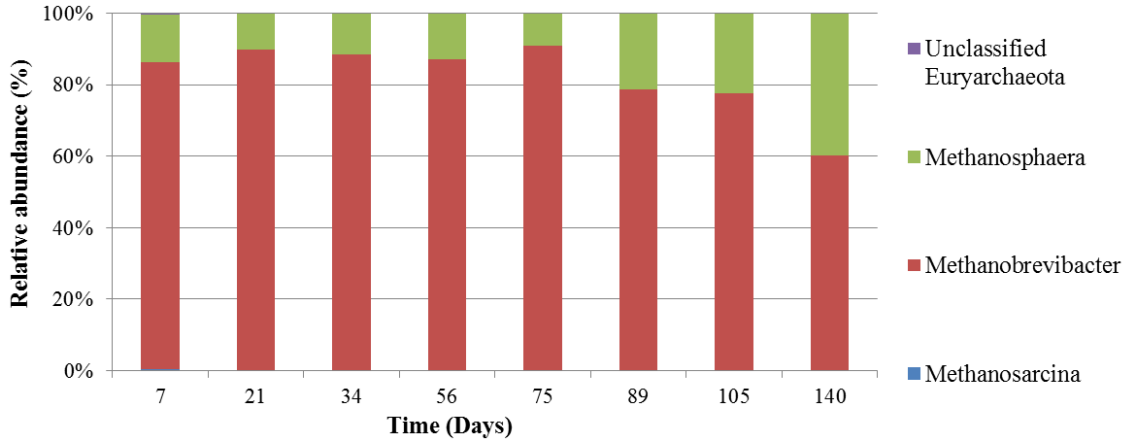


Figure 7.6 Archaeal community in CM

While CM was mixed with water, the dominating phyla were *Firmicutes*, *Bacteroidetes* and *Proteobacteria*. These phylas were mainly represented by *Moraxellaceae (Proteobacteria*), *Prevotellaceae* (*Bacteroidetes*) and *Clostridiaceae* (*Firmicutes*). When urine was added instead of water, *Moraxellaceae* and *Clostridiaceae* disappeared and *Firmicutes* became the most dominant phyla represented by the families *Carnobacteriaceae* and *Peptostreptococcaceae*. This change in the microbial community did not affect the reactors performance. When CM started to be mixed with urine only PA and pH changed but only because of the chemical characteristics (high pH and PA) that the substrate presented (Fig. 7.3c and d).

7.3.2.2 Reactors

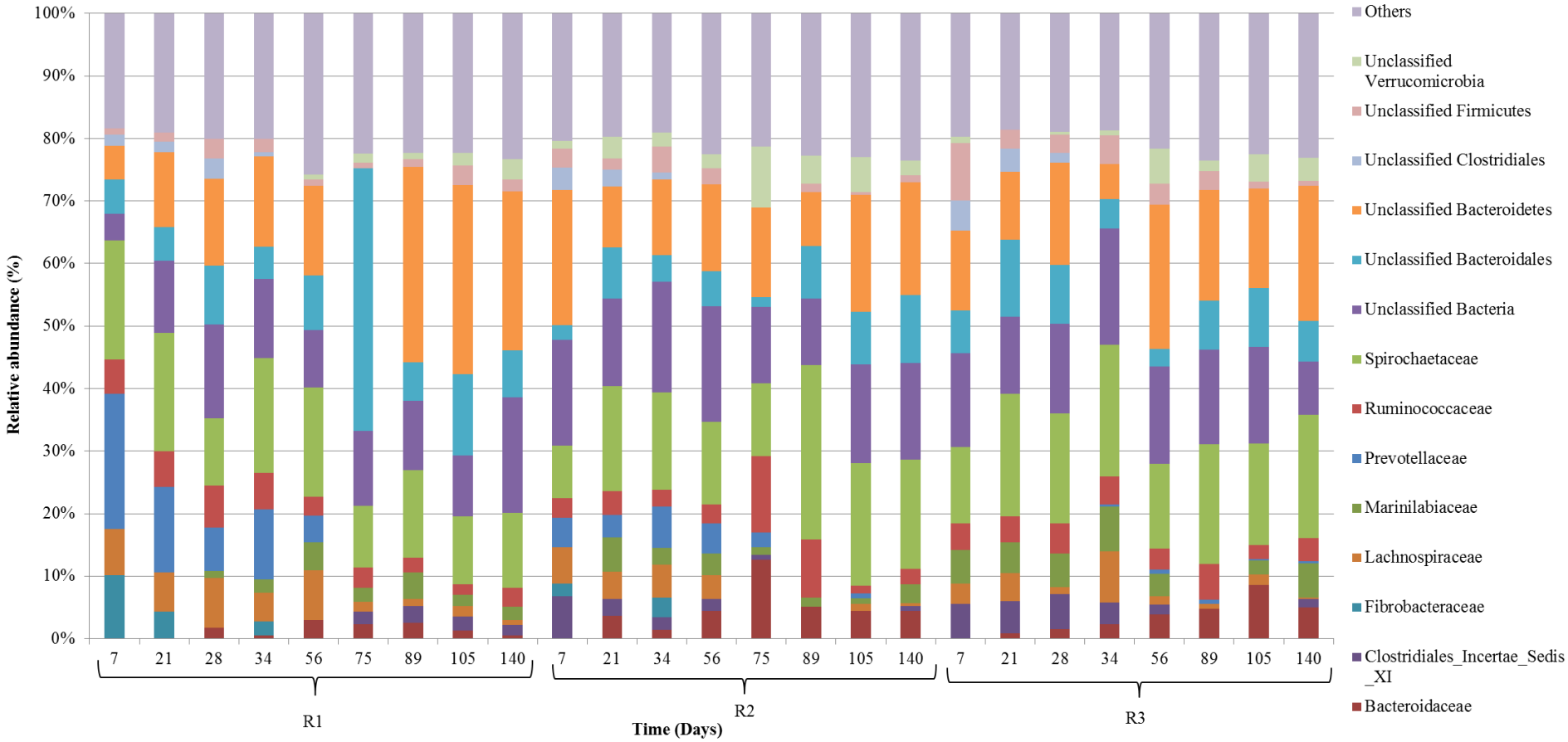


Figure 7.7 Bacterial community in Reactors

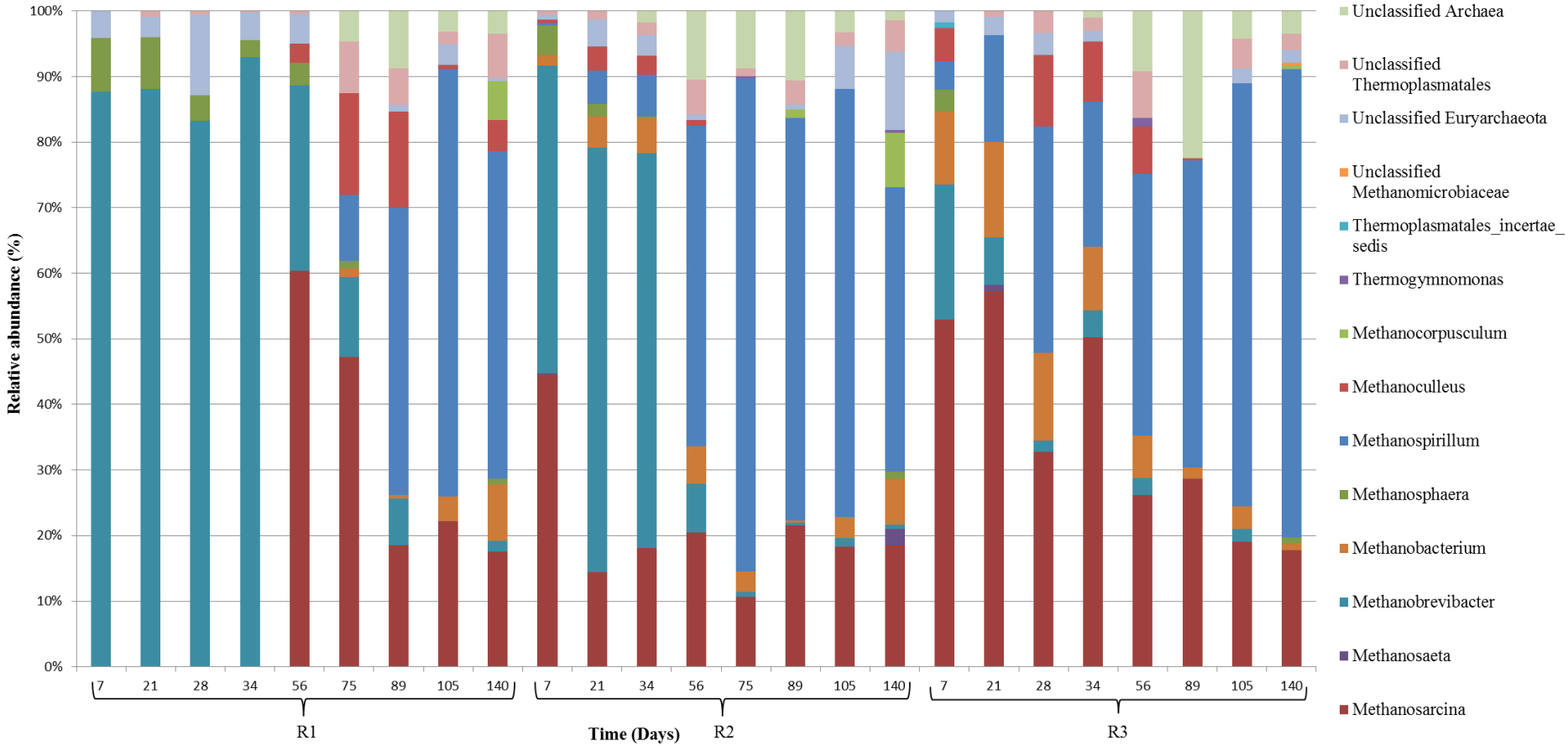


Figure 7.8 Archaeal community in Reactors

During the whole experiment the most dominating phyla in all three reactors were *Bacteroidetes*, *Firmicutes* and *Spirochaetes* For the reactors, only 30–50% of samples could be classified in the family section. During the first 34 days R1 presented a bacterial community dominated by *Spirochaetaceae* and by hydrolytic bacteria such as *Fibrobacteres*, *Bacteroidetes*, *Clostridium*, *Lachnospiraceae* and *Ruminococcaceae* (Azman et al., 2015) and an archaeal community dominated by *Methanobrevibacter*. The fact that the community was highly dominated by unclassified *Bacteroidales* and *Bacteroidetes* suggest that this reactor had a good hydrolytic and fermentative activity. Nevertheless, the community presented a lot of variation probably due to the VFA build-up, the low pH levels and the change in the HRT. *Spirochaetes* was represented by the genus *Treponema* which is a homo-acetogen able to produce acetate from H_2 and CO_2 (Brune, 2014). This genus was increasing its dominance during this period explaining the acetate build-up. Apparently, due to the presence of hydrolytic and fermentative bacteria such as the genus *Fibrobacter*, the production of H_2 should be very high during the first 34 days being the responsible for the high biogas production at the first week (Ransom-Jones et al., 2012). However, since the Archaeal Shannon index for this reactor was quite low and no acetoclastic methanogen was present, the acetate produced by *Treponema* genus accumulated in the reactor having bad consequences such as very low methane production and VFA levels around 14000 g L^{-1}. The equilibrium between hydrolysis and Methanogenesis was not present.

R2 and R3 had a similar bacterial community with the clear dominance of *Treponema*, and unclassified *Bacteroidales* and *Bacteroidetes* suggesting, that the production of H2 was also high. Nevertheless, R2 presented a difference from R3, the presence of the genus *Fibrobacter*. *Fibrobacter* is a genus quite common in RC microorganisms and is, with other populations, the responsible for the cellulose degradation. Only R1 and R2, the reactors inoculated with RC, presented this genus, which confirm the high hydrolytic activity and the VFA build-up that both reactors experimented at the beginning of the experiment. However, due to the presence of *Methanosarcina* in MSU inoculum, acetate could be degraded faster in R2 than R1. Besides, the archaeal Shannon index in R2 and R3 was higher than the one presented by R1. Another indicator that H_2 production was high was propionate which levels were very high in all three reactors. Due to a high Gibbs free energy, the degradation of propionate can only be performed under low concentrations of H_2 (McCarty and Mosey, 1991; Mosey and Fernandes, 1989).

After this period of 34 days, the relative abundance of *Treponema* decreased so the VFA started to be degraded in R1 and, at the same time, propionate was also degraded in R2 and R3. These facts suggest that the production of H_2 decreased, probably, because the mixing was stopped. As it was mentioned before, different studies have shown that hydrogen build-up might occur when the system is mixed. When the system was not stirred anymore, less H_2 was produced and therefore the levels of acetate and propionate could decrease. The pH was higher than 6.5 at that time due to the buffer addition. Because of these two mentioned facts, the archaeal Shannon index increased and *methanosarcina* genus started to appear in R1.

During the rest of the experiment the bacterial community was dominated by unclassified *Bacteroidetes* and *Treponema*. In R1, the relative abundance of some of the hydrolytic bacteria that were present at the beginning like *Fibrobacteres, Lachnospiraceae* and *Ruminococcaceae* decreased or were null. The fact that the genus *Fibrobacter*, typical in rumen, was not present when all three reactors were presenting the same conditions and low hydrolysis rate towards the end of the experiment, suggest that this genus is really important to maintain a high hydrolytic activity. *Fibrobacter*, which was present in R1 and R2 at the beginning, might be washed out from the system or it just disappeared because of the reactor conditions were not suitable for its development.

Table 7.5 Shannon index for the bacterial and archaeal community in the three reactors at different days

Shannon index	Day 7	Day 21	Day 28	Day 34	Day 56	Day 75	Day 89	Day 105	Day 140
Bacteria R1	2.49	2.52	2.80	2.70	2.80	2.06	2.31	2.36	2.42
Bacteria R2	2.77	2.87	n.a.	2.76	2.62	2.27	2.31	2.41	2.49
Bacteria R3	2.75	2.63	2.52	2.61	2.44	n.a.	2.46	2.56	2.47
Archaea R1	0.45	0.46	0.57	0.31	1.05	1.58	1.59	1.06	1.61
Archaea R2	1.05	1.24	n.a.	1.33	1.50	0.88	1.13	1.12	1.72
Archaea R3	1.42	1.27	1.54	1.45	1.64	1.13	n.a.	1.15	0.98

n.a. Non analyzed

7.3.3 The inoculum effect

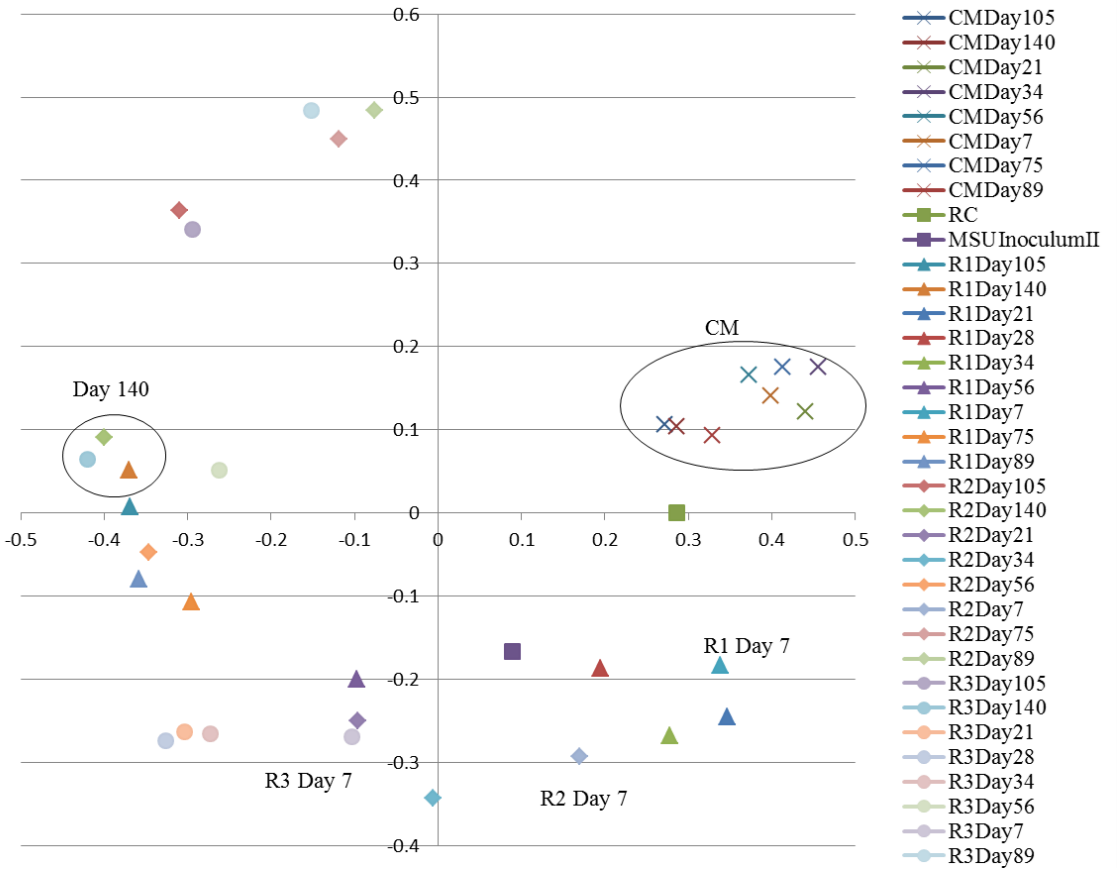


Figure 7.9 PCoA plot of microbial communities

When interpreting an ordination plot such as the one in Figure 7.9, it is important to realize that the two separate axes are meaningless. The important quantitative relationships in Figure 7.9 are the distances between points. The goal is to visualize distances between samples, in this case, phylogenetic distances. Samples that appear closer together in the plots were more similar in community structure, and samples that are plotted farther apart had greater differences in community structure.

Figure 7.9 shows how the microbial community in R1 was very different from R2 and R3 and how close to the RC populations it was, explaining, the enormous difference in the performance. Towards the end, all three reactors ended up into the same point. Therefore, at the 140th day, the microbial community was independent of the inoculum used, being governed by the substrate and the digester parameters. The change in the microbial community can be the explanation because of the low SMP and VS removal. Apparently, R1 and R2 presented a high hydrolytic activity at the beginning since the biogas production was high during the first week. According to the microbial analysis

(Fig 7.7) R1 presented populations with good hydrolytic activity like *Fibrobacter*. However, all three digesters presented low SMP and VS removal. The drastic change in the microbial populations (Fig. 7.9) can be the explanation of this behavior, suggesting that hydrolytic populations could not remain in the system.

Even though the SMP was around 0.16 $LCH_4\ gVS^{-1}\ day^{-1}$, the AD of NG could be suitable due to its high biomass yield (Ahring and Uellendahl, 2013; Song et al., 2014; Waramit and Chaugool, 2014). Nevertheless, increasing the production of methane is very important to ensure the application of this renewable technology instead of the energy production through the use of fossil fuels. Therefore, the idea of integrating RC into the AD of lignocellulosic substrates needs to be further developed with the optimal use of hydrolytic bacteria through AcoD or microorganisms immobilization.

7.4 Conclusions

In this work RC was used as an inoculum for the semi-continuous AD of NG to bring microbial populations with a high hydrolytic. This strategy was evaluated by analyzing the digester performance and the microbial community involved in the experiment. The main conclusions extracted from the study are summarized as follows:

- The inoculation with only Rumen content performed a fast hydrolysis, but, due to the low pH and alkalinity presented and the absence of acetoclastic methanogens, the equilibrium between hydrolytic bacteria and methanogens could not be accomplished, leading to a very poor performance.
- Mixing a conventional inoculum with rumen content was a good strategy. Hydrolytic microorganisms were brought from rumen into the system improving the biogas production, and, the conventional inoculum allowed the reactor stabilization under a VFA build-up
- When Rumen is used as inoculum, a co/substrate with a high buffer capacity is highly recommended to ensure a good performance
- Towards the end of the experiment the microbial community was quite similar between all three reactors and *Fibrobacter* was not present anymore. Therefore, low SMP and VS removal were registered.

8. Anaerobic digestion of lignocellulosic substrates with cow manure and rumen as potential co-substrates

Abstract

To improve the methane production during the Anaerobic digestion of Napier grass, a second generation biomass with a high concentration in lignocellulosic components, cow manure and rumen were used as co-substrates. Cow manure is an agricultural waste which presents a good buffer capacity and a high amount of nutrients for anaerobic digestion. Rumen is a waste from the slaughterhouses which harbors a microbial community able to degrade lignocellulose.

Three reactors, inoculated with the same inoculum, were used to study the effect of adding rumen as a co-substrate. R1 used only cow manure as a co-substrate, R2 used cow manure and rumen and R3 used only rumen.

The results showed that the cow manure is really important to maintain a good buffer capacity and pH in the system. Alkalinity needed to be added to R3 at the 44^{th} day because the pH and partial alkalinity reached 6.9 and 2.7 g_{CaCO3} L^{-1} respectively. However, the addition of rumen did not induce a fast hydrolysis and the SMP (0.14 LCH4 gVS^{-1} day^{-1}) and the VS removal (20-30%) remained quite low during the whole experiment. The microbial analysis demonstrated that , the digester conditions, specially pH, were not suitable for the development of rumen hydrolytic bacteria.

This chapter was presented as oral <u>communication</u> in:

- **Anaerobic digestion of lignocellulosic substrates with cow manure and rumen as potential co-substrates.** 14th World Congress on Anaerobic Digestion, Viña del Mar, Chile, November 2015.

And then in preparation for <u>publication</u> as:

- Fonoll, X., Shrestha, S., Dosta, J., Mata-Alvarez, J., Khanal, S., Raskin, L. **Anaerobic digestion of lignocellulosic substrates with cow manure and rumen as potential co-substrates.**

8.1 Introduction

Recent concerns about climate change, resource limitation, and energy independence have served as a catalyst for the development of renewable energy, including the production of biofuels. Most of the growth in this area has been in the production of so-called first generation biofuels from carbohydrates and oils found in crops such as corn, sugar cane, and soybean. However, the increased production of these crops for fuel production has environmental consequences through competition with food production, changing land-use practices, and impacts on water resources and water quality (Fargione et al., 2008; Searchinger et al., 2008).

SGB, which are derived from agricultural residues/wastes as well as lignocellulosic energy crops such as switchgrass and NG, are more suitable for sustainable bioenergy production compare to energy crops. So far, the focus of first- and second-generation biofuel production has been on biomass conversion for the production of ethanol and biodiesel. However, AD of biomass using mixed communities of anaerobic microorganisms has the potential to generate high amounts of renewable energy as biogas, which is rich in methane. Biogas production from energy crops is thermodynamically more efficient than converting plant matter into liquid fuels (J. Frigon and Guiot, 2010). In addition, AD provides more flexibility since it can be used at different scales (household to large industrial scale), allows the use of a variety of feedstock with AcoD, and biogas can be used to produce heat, electricity, or compressed natural gas for use as vehicle fuel.

In recent years, there has been increasing interest in the use of NG as a potential energy crops due to its high biomass yield potential, high lignocellulose content, low water demand, and positive environmental impact (Waramit and Chaugool, 2014). However, its methane potential cannot be exploited completely due to the presence of recalcitrant components such as lignin, hemicellulose and cellulose (Raposo et al., 2012). As was mentioned before, the integration of RC in the AD of lignocellulosic substrates would bring different microbial populations able to perform the hydrolysis of these kind of substrates into the system. It was observed in the previous chapter that the SMP of digesters inoculated with RC did not differ from the SMP produced from a digester inoculated with a conventional inoculum because, in a long term, both microbial communities were similar. Using RC as a co-substrate instead of using it as an inoculum could be a better approach since the microbial populations mentioned before could be

introduced continuously. Since now there are no studies focusing on the use of rumen as a co-substrate.

In the present study RC was used as a co-substrate to enhance the methane production during the AcoD of NG and CM. Molecular tools were also performed to evaluate the changes in the microbial community structure when different co-substrates are used.

8.2 Materials and methods

8.2.1 Substrates and inoculum

Napier grass (2 months old) was harvested from Waimanalo Research Station (Waimanalo, HI, USA). The biomass samples were shredded using a cutting mill (Vincent Corporation, Tampa, FL, USA) and air dried to reduce the moisture content to less than 10%. It was then passed through a second laboratory cutting mill with a screen size of 2mm. 30% (ww) of CM, obtained from a dairy farm in Michigan State University (MSU), was blended with 70% (ww) of urine also obtained from MSU farm. The inoculum used (MSU inoculum) was obtained from a full-scale AD plant at MSU treating a mixture of food waste and cow manure at mesophilic conditions. Solid and liquid samples of RC were obtained from a fistulated cow in MSU farm and stored at 4ºC during the transportation (Hervás et al., 2005). Once in the lab both samples were mixed together to get a TS content of 12% and simulate better RC from slaughterhouses (Tritt and Schuchardt, 1992). RC mixtures were stored at -20ºC and thaw during 10 minutes in a water bath at 37ºC before its use. The substrates and inoculum characteristics can be seen in Table 8.1. NG, CM mixtures were stored at 4ºC and the inoculum was immediately used.

Table 8.1 Inoculum and substrates characteristics

		NG	CM + Urine	RC	MSU Inoculum
TS	mg L^{-1}	886 ± 19	68 ± 3	117 ± 7	32 ± 0
VS	mg L^{-1}	791 ± 17	48 ± 3	107 ± 6	22 ± 0
pH	-	n.a.	8.9 ± 0.1	6.0 ± 0.0	8.1 ± 0.1
PA	gCaCO3 L^{-1}	n.a.	26.3 ± 4.5	0.6 ± 0.0	7.7 ± 1.3
TA	gCaCO3 L^{-1}	n.a.	34.0 ± 5.6	5.2 ± 0.0	9.7 ± 0.6
VFA	g L^{-1}	n.a.	5.2 ± 1.3	8.0 ± 0.1	2.6 ± 0.1
NH_3	mg L^{-1}	n.a.	4734.4 ± 1674.5	0.2 ± 0.1	163 ± 25

n.a. Non analyzed

8.2.2 Experimental design

Two semi-continuous stirred tank reactors with a working volume of 1.0 L were run at mesophilic conditions (37°C). Reactor 1 (R1) was fed a mixture of NG and CM (30:70, w:w), Reactor 2 (R2) was fed with a mixture of NG, CM, and RC (25:60:15, w:w:w) and reactor 3 (R3) was fed with a mixture of NG and RC (85:15, w:w). All the mixtures were diluted with water until getting a TS content of 9%. During the start-up the OLR was gradually increased to 4.0 g VS L^{-1} day^{-1} and the HRT decreased to 20 days. Table 8.2 shows all the changes made on design parameters during this stage. The inoculum used in both reactors was MSU inoculum and the reactors were shaken once a day until the 40^{th} day when a shaker water bath started to be used. The HRT was increased up to 30 days to improve the solid degradation at the 60^{th} day. The biogas collected in Tedlar gas bags was measured by a gas meter daily.

Table 8.2 Reactors design parameters

	R1			R2			R3		
Period of days	OLR ($gVS\ L^{-1}\ day^{-1}$)	HRT (days)	TS content (%)	OLR ($gVS\ L^{-1}\ day^{-1}$)	HRT (days)	TS content (%)	OLR ($gVS\ L^{-1}\ day^{-1}$)	HRT (days)	TS content (%)
$1 – 3^{rd}$	1.0	79	9.1	1.1	75	9.3	1.0	82	9.1
$3^{rd} – 14^{th}$	2.0	39	9.1	2.0	38	9.3	2.0	41	9.1
$14^{th} – 23^{rd}$	3.0	26	9.1	3.2	25	9.3	3.0	27	9.1
$23^{rd} – 60^{th}$	4.0	20	9.1	4.3	19	9.3	4.0	20	9.1
$60^{th} – 113^{rd}$	2.6	30	9.1	2.8	29	9.1	2.6	31	9.1

8.2.3 Analytical methods

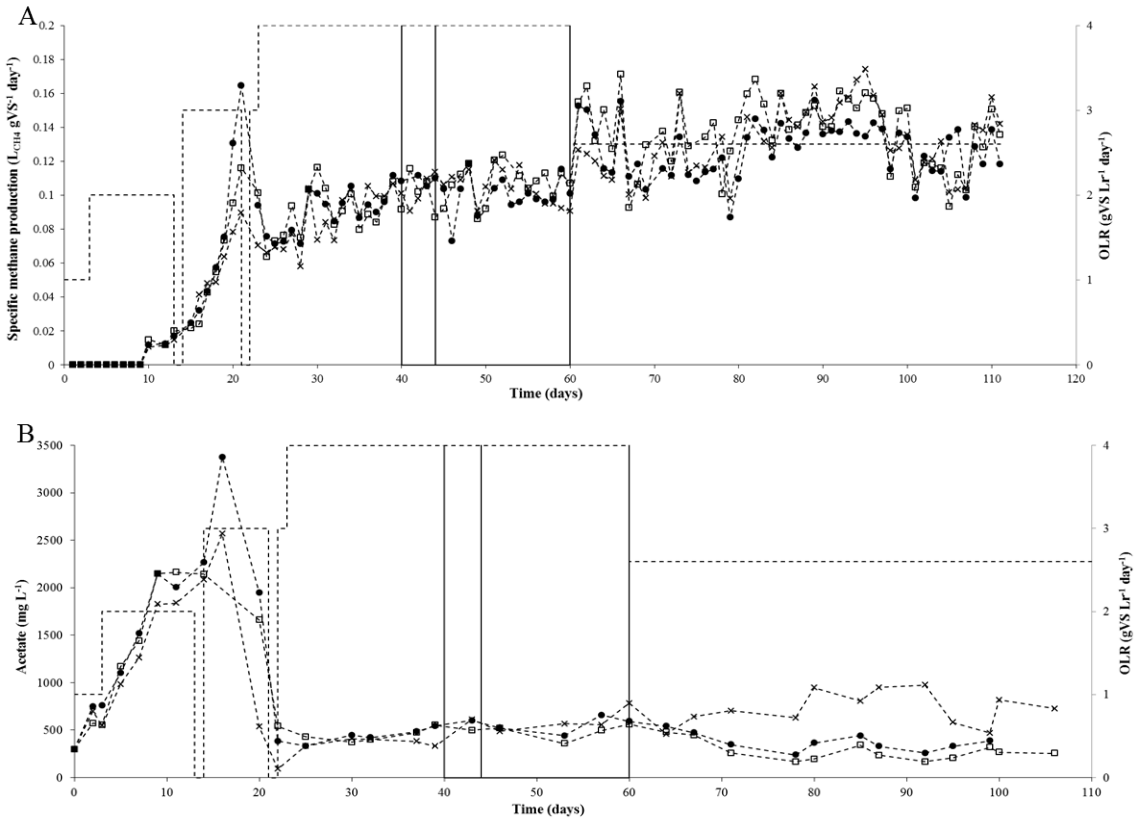
TS, VS, TA, PA, IA, NH_3, VFA and biogas composition were measured as stated in the section 3.1.2.

8.2.4 Microbial analysis

All the microbial analyses were performed as stayed in section 3.3. However, due to the low number of reads of some sequences, while MOTHUR was running the subsampling step was skipped (McMurdie and Holmes, 2014).

8.3 Results and discussion

8.3.1 Reactors Performance



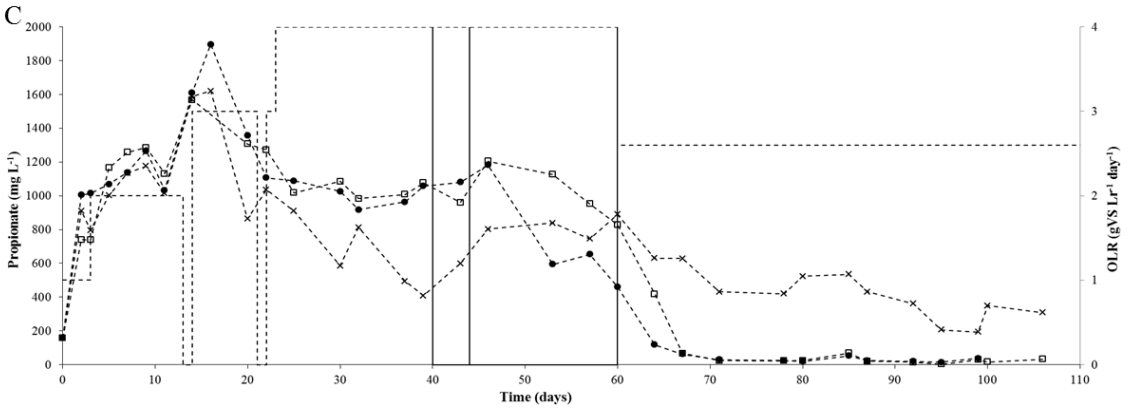


Figure 8.1 SMP (A), Acetate (B) and Propionate (C) levels from R1 (●), R2 (□) and R3 (×) Change of stages (▬▬) and OLR (▬ ▬).

The start-up of three reactors took 23 days. In this period, the OLR increased from 1.0 gVS L^{-1} day^{-1} to 4.0 gVS L^{-1} day^{-1} and the HRT decreased from 79 to 20 days. The biogas production was very low until the OLR was increased to 3.0 gVS L^{-1} day^{-1} in the 14th day. Since that day, the SMP in all three reactors could increase and remained constant (0.10 LCH_4 gVS^{-1} day^{-1}) until the 60th day. The VFA increased up to 6000 mg L^{-1} in 16 days and therefore the feeding was stopped at the 14th and 21th day. Due to this action, between the 16th and the 20th day, the VFA started to decrease, increasing the SMP up to 0.16 LCH_4 gVS^{-1} day^{-1}. VFA peaks are quite common during the start-up of AcoD (Fonoll et al., 2015). By the 25th day VFA levels could be around 1600 mg L^{-1} and constant during the next 35 days in all three reactors due to acetate degradation (Fig 8.1b). However, propionate remained constant at 1000 mg L^{-1} in R1 and R2 and 700 mg L^{-1} in R3. In fact, the P/A ratio was between 1.5 and 2.3 in all three reactors which is a sign of process instability (Hill et al., 1987). Nevertheless, only in R3 PA and pH decreased continuously. In R1 and R2, due to the low start-up, the good conditions of MSU inoculum (pH=8.1 and PA=7.7 gCaCO3 L^{-1}) (Table 8.1) and the addition of CM, the stability parameters such as PA and pH were maintained in a good range (pH=7.4 and PA=7.7 gCaCO3 L^{-1}) (Fig 8.2b). R3 presented such a different frame because its feedstock, constituted by NG and RC, had a very low buffer capacity (pH=6.0 and PA=0.6 gCaCO3 L^{-1}) (Table 8.1). At the 44th day, buffer in form of NH_4HCO_3 started to be added continuously to R3 and the pH and the PA could remain constant (PA= 3.3 ± 0.3 gCaCO3 L^{-1}; pH=7.0 ± 0.1) (Fig. 8.2b).

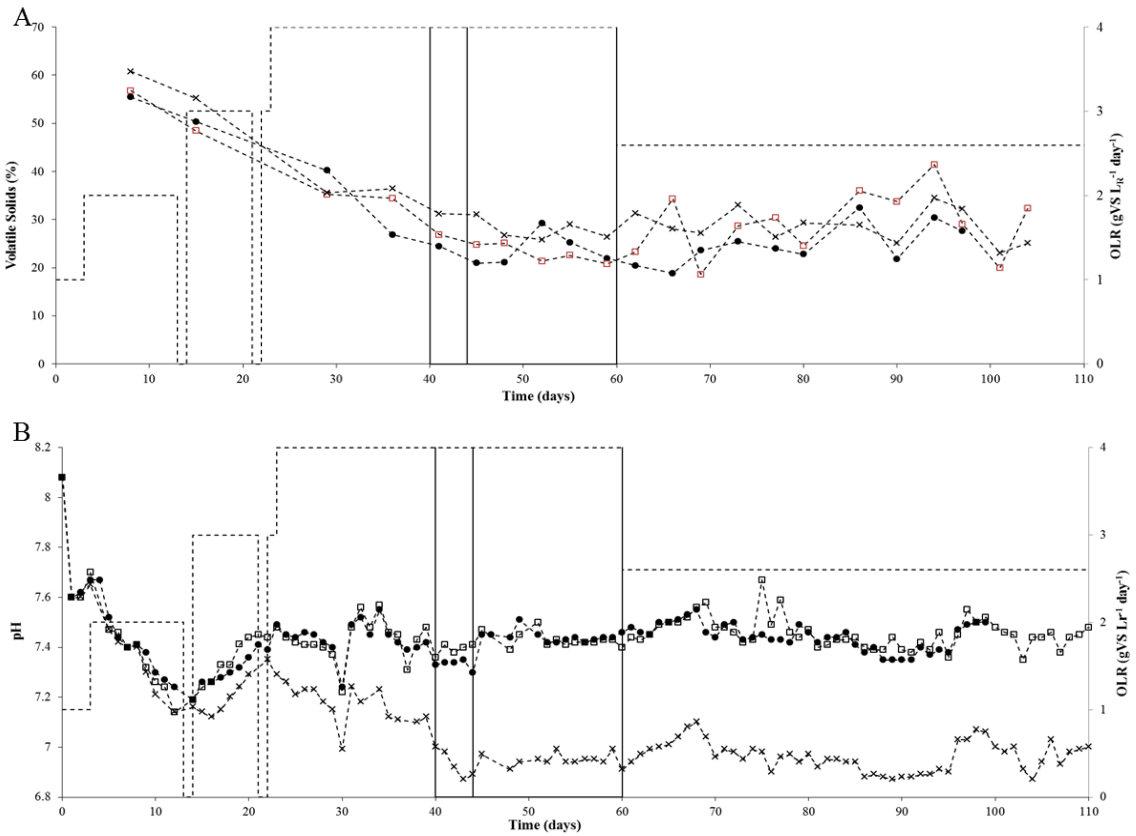


Figure 8.2 VS removal (A) and pH levels (B) from R1 (●), R2 (□) and R3 (×). Change of stages (▬▬) and OLR (▬ ▬).

The main problem was the low levels of SMP (0.10 LCH_4 gVS^{-1} day^{-1}) and VS removal (40%) (Fig. 8.1a and 8.2a). Vavilin et al. (2002) demonstrated that a gradual increase in the OLR during the start-up can allow the growth of methanogenic archaea, but, after the initial startup phase, an increase in mixing aids the mass transfer of nutrient and accelerates solid waste digestion. Therefore, at the 40th day it was decided to mix the reactors continuously, but this action did no improve neither the SMP, which did not change at all, nor the VS removal, which decreased between 20 and 30%. At the 53rd day propionate started to decrease in R1 and later on in R2. Usually, propionate degradation is not performed under high concentrations of H_2 but, somehow, the H_2 levels could decrease. Perhaps, mixing could have developed populations able to use H_2 as substrate. However, after mixing the propionate levels increased in R3, maybe, due to the low pH presented in this reactor at 40th day (fig. 8.1c and 8.2b).

As it was mention in chapter 7, HRT can be a crucial parameter to improve the degradation of lignocellulosic biomass. One HRT after the mixing started, it was decided to increase the HRT to 30 days which, at the same time, decreased the OLR to

2.6 gVS L^{-1} day^{-1} (Table 8.2). After the HRT increase, the SMP could only increase to 0.13 LCH_4 gVS^{-1} day^{-1} and the VS removal remained very low (around 30%) (Fig. 8.1a and 8.2b). The hydrolysis activity could not be enhanced, but, propionate could be degraded in R3 up to 350 mg L^{-1}.

8.3.2 Microbial analysis

8.3.2.1 Inoculum and substrates

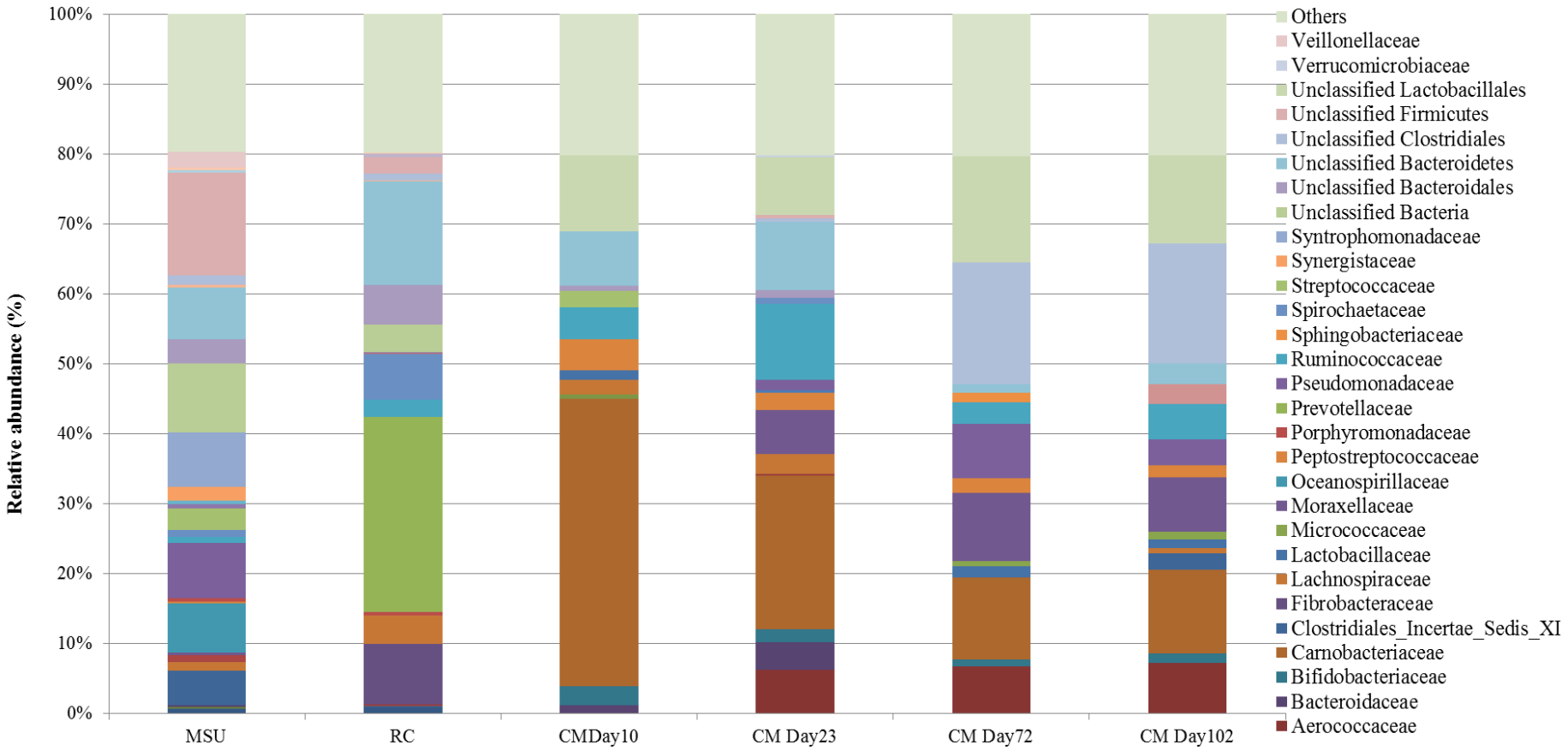


Figure 8.3 Bacterial community in the inoculum and substrates

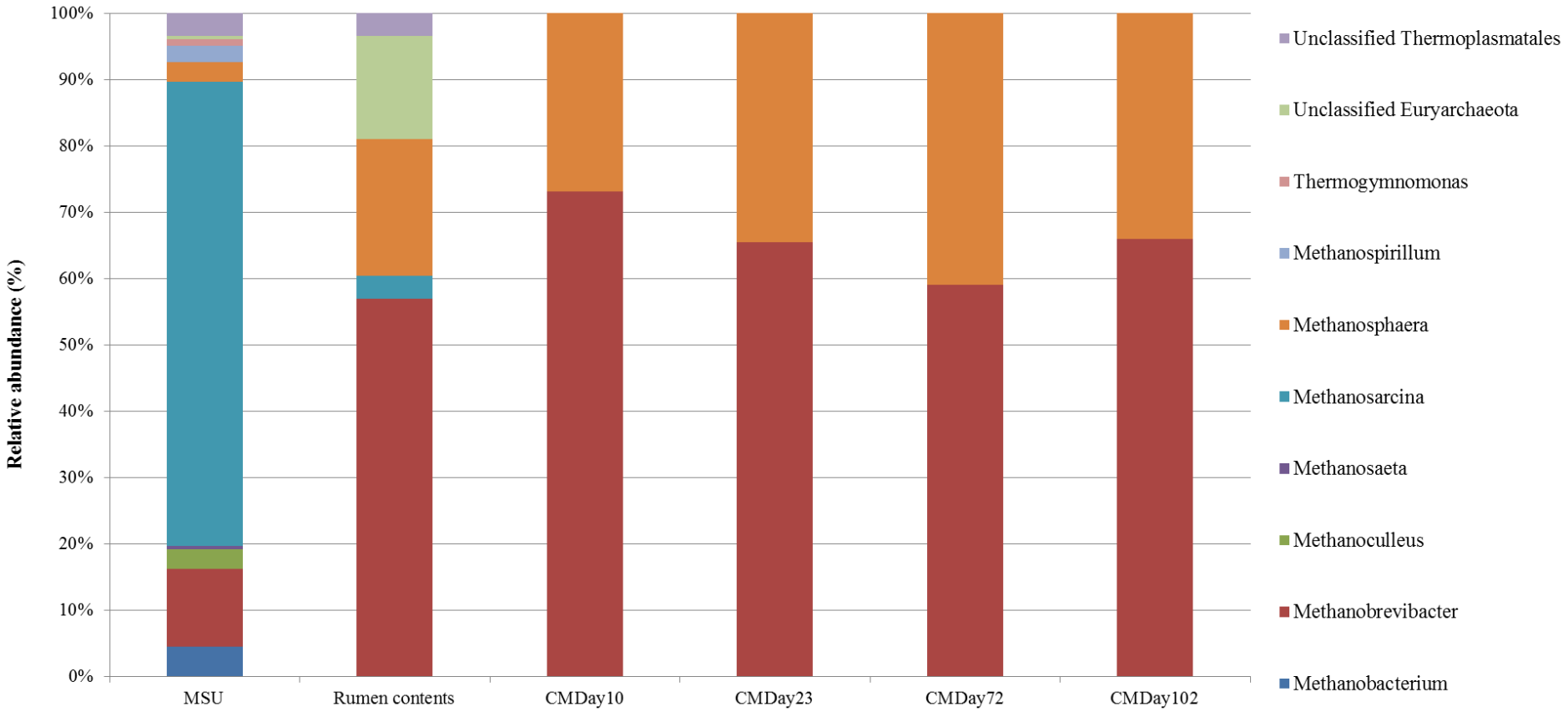


Figure 8.4 Archaeal community in the inoculum and substrates

The bacterial community in the inoculum and substrates was dominated by *Bacteroidetes* and *Firmicutes* (Fig. 8.3). Those phyla are rich in anaerobic bacteria able to degrade lignocellulose components, and to perform fermentation using hydrolysis products such as amino-acids, carbohydrates or long- and mid-chain fatty acids (Ren et al., 2014; Sun et al., 2013; Wang et al., 2014; Zheng et al., 2015). In those samples, between the 60 and 90% of the populations could be classified in the family subcategory.

As happened in chapter 7, the bacterial community in the inoculum was again dominated by *Syntrophomonadaceae*, a family of bacteria able to degrade butyric and propionic acid into acetate (Smith et al., 2015) and unclassified *Firmicutes*. However, in this case *Oceanospirillaceae*, which normally lives in salty environments and *Pseudomonadaceae* were dominant at the same level than *Syntrophomonadaceae*. RC families were dominated by microorganisms typically found in rumen such as: *Prevotellaceae*, *Spirochaetaceae*, a homo-acetogen that produces acetate from H_2, and hydrolytic bacteria such as *Lachnmospiraceae* and the genus *Fibrobacter*. According to the results obtained in chapter 7 and other studies (Azman et al., 2015), Fibrobacter has a high hydrolytic activity. The microbial community in CM varied with time. At the beginning the community was clearly dominated by *Carnobacteriaceae*, but the presence of this family decreased with time and unclassified *Clostridiales* and *Lactobacillales* increased.

Regarding the archaeal community, MSU inoculum was clearly dominated by acetoclastic methanogens (*Methanosarcina*) (Fig. 8.4). RC had a better archaeal diversity compared to the RC used in chapter 7. Again, the community was dominated by *Methanobrevibacter* and *Methanosphaera*, typical methanogens in rumen (McKain et al., 2013), and the presence of acetoclastic archaeas was negligible. CM also presented a microbial community with only hydrogenotrophic archaea (Fig. 8.4).

According to the microbial community analysis, MSU was a good inoculum for the AcoD of NG since it contained families with syntrophic and acetoclastic activity. RC appeared to be a better co-substrate than CM in terms of microbial community since bacteria with a high hydrolytic activity like *Fibrobacter* was present in a high percentage. The dominating families in CM were not related to hydrolytic bacteria, although, towards the end, the CM microbial community was dominated by the order *Clostridiales*, which could be composed of hydrolytic strains. Nevertheless, as was

mentioned in the previous section, CM presented better chemical characteristic such a high buffer capacity (Table 8.1).

8.3.2.2 Reactors

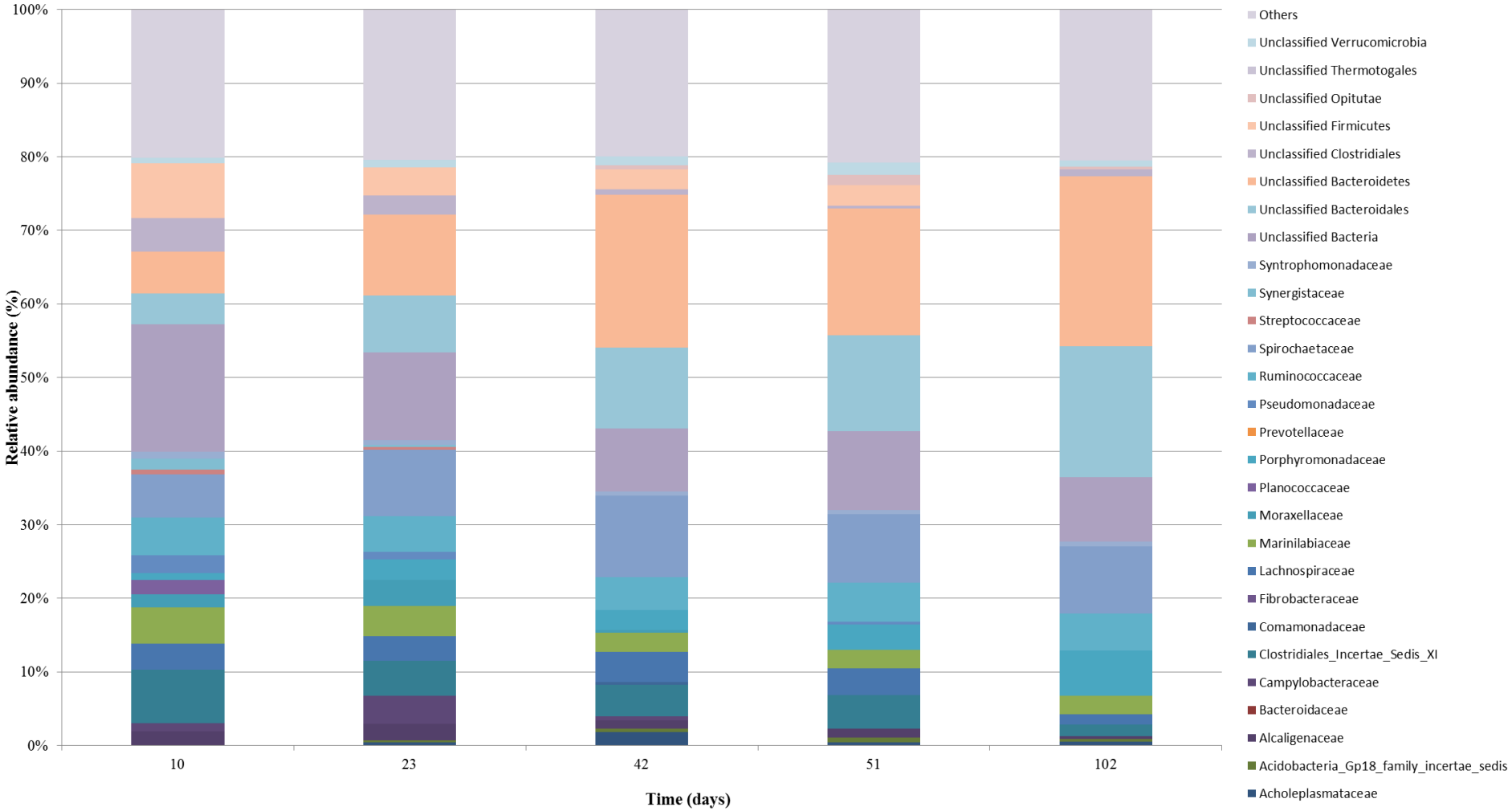


Figure 8.5 Bacterial community in R1

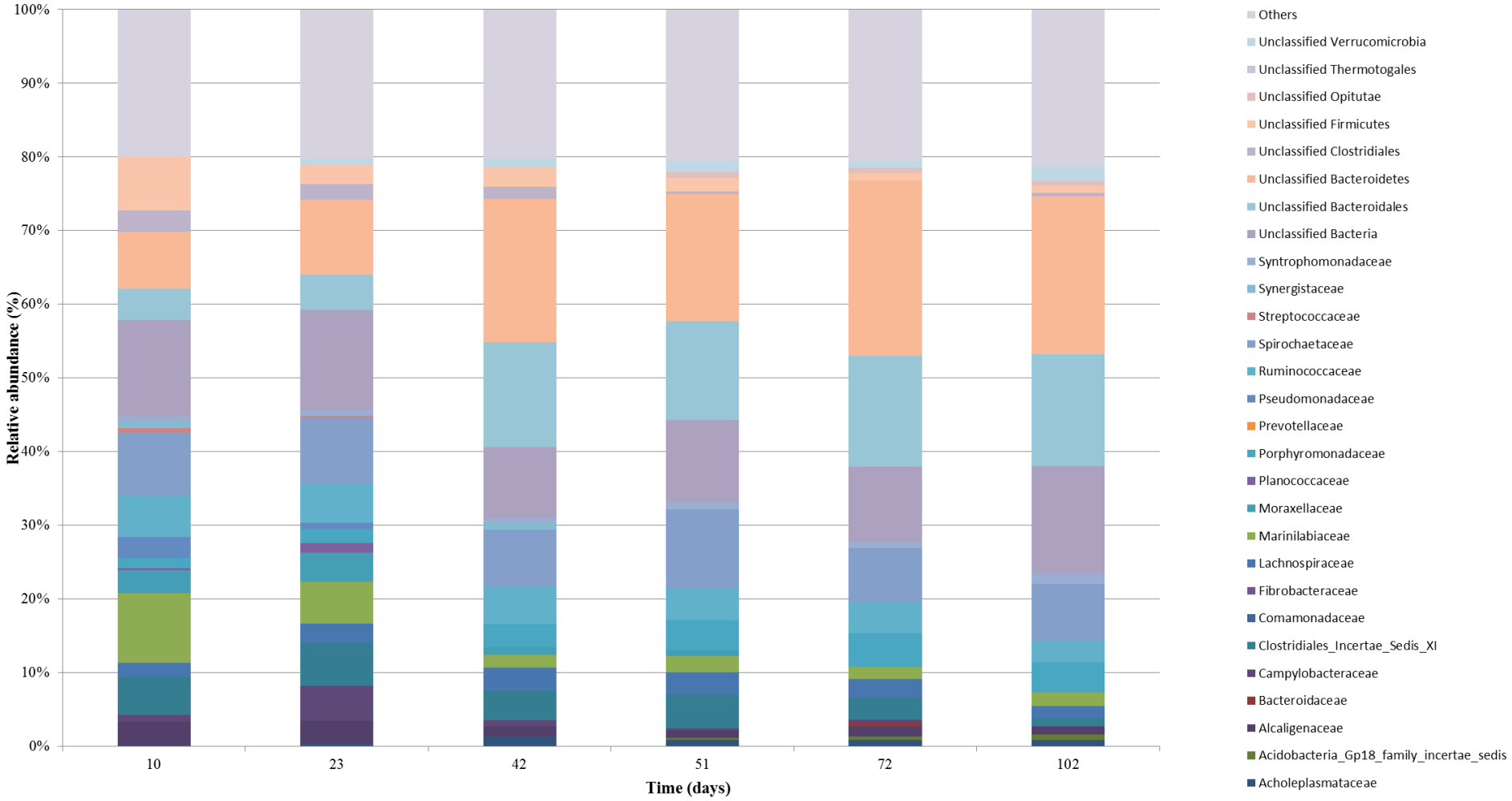


Figure 8.6 Bacterial community in R2

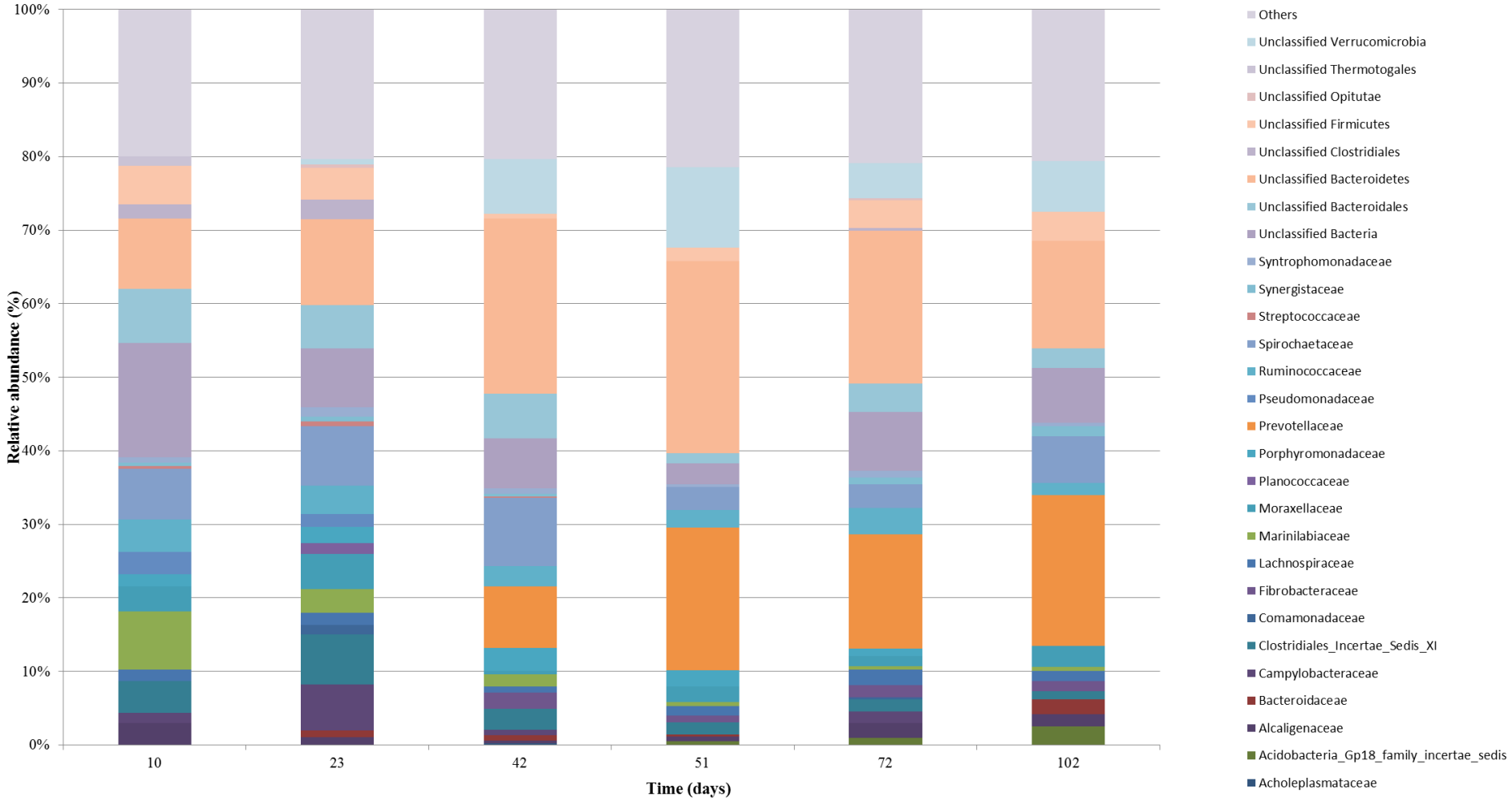


Figure 8.7 Bacterial community in R3

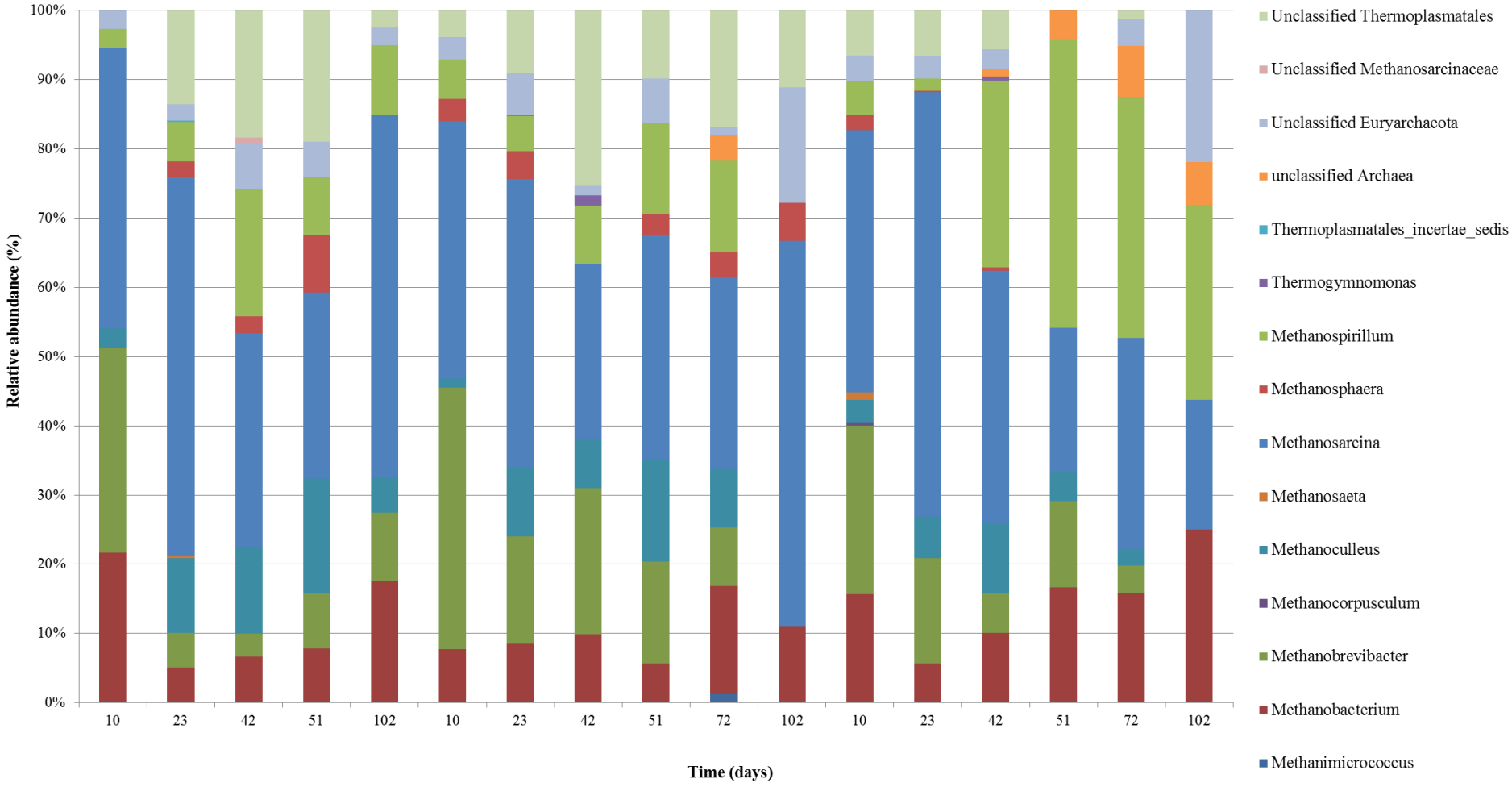


Figure 8.8 Archaeal community in R1, R2 and R3

During the whole experiment the most dominating phyla in all three reactors were *Bacteroidetes*, *Firmicutes* and *Spirochaetes* for Bacteria (Fig. 8.5, 8.6 and 8.7). Even though the 40-50% of the populations could not be classified in the family subcategory, the bacterial community was quite similar between in all three reactors but, for R3, the microbial community started to be different compared to R1 and R2 due to decrease in pH.

In R1 and R2 the microbial community was dominated byunclassified *Bacteroidetes*. *Ruminococcaceae* and *Spirochaetaceae*. At the beginning *Clostridiales and Marinilabiaceae* were present, but, the community changed at the 42nd day and the relative abundance of unclassified Bacteroidetes increased. This change is also connected to a change suffered by the methanogenic populations (Fig. 8.8). The acetoclastic populations were the most present genus at the beginning explaining the low levels of acetate. However, at the 42nd day hydrogenotrophic populations increased. This change could be the reason for the propionate degradation at the 55th day. H_2 levels seemed to be high at the beginning since the relative abundance of hydrogenotrophic archaea was low and propionate levels were high. Apparently, the decrease in the H_2 levels could be the explanation for the increase of the unclassified *Bacteroidetes* relative abundance. Actually, the Shannon index for the archaea community in the inoculum was low (1.2). The use of an inoculum whit a highest evenness and diversity is important to avoid propionate accumulation during AcoD. However, RC did not affect the microbial community in R2 even though this co-substrate presented a high dominance of hydrolytic bacteria (Fig. 8.3).

At the beginning, the microbial community in R3 was similar than the community in R1 and R2, being governed by *Clostridiales*, *Marinilabiaceae*, *Ruminococcaceae* and *Spirochaetaceae* (Fig. 8.5, 8.6 and 8.7). Besides, the methanogenic community was also dominated by *Methanosarcina* (Fig. 8.6). The community started to be different at the 42nd day when the pH was constant around 7.0 (Fig.8.2b). When the pH was low, typical microorganisms from rumen started to appear like the genus *Fibrobacter* and the family *Prevotellaceae* (Fig. 8.7). Nevertheless, the relative abundance of *Fibrobacter* was very low and, therefore, the VS removal and SMP could not be very high as it was expected when RC was used as co-substrate (Fig. 8.1a and 8.2a). Actually, the conditions in that reactor were not optimal for the development of *Fibrobacter* since the pH in rumen is usually around 6.0 (Tritt and Schuchardt, 1992). In R3 propionate could also be degraded due to an increase of hydrogenotrophic archaea, however, due to the

low pH, the relative abundance of *Methanosarcina* (18%) was lower than the relative abundance in R1 or R2 (55%) making the acetate levels high at the end of the experiment in R3.

Even though rumen microorganisms were added continuously into the system the reactor conditions did not seemed to be suitable for them and the hydrolysis was slow. Actually rumen conditions are: pH= 6.0, T= 35 °C and SRT= 2 days (Tritt and Schuchardt, 1992). However in AD the pH should be higher than 6.5, otherwise, the digester conditions would be harmful for the archaea community (Appels et al., 2008) To improve the hydrolysis by rumen microorganisms a system with the optimal conditions for both, rumen microorganisms and archaea should be designed to perform hydrolysis and produce methane.

8.4 Conclusions

In this work RC was used as a co-substrate for the semi-continuous AD of NG to bring microbial populations with a high hydrolytic. The main conclusions extracted from the study are summarized as follows:

- An inoculum with an even archaea community between hydrogenotrophic and acetoclastic methanogens can avoid the accumulation of propionate in the system.
- The use of only RC as a co-substrate can induce to a decrease in the pH being critical for the digester.
- Even though rumen was used as a co-substrate to continuously add hydrolytic bacterial populations, the pH was very for the development of rumen hydrolytic bacteria. Therefore, the volatile solid removal and the specific methane production were very low. The digester configuration used is not suitable for the anaerobic co-digestion of Napier grass with rumen content.

9. Conclusions and recommendations

9.1 Conclusions

In this study, different strategies to improve the economic viability of Anaerobic digestion plants considering, the presence of recalcitrance compounds on the substrates used (MSW, agricultural wastes and second generation biomass), have been studied in order to contribute to the knowledge of more sustainable treatment processes.

The main conclusions extracted from this work are compiled in this section:

Chapter 4: Anaerobic co-digestion of sewage sludge and fruit wastes: Evaluation of the transitory states when the co-substrate is changed

- When the co-substrate was changed the process stability was not affected. Only volatile fatty acids concentration increased during a short period of time when Anaerobic co-digestion started due to the sudden increase in the organic loading rate.
- Due to the high biodegradability of some fruit wastes, the alkalinity decreased during Anaerobic co-digestion. To avoid the process failure, the sewage sludge used must have a good buffer capacity.
- The methane production could be very low if the co-substrate contains a high amount of fibers. A strategy to improve the hydrolysis of those co-substrates needs to be studied to avoid negative economical balances.

Chapter 5: Effect of waste paper suppression on organic fraction of municipal solid waste anaerobic digestion: Biogas and digestate evaluation

- The waste paper separation from the municipal solid wastes increased the feedstock biodegradability.
- Half or total separation of waste paper can increase the specific methane and 27% respectively.
- Due to the feedstock biodegradability increase the reactor became fragile against instability periods. In case that such episode occurs, a digester without waster paper in the feedstock will take long time to recover.
- The digestate quality gets worse when waste paper is removed if low hydraulic retention times are applied.

- The waste paper removal will decrease the absolute methane production in the plant, so, the market of biogas and by-products obtained from waste paper should be studied before removing it.

Chapter 6: Anaerobic co-digestion of barley waste and pig manure under high ammonia concentrations: Low temperature and ultrasounds pretreatment application

When low-temperature and ultrasounds pretreatment were applied on bagasse, the methane production could increase in a 26 and 12% respectively.

According the energy balance results, only the low-temperature pretreatment would be recommended for its application in a full scale system. The reasons for the positive energy balance were the increase in the methane production and the availability to recover the heat used.

The reactors methane production where the pretreatments were applied decreased in 30 days at the same level than the reference reactor. Any other parameter changed so no explanation could be obtained for this behavior.

The use of an inoculum already adapted to high ammonia levels allows performing the Anaerobic co-digestion of pig manure under high ammonia levels without toxicity problems.

Chapter 7: Anaerobic digestion of lignocellulosic substrates: Inoculation with rumen, a natural ecosystem harboring hydrolytic bacteria

The inoculation with only Rumen content performed a fast hydrolysis, but, due to the low pH and alkalinity presented and the absence of acetoclastic methanogens, the equilibrium between hydrolytic bacteria and methanogens could not be accomplished, leading to a very poor performance.

- Mixing a conventional inoculum with rumen content was a good strategy. Hydrolytic microorganisms were brought from rumen into the system improving the biogas production, and, the conventional inoculum allowed the reactor stabilization under a VFA build-up.
- When Rumen is used as inoculum, a co/substrate with a high buffer capacity is highly recommended to ensure a good performance.

- Towards the end of the experiment the microbial community was quite similar between all three reactors and *Fibrobacter* was not present anymore. Therefore, low SMP and VS removal were registered.

Chapter 8: Anaerobic digestion of lignocellulosic substrates with cow manure and rumen as potential co-substrates

- An inoculum with an even archaea community between hydrogenotrophic and acetoclastic methanogens can avoid the accumulation of propionate in the system.
- The use of only RC as a co-substrate can induce to a decrease in the pH being critical for the digester.
- Even though rumen was used as a co-substrate to continuously add hydrolytic bacterial populations, the pH was very for the development of rumen hydrolytic bacteria. Therefore, the volatile solid removal and the specific methane production were very low. The digester configuration used is not suitable for the anaerobic co-digestion of Napier grass with rumen content.

9.2 Recommendations

For further research, the following recommendations are proposed:

- To study the effect of using greases from waste water treatment plants or slaughterhouse wastes as co-substrates in municipal solid wastes anaerobic digestion plants were the waste paper was removed.
- To couple a chemical pretreatment with NaOH to the low-temperature pretreatment to increase the methane production
- To study an immobilization technique when rumen is used as inoculum or co-substrate to make the most of hydrolytic bacteria such as Fibrobacter for long periods.
- To use a two-phase system when rumen is integrated in the Anaerobic digestion of lignocellulosic substrates. In the first phase, rumen should be used as a co-substrate at high organic loading rates to decrease the pH to simulate the conditions in rumen and degrade lignocellulosic compounds. In the second phase, an inoculum and a co-substrate with high pH and buffer capacity should be used to allow the good development of archaea.

- Molecular tools are necessary to understand how to improve the hydrolysis step. For future research, the extraction of RNA instead of DNA should be implemented to know which strain is active during the process.

Publications and congress communications

Publications

Astals, S., Dosta, J., Fonoll, X., Peces, M., Romero-Güiza, M., Mata-Álvarez, J., 2013. Codigestión anaeróbica como opción de mejora de la valorización energética de los lodos de depuradora. RETEMA, Revista Técnica de Medio Ambiente nº 170, pp. 68-71

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Shrestha, S., Fonoll, X., Raskin, L., Khanal, S.K. Bioengineering strategies for enhanced hydrolysis of lignocellulosic biomass during anaerobic digestion. In preparation.

Fonoll, X., Astals, S., Dosta, J., Mata-Álvarez, J. Effect of waste paper suppression on OFMSW anaerobic digestion: Biogas and digestate evaluation. In preparation.

Fonoll, X., Roig, R., Dosta, J., Mata-Álvarez, J. Anaerobic co-digestion of barley waste and pig manure under high ammonia concentrations: Low temperature and ultrasounds pretreatment application. In preparation.

Shrestha, S., Fonoll, X., Mata-Alvarez, J., Khanal, S., Raskin, L. Anaerobic digestion of lignocellulosic substrates: Inoculation with rumen, a natural ecosystem harboring hydrolytic bacteria. In preparation.

Fonoll, X., Shrestha, S., Dosta, J., Mata-Alvarez, J., Khanal, S., Raskin, L. Anaerobic digestion of lignocellulosic substrates with cow manure and rumen as potential co-substrates. In preparation.

Congress communications

Oral presentations

Mata-Alvarez, J., Dosta, J., Astals, S., Peces, M., Fonoll, X., Romero, M.S., 2013. Anaerobic co-digestion: A review of achievements and perspectives. 13th World Congress on Anaerobic Digestion. Santiago de Compostela (Spain), June 2013.

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Fonoll, X., Astals, S., Dosta, J., Mata-Álvarez, J., 2014. Effect of paper fraction on the mesophilic anaerobic digestion of OFMSW. Biogas and digestate evaluation. 9th International Conference ORBIT 2014. New Challenges, New Responses in the 21st Century. Gödöllő (Hungary), June 2014.

Fonoll, X., Shrestha, S., Kunstman, B., Mata-Alvarez, J., Khanal, S., Raskin, L., 2015. Anaerobic digestion of lignocellulosic substrates with cow manure and rumen as potential co-substrates. 14th World Congress on Anaerobic Digestion. Viña del Mar (Chile), November 2015.

Poster communication

Fonoll, X., Astals, S., Dosta, J., Mata-Alvarez, J., 2013. Anaerobic co-digestion: focusing on the transitory state when the co-substrate is changed. 13th World Congress on Anaerobic Digestion, Santiago de Compostela (Spain), June 2013

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Abbreviations

Symbol	Description	Units
AcoD	Anaerobic co-digestion	-
AD	Anaerobic digestion	-
A_{opt}	Tank surface	m^2
AW	Apple waste	-
BioW	Bio-waste	-
BMP	Bio-methane potential	-
BW	Banana waste	-
BOD	Biological Oxygen Demand	g O_2 L^{-1}
BSG	Barley spent grain	-
CAP	Common Agricultural Policy	-
CHP	Combined heat and power unit	-
CM	Cow manure	-
COD	Chemical oxygen demand	g O_2 L^{-1}
Cpb	Heat capacity of barley spent grain	kJ Kg^{-1} $^oC^{-1}$
Cps	Heat capacity of supernatant	kJ Kg^{-1} $^oC^{-1}$
CSTR	Completely Stirred Tank Reactor	-
D_{opt}	Tank diameter	M
E_i	Energy needed	kW
$E_{i,LTP}$	Energy needed for low-temperature pretreatment	kW
$E_{i,LTP1}$	Energy needed to heat BSG up to 60ºC	kW
$E_{i,LTP2}$	Energy losses due to convection	kW
$E_{i,USP}$	Energy needed for ultrasound pretreatment	kW
E_o	Energy obtained	kW
E_s	Specific energy for ultrasounds pretreatment	kJ $kgST^{-1}$
FOG	Fat oil and grease	-
FVW	Fruit and vegetable wastes	-
FW	Fruit wastes	-
GHG	Greenhouse gasses	-
HRT	Hydraulic retention time	Days
IA	Intermediate alkalinity	

Symbol	Description	Units
LCFA	Long-chain fatty acids	-
LTP	Low-temperature pretreatment	-
m	Mass of diluted barley spent grain	G
MBT	Mechanical biological treatment	-
MSU	Michigan State University	-
MSW	Municipal solid waste	-
NG	Napier grass	-
OFMSW	Organic fraction of Municipal solid waste	-
OLR	Organic loading rate	gVS L_R^{-3} d^{-1}
P	Supplied power	kW
P/A	Propionate to acetate ratio	-
PA	Partial alkalinity	-
PM	Pig manure	-
PW1	Peach waste (Type 1)	-
PW2	Peach waste (Type 2)	-
R	Reactor	-
RDW	Residual derived fuel	-
RW	Residual waste	-
RC	Rumen content	-
SGB	Second generation biomass	-
SHW	Slaughterhouse waste	-
SMP	Specific methane production	LCH4 gVS^{-1} day^{-1}
SRT	Solid retention time	Days
SS	Sewage sludge	-
t	Time for ultrasounds pretreatment	S
TA	Total alkalinity	-
T_D	Digester temperature	ºC
T_i	Outside temperature	ºC
toe	Tones of equivalent oil	-
TS	Total solid	-
U	heat transfer coefficient	W m^{-2} K^{-1}
UB	University of Barcelona	-

Symbol	Description	Units
UM	University of Michigan	-
USP	Ultrasound pretreatment	-
V	Tank volume	m^3
VS	Volatile solids	-
VFA	Volatile fatty acids	-
w_b	Mass flow of barley spent grain	Kg day^{-1}
w_f	Mass flow of diluted barley spent grain	Kg day^{-1}
WP	Waste paper	-
w_s	Mass flow of supernatant	Kg day^{-1}
ww	Wet weight	-
WWTP	Waste water treatment plant	-
ΔP_{CH4}	Methane yield obtained after pretreatment	LCH4 $Kg_{feedstock}^{-1}$
η	Efficiency of the process	-
ξ	Methane heating value	kJ $LCH4^{-1}$
ρ_f	Density of diluted barley spent grain	Kg m^{-3}
φ_{CHP}	Efficiency of the Combined heat and power unit	-

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Resumen en Castellano

El incremento de la demanda energética y la consecuente generación de residuos ponen en peligro la sostenibilidad del futuro. Es por eso que la digestión anaeróbica resulta ser una solución factible para mitigar el problema ya que se puede generar electricidad, calor y fertilizante a partir de los residuos orgánicos. El incremento de la producción de metano se consiguió a partir de la co-digestión de residuos con características complementarias. Aun así, no siempre se consigue extraer todo el potencial metanogénico de los residuos, especialmente, en el caso de los sustratos ligno-celulósicos. Los compuestos ligno-celulósicos son estructuras complejos entre la lignina, la hemicelulosa y la celulosa con enlaces resistentes a la degradación microbiana que se encuentran en los residuos agro-industriales, los residuos municipales y los cultivos energéticos.

En la tesis, se ha buscado aumentar la viabilidad económica de las plantas de digestión anaeróbica que tratan residuos con componentes ligno-celulósicos. Se usaron distintas nuevas estrategias para aumentar la degradabilidad de la materia ligno-celulósica como los pretratamientos térmicos de baja temperatura y de ultrasonidos y la integración del rumen, un residuo de la industria cárnica, para aportar bacterias hidrolíticas. Para valorar la opción de separar estos componentes para la formación de sub-productos, se estudió su efecto sobre el rendimiento del digestor en términos de estabilidad, producción de metano y calidad del digerido para así poder implementar la producción se sub-productos.

Los estudios realizados en esta tesis doctoral se llevaron a cabo a escala de laboratorio y las conclusiones han supuesto un avance para el aprovechamiento energético de los residuos ligno-celulósicos en el futuro.

1. Introducción

1.1 Problemática relacionada con el incremento de la población y la demanda energética

La población mundial está aumentando a un ritmo frenético nunca antes visto y va alcanzar el nivel de 7 billones de personas en 2050 (Bedoussac et al., 2015). Este incremento vendrá también acompañado de una masiva urbanización y por lo tanto, de un aumento de la demanda energética que se posicionara alrededor de los 44,6 billones de t.e.p (Bilgen, 2014; Ramaswami et al., 2012). Este nivel de demanda energética va a suponer un reto ambiental de grandes características y más si se tiene en cuenta que más del 80% de la demanda energética de nuestros días está cubierto por energías fósiles que son extremadamente contaminantes (Fig. 1.1) (Guo et al., 2015).

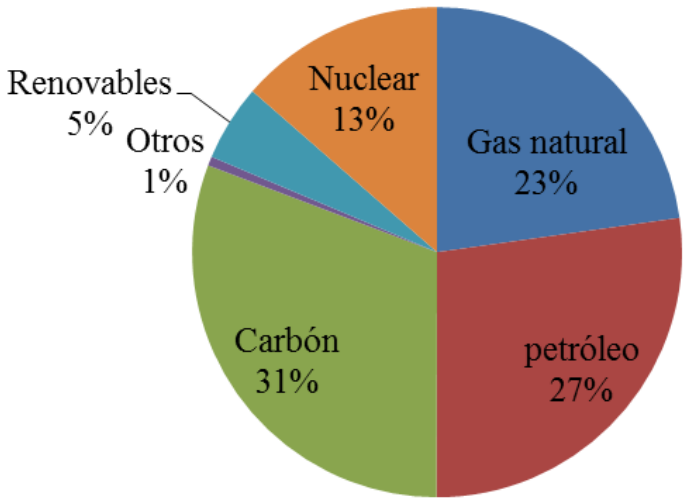

Figura 1.1 2013 Distribución de la producción energética mundial (IEA, 2015)

De hecho, el problema se agrava con los países en vías de desarrollo que, aun contando con un drástico aumento de la demanda energética en el futuro, no tienen, a día de hoy, una legislación estricta hacia la emisión de contaminantes (Nejat et al., 2015).

1.1.1 Generación de residuos a nivel mundial

Alrededor de 1,3 billones de toneladas de residuos sólidos urbanos (RSU) se generaron en 2010 en 161 países y se espera que este número se incremente a 2,2 billones en 2025 debido al incremento de la población mundial y de la economía (Tabla 1.1) (Hoornweg

and Bhada-Tata, 2012). Este último factor es también responsable de la agricultura intensiva que en 2011 generó 140 billones de toneladas en el mundo según la (UNEP, 2011). Los residuos también contribuyen en las emisiones de gases de efecto invernadero, sobre todo si no se tratan de la manera adecuada. Por eso, la unión europea ha aprobado distintas legislaciones sobre el tratamiento de residuos como la directiva 2008/98/CE que presenta la jerarquía de los residuos (Prevención, re-utilización, reciclaje, otras formas de recuperación y deposición en vertederos).

Tabla 1.1 Generación de residuos mundial y proyección para el 2025 (Ross and Rogoff, 2012)

Región	Datos actuales			Proyecciones para el 2025			
	Población urbana total (millones)	Generación de RSU		Proyección sobre la población		Proyección sobre la generación de RSU	
		Per cápita (kg/cápita/día)	Total (Toneladas/día)	Población total (millones)	Población urbana (Millones)	Per capital (kg/cápita/día)	Total (Toneladas/día)
África	260	0,65	169.119	1152	518	0,85	441.840
Este Asiático	777	0,95	738.958	2124	1229	1,5	1.865.379
Asia Central	227	1,1	254.389	339	239	1,5	354.810
América Latina	399	1,1	437.545	681	466	1,6	728.392
Oriente Medio Norte de África	162	1,1	173.545	379	257	1,4	369.320
OECD	729	2,2	1.566.286	1031	842	2,1	1.742.417
Asia Sur	426	0,45	192.410	1938	734	0,7	567.545
Total	2980	1,2	3.532.252	7644	4285	1,4	6.069.703

1.1.2 Los residuos como fuente de recursos. La biotecnología ambiental como una de las soluciones

Es bien sabido que los combustibles fósiles como el petróleo o el gas natural se están agotando (Guo et al., 2015). Debido a que la gran mayoría de residuos generados a nivel global son orgánicos, la legislación europea está considerándolos como un recurso energético. La digestión anaeróbica (DA) tiene la habilidad de recuperar la energía de una amplia variedad de residuos orgánicas en forma de biogás (CH4 y CO2) y además de producir un digestado con altas propiedades fertilizantes (Mata-Alvarez et al., 2000).

1.2 Digestión anaeróbica

La DA se ha implementado para el tratamiento de una gran variedad de residuos orgánicos como los lodos provenientes de estaciones depuradoras del agua residual (EDAR), la fracción orgánica de los RSU (FORSU) o los residuos agrícolas. En Europa ya hay 14.000 plantas en operación que en el 2013 generaron 13,4 Mt.e.p. de biogás (EurObserv'ER, 2014). Pero la DA también resulta atractiva para los países en vías de desarrollo dado que disponen de gran cantidad de residuos orgánicos. China construyó 3,5 millones de digestores a bajo coste para suministrar electricidad a las familias que habitan en el ámbito rural (Surendra et al., 2013).

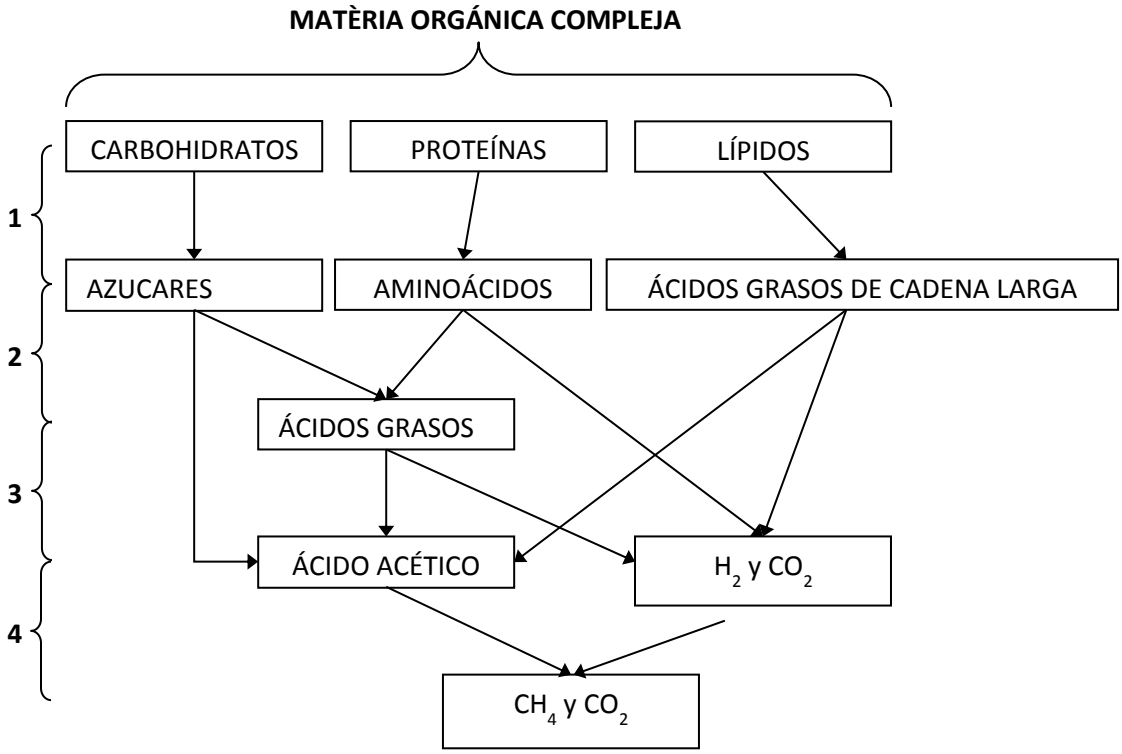

Figura 1.2 Etapas de la DA. 1-Hidrolisi 2-Acidogenesis 3-Acetogenesis 4-Metanogénesis

Des del punto de vista técnico, una de las mejores y simples opciones para mejorar el rendimiento de la DA, y consecuentemente su viabilidad económica, es la co-digestión de residuo. La co-digestión anaeróbica (CoDA) consiste en digerir una mezcla de dos o más sustratos de origen diferente para aprovechar la sinergia de las mezclas y compensar las carencias que los sustratos presentan cuando son digeridos individualmente (Mata-Alvarez et al., 2011). De hecho, el interés científico hacia la CoDA ha aumentado exponencialmente en los últimos años (Fig. 1.3). Hasta el momento, el coste de transportar el residuo es uno de los principales factores para la selección del co-sustrato. No obstante, sigue siendo importante elegir el co-sustrato que mejor favorezca las sinergias durante la digestión, que diluya compuestos inhibitorios o tóxicos y que optimiza la producción de metano sin afectar la calidad del digestado.

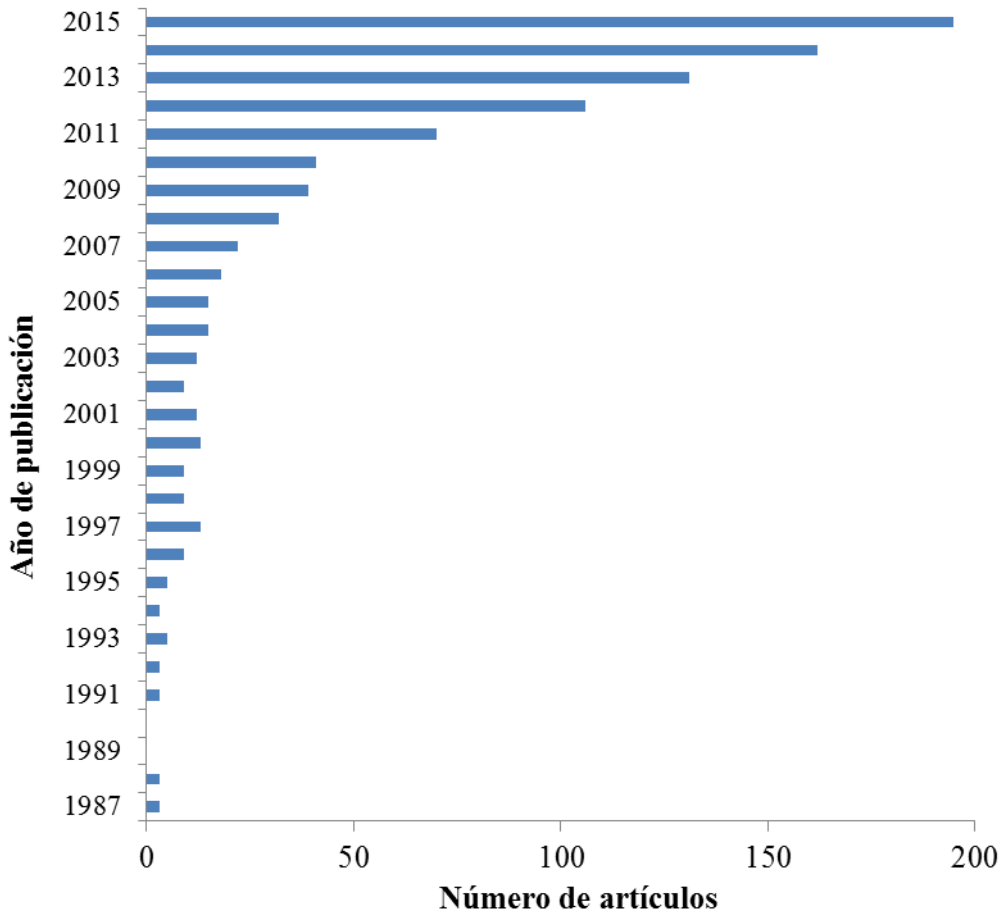

Figura 1.3 Evolución del número de artículos publicados con las palabras "*Co-digestión*" y "*Codigestión*" en el titulo

1.3 El reto de los sustratos ligno-celulósicos

1.3.1 Celulosa, hemicelulosa y lignina: Características recalcitrantes

Compuestos recalcitrantes como la lignina, la celulosa o la hemicelulosa están presentes en los residuos agrícolas, los cultivos energéticos y en los RSU. (Azman et al., 2015; Baba et al., 2013; Yuan et al., 2014). Estos compuestos enmascaran el potencial metanogénico de los residuos reduciéndolo en un 40% (J. C. Frigon and Guiot, 2010). La conversión de estos sustratos a CH4 se ve bloqueada ya en la etapa de hidrolisis que suele ser la etapa limitante en la degradación de este tipo de sustratos. La secuenciación mediante técnicas de biología molecular de alto rendimiento son altamente útiles para tratar de entender quiénes son los microorganismos involucrados en la hidrolisis y para mejorar su rendimiento aportando las condiciones óptimas para la elaboración de su actividad hidrolítica.

Los compuestos ligno-celulósicos se encuentran principalmente en la pared celular de los plantas junto con otros carbohidratos (glucosa, fructosa, sacarosa), proteínas y

lípidos. Su composición depende de la especie y del tiempo de cultivo. La celulosa es un polisacárido compuesto exclusivamente de moléculas de β-glucosa que en la mayoría de los casos se encuentra envuelta en una matriz creada por otros biopolímeros como la hemicelulosa o la lignina. La hemicelulosa, que restringe el acceso a la celulosa, es polisacárido complejo y heterogéneo compuesto por polímeros de pentosas (xilosa, arabinosa), hexosas (Glucosa, galactosa, manosa), ácido glucurónico o una combinación de estos (Pérez et al., 2002). La degradación de ambos polisacáridos es más eficiente de manera conjunta que por separado (Zverlov et al., 2010). La lignina ofrece un buen soporte estructural para las plantas y resistencia hacia ataques microbianos o enzimáticos. Es un complejo molecular formado por tres monómeros diferentes (alcohol p-cumarílico, alcohol coniferílico y alcohol sinapílico) unidos mediante enlaces éter y de carbono formando una estructura tridimensional (Martínez et al., 2005; Pérez et al., 2002; Zeng et al., 2014). Dichos enlaces hacen que su degradación en condiciones anaeróbicas sea extremadamente difícil. El incremento de la producción de metano se obtiene gracias a la escisión de los enlaces lignina-hemicelulosa-celulosa. Entre la lignina y la hemicelulosa y celulosa son los responsables de su resistencia. Por otra parte, el contenido de lignina en las plantas incrementa durante la transición del estado vegetativo al estado reproductivo de las plantas, con lo cual resulta recomendable recolectar las plantas antes de dicha transición para obtener altos rendimientos de metano.

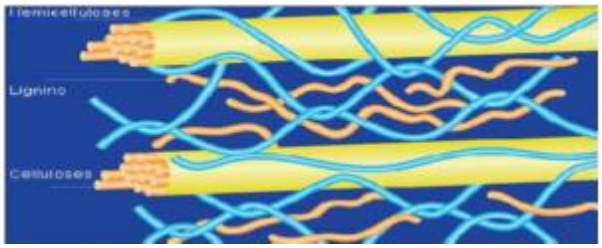

Figura 1.4 Ejemplo de estructura ligno-celulósica (Abdullah et al., 2013)

1.3.2 Estrategias para la degradación de los compuestos ligno-celulósicos

1.3.2.1 Pretratamientos

Mediante los pretratamientos se puede lograr la solubilización de los polímeros mencionados en la sección anterior. Estos tratamientos deben de: i) tener costes de operación y de inmovilizado bajos, ii) ser aplicables en una gran variedad de sustratos ligno-celulósicos, iii) evitar la producción de compuestos tóxicos a partir de la degradación de la lignina y iv) evitar la degradación de los productos obtenidos (Agbor

et al., 2011; Zheng et al., 2014). Los pretratamientos físicos, químicos, físico-químicos y biológicos han sido ampliamente estudiados incrementando a veces la producción especifica de metano (PEM) en un 265% (Gianico et al., 2013; Hendriks and Zeeman, 2009; Menardo et al., 2012; Neves et al., 2006; Zheng et al., 2014). No obstante, la mayoría de los estudios se realizó en reactores en discontinuo mientras que los experimentos en continuo son más apropiados ya que la comunidad microbiana se adapta a la condiciones del sustrato y no a las del inoculo.

1.3.2.2 Co-digestión anaeróbica

Las deyecciones ganaderas han sido el co-sustrato más usado para la CoDA de los residuos agrícolas ya que aportan los nutrientes necesarios de los cuales no disponen (Estevez et al., 2014; Jiménez et al., 2014; Molinuevo-Salces et al., 2015; Nakakihara et al., 2014; Pagés-Díaz et al., 2015, 2014). Los estudios han reportado PEM bastante altas (0,40 L_{CH4} gSV^{-1}) en algunos casos pero bajas en otros (0,15 L_{CH4} gSV^{-1}) donde las cantidades de componentes ligno-celulósicos era bastante alta. No obstante, la CoDA con residuos que contienen una comunidad microbiana ya adaptada a la degradación de compuestos ligno-celulósicos supondría una excelente estrategia para aumentar la velocidad de la etapa hidrolítica. Un ejemplo de residuo podría ser el rumen proveniente de mataderos puesto que las vacas tienen la celulosa, hemicelulosa y lignina como la base de su alimentación (Cheng et al., 2009; Z.-B. Yue et al., 2013).

1.3.2.3 Inoculación

La inoculación de los reactores es muy importante para evitar largos de aclimatación. No basta con solo disponer de un inoculo con una comunidad microbiana diversa y de gran actividad. También debe de ser flexible, dinámica capaz de soportar periodos de inestabilidad (De Vrieze et al., 2014; Keating et al., 2013; Saady and Massé, 2013). Inóculos provenientes de la degradación de deyecciones ganaderas y de lodos de EDAR han sido los inóculos más usados ya que contienen una alta cantidad de nutrientes capaces de promover la actividad enzimática (Gu et al., 2014; Li et al., 2010, 2013a; Xu et al., 2013). Tal y como se ha mencionado en la sección anterior, el uso de comunidades microbianas ya adaptadas a este tipo de residuo pueden brindar una alta actividad hidrolítica al sistema. Por ejemplo, Quintero et al. (2012) estudió el PEM del bagazo de fique usando distintos inóculos. Mediante la utilización de rumen como

inoculo obtuvo la mayor PEM (0,3 LCH4 gSV^{-1}), siendo un 75% más alta que la PEM obtenida usando un inoculo convencional.

2 Objetivos

La presente tesis doctoral tiene como finalidad estudiar diferentes estrategias para mejorar la viabilidad económica de las plantas de DA considerando especialmente la presencia compuestos lignocelulósicos en los sustratos usados (RSU, residuos agrícolas y cultivos energéticos de segunda generación).

Los objetivos específicos de este proyecto son:

- La implementación de la CoDA en plantas de DA de lodos de EDAR usando residuos agrícolas como co-sustrato.
- Identificar cuáles son los efectos sobre la DA cuando los componentes ligno-celulósicos de los RSU son separados para la producción de subproductos de alto valor económico.
- Aumentar la producción de metano en la CoDA de los residuos agrícolas mediante el uso de dos pretratamientos: Ultrasonidos y térmico a baja temperatura.
- Evaluar por separado la implementación de los pretratamientos térmico de baja temperatura y ultrasonido considerando la sobreproducción de metano
- Promover la degradación de la lignina usando un inoculo rico en poblaciones hidrolíticas
- Estudiar la comunidad microbiana involucrada en la degradación de compuestos ligno-celulósicos mediante el uso de rumen
- Usar rumen como co-sustrato para aportar de manera continua poblaciones microbianas con alta actividad hidrolítica para la degradación de sustratos ligno-celulósicos.

3 Materiales y métodos

Los métodos analíticos de la tesis doctoral se han realizado siguiendo los procedimientos del *Standard methods for the examination of water and wastewater* (APHA et al., 2012) tal y como se detalla en la Tabla 3.1.

Tabla 3.1 Métodos analíticos

Parámetro	**Método**
Sólidos Totales (ST) y volátiles (SV)	Método estándar 2540G
Demanda Bioquímica de Oxígeno (BOD)	Método estándar 5210D
Alcalinidad parcial (AP) Alcalinidad Total (AT) Alcalinidad Intermedia (AI)	(Ripley et al., 1986)
Ácidos Grasos Volátiles (AGV)	Cromatógrafo de gases (UB y UM) Cromatógrafo iónico (UM)
Amonio	Cromatógrafo ionic 863 Advanced compact Metrohm
Poblaciones bacterianas	Secuenciación por Ilumina (Kozich et al., 2013)
Potencial de biometanización (PBM)	(Angelidaki et al., 2009; VDI-4630, 2006)
Composición del biogás	Cromatógrafo Shimadzu GC-2010+

4 Resultados y discusiones

4.1 Co-digestión anaeróbica entre lodos de depuradora y residuos de fruta: Estudio del estado transitorio cuando el co-sustrato cambia

En Catalunya se generan una gran cantidad de residuos provenientes de la industria alimentaria y de las EDAR (Llena i Cortina, 2010; Obis et al., 2008). La restricción a la hora de verter residuos orgánicos en el vertedero hace que se tenga que buscar una alternativa cuanto más sostenible mejor. Teniendo en cuenta que los residuos provenientes de la industria frutera (RF) son altamente biodegradables y que los lodos de EDAR contienen una alta capacidad buffer y alta concentración de nitrógeno, la CoDA, es la mejor alternativa para tratar los residuos. Además muchas de las plantas de DA en las EDAR cuentan con una capacidad extra de un 30% (Di Maria et al., 2014; Montusiewicz and Lebiocka, 2011; Pagés-Díaz et al., 2014). No obstante, la industria frutera es también estacional y por lo tanto es imposible aportar el mismo tipo de residuo durante todo el año. El objetivo del estudio se evaluar como se ve afectado el rendimiento del digestor cuando el tipo de co-sustrato cambia.

Para ello se pusieron en marcha dos reactores agitados a escala laboratorio en semi-continuo bajo condiciones mesófilas (37 ºC). Los reactores fueron inoculados con lodo digerido. Los sustratos empleados fueron el lodo de EDAR como sustrato principal y luego dos tipos de residuo de melocotón (RM1 y RM2), residuos de plátano (RP) y de manzana (RMa). El reactor (R2) solo trataba lodos de EDAR como referencia a una velocidad de carga orgánica (VCO) de 1,2 g SV L_R^{-1} $día^{-1}$ y el reactor de co-digestión (R1) tenía una VCO de 3,0 g SV L_R^{-1} $día^{-1}$. Ambos digestores trabajaron a un tiempo de residencia hidráulica (TRH) de 20 días. Las condiciones de trabajo de R2 se muestran en la Tabla 4.1.

Tabla 4.1 Condiciones de trabajo de R1

Fases	Fase I	Fase II	Fase III	Fase IV	Fase V
Periodo de días	0-60	61-130	131-189	190-240	241-280
Alimentación	SS	SS + PW	SS + BW	SS + AW	SS
Lodos/RF mezcla (ww/ww)	100/0	87/13	79/21	70/30	100/0

Tabla 4.2 PEM y AP media de R1 y R2.

	Unidades	**Fase I**	**Fase II**	**Fase III**	**Fase IV**	**Fase V**
SMP R1	L_{CH4} g^{-1} SV	0,28 ± 0,04	0,20 ± 0,03	0,30 ± 0,02	0,26 ± 0,03	0,28 ± 0,03
SMP R2	L_{CH4} g^{-1} SV	0,25 ± 0,03	0,23 ± 0,03	0,24 ± 0,01	0,26 ± 0,02	0,27 ± 0,02
Producción de metano R1	L_{CH4} $día^{-1}$	0,67 ± 0,10	1,16 ± 0,10	1,72 ± 0,09	1,45 ± 0,2	0,69 ± 0,25
Producción de metano R2	L_{CH4} $día^{-1}$	0,6 ± 0,08	0,55 ± 0,06	0,58 ± 0,02	0,62 ± 0,05	0,66 ± 0,05
AP R1	g CaCO3 L^{-1}	2,2 ± 0,2	2,0 ± 0,3	1,0 ± 0,4	1,3 ± 0,2	2,1 ± 0,5
AP R2	g CaCO3 L^{-1}	2,2 ± 0,2	2,1 ± 0,6	2,4 ± 0,6	3,1 ± 0,1	3,0 ± 0,1

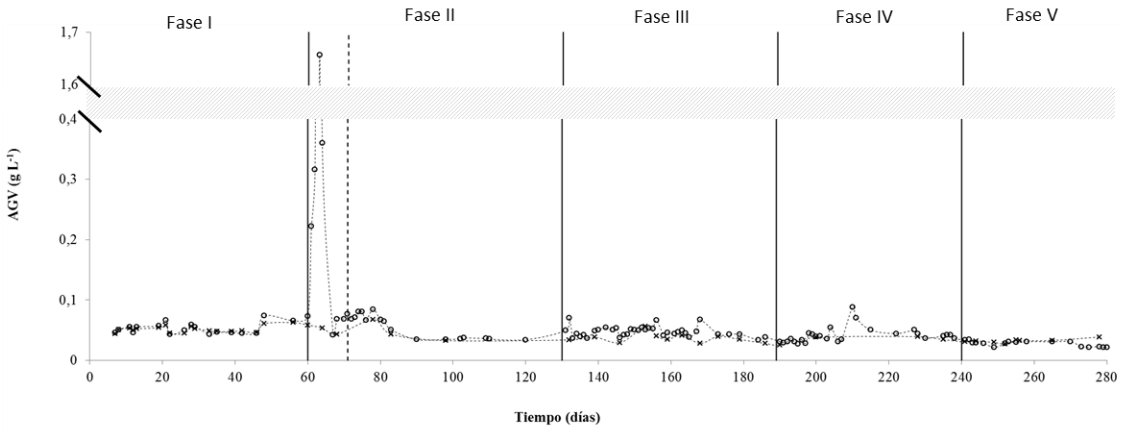

Figura 4.1 Concentración de AGV en R1 (○) y R2 (×)

La puesta en marcha con lodo de EDAR se hizo sin ningún problema. Cuando RM1 se incorporó como co-sustrato la concentración de AGV, especialmente la de propiónico, se disparó alcanzando niveles de 1,8 g L^{-1} (Fig. 4.1). Gracias a la alcalinidad aportada por los lodos este cambió solo duró 5 días. La PEM que se obtuvo con este residuo fue bastante alta (0,45 L_{CH4} gSV^{-1}) pero debido a la alta cantidad en fibras en RM2 la PEM se redujo al introducir dicho residuo (0,20 ± 0,03 L_{CH4} gSV^{-1}) siendo incluso más baja que la del reactor de referencia (0,23 ± 0,03 L_{CH4} gSV^{-1}) (Tabla 4.2). En los siguientes cambios la concentración de AGV no volvió a aumentar, pero, la PA disminuyó durante la CoDA. La biodegradabilidad de RB y RMa, que fue más alta que la de RP2 como se puede ver en la producción de metano, seguramente generó una concentración alta de H^+ que luego tuvo que ser contrarrestada por la PA. Al volver a la mono-digestión, los niveles de PEM y de PA de R1 volvieron a ser iguales a los de R2.

El cambió de co-sustrato no altero el rendimiento de la CoDA, no obstante, es importante que el sustrato principal empleado tenga una alcalinidad alta para soportar los cambios bruscos de biodegradabilidad entre sustratos. Debido a la alta cantidad de material ligno-celulósico que se puede encontrar en algunos RF, es posible que el incremento de metano no sea suficiente para amortiguar los costes de transporte del co-sustrato.

4.2 Separación del papel residual de la fracción orgánica de los residuos sólidos urbanos: Efecto en el rendimiento del digestor (Biogás y digestado)

Los RSU pueden ser tratados en plantas de tratamiento mecánico-biológico (TMB) donde se realiza una separación de materiales (Abeliotis et al., 2012; Beylot et al., 2015;

Pires et al., 2011). Materiales de plástico, vidrio o metal se separan para una valorización material y los elementos orgánicos se tratan biológicamente en un digestor anaeróbico o mediante compostaje. La fracción orgánica que más componentes ligno-celulósicos contiene y que por lo cual puede ralentizar la etapa de hidrolisis y disminuir el potencial metanogénico es el papel residual (PR) (Saint-Joly et al., 2000). En la planta TMB de Barcelona (Ecoparc 3) se instaló un separador óptico que aumentó notablemente el rendimiento de la planta (Romero-Güiza et al., 2014b). Debido a esto, la unidad de separación puede ser instalada en otras plantas de tratamiento de RSU para aumentar su viabilidad económica. Puesto que el separador óptico es capaz de separar el PR de la FORSU, este se puede emplear para la producción de sub-productos como el bioetanol o el combustible sólido recuperado (CDR) (Wang et al., 2012b). Depende del escenario económico en el que se encuentre la planta de TMB esta opción podría resultar interesante. En dicho caso, resultaría útil como podría afectar al digestor la separación del PR.

En el siguiente estudio se pusieron en marcha dos digestores semi-continuos a escala laboratorio en condiciones mesófilas. La VCO y el TRH de los reactores fueron de 2,9 gSV L_R^{-1} $día^{-1}$ y 15 días respectivamente. El Reactor R1 se usó como de referencia y solo se le alimentó materia orgánica y el reactor R2 se usó para evaluar el efecto de la separación del papel añadiendo, en dos fases distintas, dos porcentajes de papel distintos (15% primero y luego 30%). En los primeros 40 días se alimentó en ambos digestores el mismo alimento que se usa en el ecoparque 3 para el digestor para tener la PEM real de la planta y en los siguientes 40 días se alimentó solo materia orgánica para adaptar a los digestores al nuevo alimento.

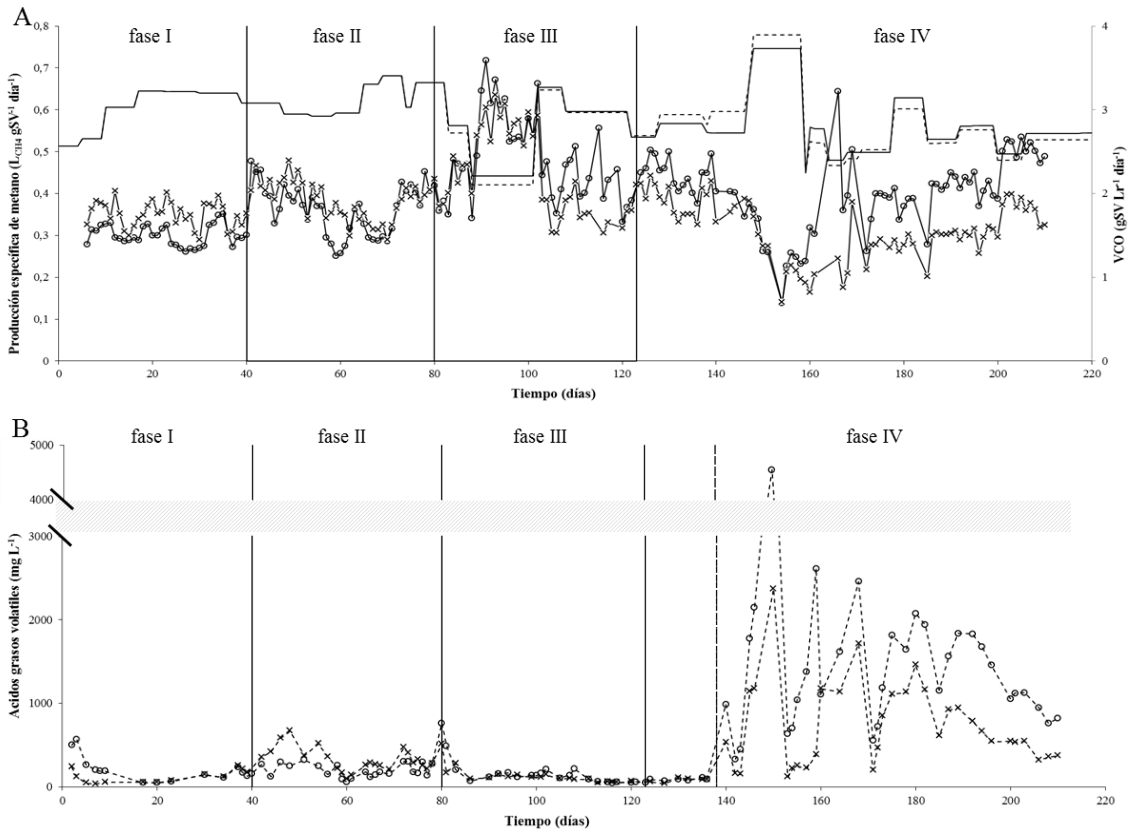

Figura 4.2 PEM (A) y concentraciones de AGV (B) de los reactores R1 (○) y R2 (×)

La figura 4.2 muestra la PEM y la concentración de AGV de ambos reactores. Las líneas negras marcan la separación entre las diferentes fases del proyecto. La línea de puntos de la figura 4.2 marca el día en que el agua de proceso (obtenida en el ecoparque 3 para homogeneizar y triturar el alimento) se acidificó. Cuando se alimentó el 15% de PR (Fase III) los reactores tenían un comportamiento muy similar excepto en la PEM, donde era un 17% más alta en R1 (0,43 ± 0,06 L_{CH4} gSV^{-1}) (Fig. 4.2a). Cuando se acidificó el agua de proceso en la fase IV ambos reactores sufrieron un episodio de inestabilidad donde la PEM disminuyó y los niveles de AGV aumentaron (Fig. 4.2b). Debido a la baja biodegradabilidad que tenía el alimento con PR, se generaron menos ácidos en R2 y se pudo recuperar más rápidamente que R1 aunque, por el mismo motivo, su PEM resultó ser un 27% más baja (Fig. 4.2).

Tabla 4.3 Resultados de los test BOD_5 y de post-metanización para analizar la estabilidad del digestado en R1 y R2

	BDO_5 (mg O_2 gSV^{-1})		Post-metanización (mg O_2 gSV^{-1})	
	R1	R2	R1	R2
Fase I	238 ± 84	213 ± 108	478 ± 40	488 ± 40
Fase II	264 ± 16	260 ± 20	595 ± 34	617 ± 43
Fase III	281 ± 6	255 ± 17	669 ± 34	584 ± 20
Fase IV	267 ± 17	191 ± 18	561 ± 37	466 ± 11

La estabilidad del digestado se estudió mediante un test de post-metanización y una BDO_5. El digestado que presentó peor estabilidad fue el del reactor alimentado con materia orgánica (Tabla 4.3). Pese a tener una PEM más alta que los digestores alimentados con fracciones menos biodegradables el potencial metanogénico del sustrato no pudo ser extraído al 100% debido al corto TRH. El test de post-metanización dio valores más altos porque, al tener un tiempo de duración más alto que el test de BDO_5 pudo degradar compuestos con una velocidad de degradación más lenta y extraer todo el potencial metanogénico restante. Por eso, el test de post-metanización parece ser más adecuado para estudiar la calidad del digestado que una BOD_5 que normalmente es el método que más se usa.

Por lo tanto, cuando el PR se separa de la FORSU la biodegradabilidad del alimento aumenta y también lo hace la PEM. No obstante, la estabilidad del digestor se vuelve más frágil ante episodios de inestabilidad y, es muy probable también, que el TRH se deba de aumentar disminuyendo el caudal de entrada al digestor para asegurar la misma calidad en el digestado.

4.3 Co-digestión anaeróbica entre bagazo y purines de cerdo bajo altas concentraciones de amonio: Comparación en la aplicación de un pretratamiento térmico a bajas temperaturas y de ultrasonidos

El aumento de la población ha conducido al sector de la agricultura hacia una actividad intensiva generadora de altas cantidades de residuo (EC, 2013b). Entre ellos las deyecciones ganaderas son un peligro ambiental porque se producen en grandes cantidades y contribuyen a las emisiones de gases de efecto invernadero. Debido a su bajo ratio C/N, el tratamiento mediante DA no solo genera poco metano, sino que

además los problemas de toxicidad debido al amonio son bastante comunes (Angelidaki and Ellegaard, 2003). Por ello, la CoDA de estos residuos junto al bagazo (B), un residuo que se genera en grandes cantidades en España, puede aumentar el ratio C/N mejorando el rendimiento del proceso (Mata-Alvarez et al., 2014b). No obstante, la PEM de la CoDA de estos dos residuos podría aumentar si se logra degradar los compuestos ligno-celulósicos que contiene el B y que son altamente recalcitrantes (Azman et al., 2015). Los pre-tratamientos químicos con NaOH y térmicos a altas temperaturas se han empleado en este tipo de residuos con muy buenos resultados pero requieren de unos costes de operación bastante altos (Zheng et al., 2014). No obstante los pretratamientos térmico de baja temperatura (TBT), que puede resultar atractivo bajo un punto de vista económico y de ultrasonido (US) no han sido estudiados tan ampliamente sobre estos residuos.

En este estudio se llevó a cabo la co-digestión del B y de los purines de cerdo (PC) en el laboratorio usando tres reactores semi-continuo a temperaturas mesófilas. Primero hubo una etapa de 109 días donde solo se alimentó B y PC y luego se le aplicó los 2 pretratamientos por separado al B. R1 fue el reactor de referencia donde no se aplicó el pretratamiento a B, en R2 se alimentó el B pretratado por US y en R3 el B pretratado por TBT. La energía específica aplicada por el US fue de 5000 kJ $kgST^{-1}$ y para el pretratamiento térmico la muestra se calentó a 60ºC durante 24 horas. Para aplicar los pretratamientos el B se diluyó con el sobrenadante del efluente del reactor hasta tener una cantidad de TS de 10%.

Tabla 4.4 Media de los parámetros de R1, R2 and R3

	Unidades	**Puesta en marcha (Días 90 – 110)**			**Etapa de pretratamiento (Días 150 – 170)**		
		R1	R2	R3	R1	R2	R3
PEM	L_{CH4} g^{-1} SV	0,32 ± 0,02	0,34 ± 0,03	0,32 ± 0,02	0,33 ± 0,02*	0,37 ± 0,02*	0,42 ± 0,03*
Producción de metano	L_{CH4} $día^{-1}$	2,86 ± 0,13	3,02 ± 0,15	2,90 ± 0,22	2,81 ± 0,15*	3,15 ± 0,21*	3,55 ± 0,22*
Eliminación de SV	%	56 ± 2	58 ± 3	60 ± 4	49 ± 1*	56 ± 2*	55 ± 1*
pH	-	8,3 ± 0,1	8,3 ± 0,1	8,3 ± 0,1	8,2 ± 0,1	8,2 ± 0,1	8,2 ± 0,1
AP	gCaCO3 L^{-1}	8,6 ± 0,9	10,7 ± 0,3	8,4 ± 0,6	8,1 ± 0,3	10,3 ± 0,2	8,0 ± 0,6
AGV	mg L^{-1}	118 ± 33	172 ± 76	160 ± 55	133 ± 53	155 ± 78	115 ± 77
Acetato	mg L^{-1}	83 ± 8	73 ± 23	65 ± 5	90± 9	96 ± 32	77 ± 36
Propiónico	mg L^{-1}	3 ± 2	2 ± 2	1 ± 0	3 ± 1	3 ± 1	2 ± 1
AGV/AT	-	0,01 ± 0,00	0,01 ± 0,01	0,01 ± 0,01	0,01 ± 0,00	0,01 ± 0,01	0,01 ± 0,00
NH_3	g L^{-1}	0,7 ± 0,2	0,5 ± 0,1	0,6 ± 0,1	0,5 ± 0,1	0,5 ± 0,1	0,5 ± 0,1

* Estos valores fueron calculados entre los días 112 y 132, cuando los pretratamientos dieron un efecto positivo sobre la PEM.

La puesta en marcha tuvo algunos problemas debido a una elevada VCO (3,10 gSV L_R^{-1} day^{-1}), una TRH baja (20 días) y a la formación de una capa de espuma. Los problemas se solventaron cambiando los parámetros (VCO=2,40 gSV L_R^{-1} day^{-1}; TRH=30 días) y cambiando las palas de agitación. No obstante, los niveles de amonio durante todo el experimento fueron muy altos sin que hubiera problemas de toxicidad en los digestores. Posiblemente, poblaciones microbianas sintróficas capaces de oxidar el acetato fueron las responsables de que el amonio no afectara el rendimiento del digestor (Tabla 4.4).

Cuando se aplicaron los pretratamientos de US y TBT la PEM aumento un 26% y un 12% y la eliminación de VS aumentó en un 14% (Tabla 4.4). El incremento al aplicar el pretratamiento TBT seguramente fue debido a la re-activación de las enzimas que intervinieron en el proceso de fermentación de la cebada para la producción de cerveza. En el caso de los US los compuestos ligno-celulósicos pudieron ser destruidos por cavitación o por la formación de radicales libres. Desgraciadamente, al cabo de 30 días la PEM de R2 y R3 volvió a ser igual a la de R1 sin ninguna explicación puesto que ningún otro parámetro varió.

Se aplicó un balance energético en cada caso usando los valores obtenidos cuando el pretratamiento dio una mejora en la PEM de los reactores. Para el balance energético se tuvo en cuenta la energía que se debía aplicar para llevar a cabo los pretratamientos, la energía obtenida a partir del incremento de la producción de metano y, en el caso del pretratamiento TBT, se consideró el calor que se podía recuperar para calentar el digestor a 37°C. Con los resultados del balance energético se pudo concluir que solo la implementación del pretratamiento TBT podía generar energía mientras que la implementación de un pretratamiento US daba un balance negativo de energía.

4.4 La digestión anaeróbica de sustratos ligno-celulósicos: Inoculación con rumen, un ecosistema natural concentrado en bacterias hidrolíticas

Alemania es el país de Europa que más biogás produce de toda la Comunidad Europea. La mayoría del biogás producido es a base de usar cultivos energéticos como sustratos. No obstante, al competir con productos alimenticios, esta fuente de energía se ha visto restringida mediante la legislación alemana actual (EurObserv'ER, 2014). Una alternativa puede ser el uso de cultivos energéticos de segunda generación ya que estos no compiten con los productos alimenticios (de Souza et al., 2014; Del Grosso et al., 2014; López-Bellido et al., 2014). Por ejemplo, en Tailandia se está introduciendo en su política energética la obtención de biogás a partir de pasto de Napier (PN) cuyo

rendimiento de biomasa es muy elevado (87 tn $año^{-1}$ ha^{-1}) (Waramit and Chaugool, 2014). La CoDA de estos sustratos con co-sustratos con una alta cantidad de nutrientes aumenta el rendimiento de la producción de biogás. No obstante, en ocasiones la PEM sigue siendo baja debido a la alta concentración de componentes ligno-celulósicos contenida en los cultivos energéticos de segunda generación. El rumen R de las vacas contiene una gran concentración de poblaciones microbianas hidrolíticas y además resulta ser un residuo en los mataderos. La introducción de este residuo en el sistema podría suponer un aumento en la PEM (Z.-B. Yue et al., 2013).

Se usaron tres co-digestores alimentados con PN y estiércol de vaca (EV) en un régimen semi-continuo. Trabajaron en condiciones mesófilas y fueron inoculados con R (R1), con un inoculo proveniente de un digestor que trataba EV y residuos alimenticios (MSU) (R3) y, para aportar las bacterias hidrolíticas del rumen y la alcalinidad del inoculo MSU, ambos inóculos se combinaron para inocular R2. En la tabla 4.5 se puede ver el régimen al que trabajaron los reactores:

Tabla 7.2 Parámetros de diseño de los reactores

	R1			**R2**			**R3**		
Periodo de días	VCO (gSV L^{-1} day^{-1})	TRH (días)	ST contenido (%)	VCO (gSV L^{-1} day^{-1})	TRH (días)	ST contenido (%)	VCO (gSV L^{-1} day^{-1})	TRH (días)	ST contenido (%)
0 – 14	0,75	20	3,6	0,75	20	3,6	0,75	20	3,6
14 – 22	1,5	20	3,6	1,5	20	3,6	1,5	20	3,6
22 – 89	1,0	35	3,6	1,5	20	3,6	1,5	20	3,6
89 – 96	2,0	20	4,8	2,0	20	4,8	2,0	20	4,8
96 – 143	3,0	20	6,8	3,0	20	6,8	3,0	20	6,8

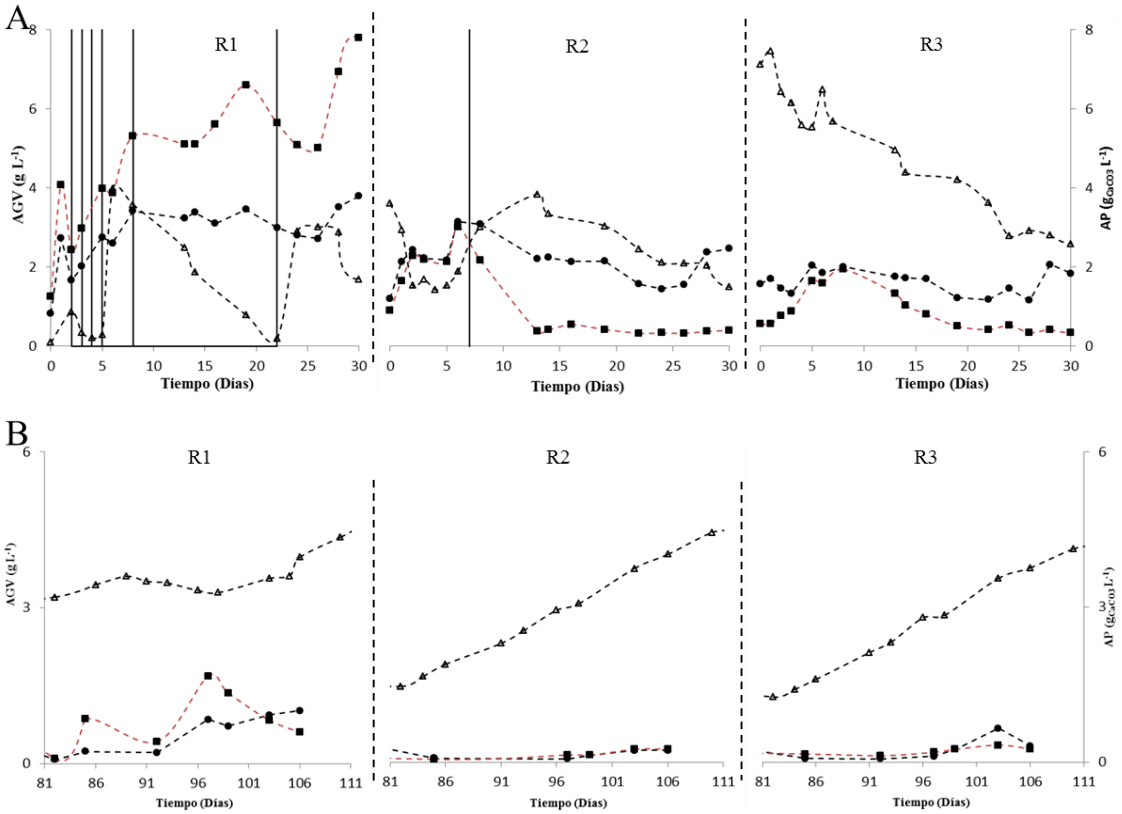

Figura 4.3 Acetato (■), Propiónico (●), y AP (△) en R1, R2 and R3 durante los primeros 30 días de experimento (A) y al final (B). Las líneas negras muestran los días en los que se añadió alcalinidad a los reactores

Nada más empezar el experimento la producción de biogás fue mucho más alta en R1 (0,3 L día^{-1}) y R2 (0,6 L día^{-1}) y en ambos reactores se encontraron activas bacterias hidrolíticas, en especial, las del genero *Fibrobacter*. No obstante la producción cayó en los días siguientes, la concentración de AGV aumentó y los niveles de AP y pH disminuían continuamente (Fig. 4.3). Los niveles de ácido acético en R2 y R3 disminuyeron a los 13 días de empezar el experimento aunque los la concentración de ácido propiónico se mantuvo alta (1.5-2.0 g L^{-1}) y constante y los niveles de pH y de AP no dejaron de disminuir. R1 tuvo unas condiciones de estabilidad mucho más drásticas (Fig. 4.3) aun cuando se añadía alcalinidad en forma de $NaHCO_3$ o NH_4HCO_3 para subir el pH que llegó a estar por debajo de 6,0. En los tres reactores se encontró que el género Treponema, un homoacetogeno, estaba altamente presente. En especial, R1 tenía una población metanogénica muy poco diversa con la ausencia de archaeas acetogénicas para degradar el acetato. La presencia del genero Treponema indicó que la causa de la inhibición en R1 fue una alta concentración de H2 debido a una potente fermentación.

Al parar la agitación en el trigésimo día la fermentación se ralentizo, disminuyendo así los niveles de H2 y la cantidad de Treponemas y desarrollando una nueva población de methanosarcina capaces de degradar el acético. En el día 51 el acético empezó a disminuir en R1 y además, al parar la agitación, el ácido propiónico acumulado en R2 y R3 también empezó a degradarse. Desde ese momento la PEM en R1 (0,22 ± 0,02 LCH_4 gVS^{-1} day^{-1}) aumentó situándose incluso por encima de la de R2 (0,15 ± 0,02 LCH_4 gVS^{-1} day-1) y R3 (0,12 ± 0,02 LCH_4 gVS^{-1} day-1). Aun así la AP (1,6 gCaCO3 L^{-1}) y el pH (6,8), se siguieron manteniendo a niveles muy bajos mostrando la fragilidad del digestor. Para poder aumentar AP y pH y conducir los reactores hacia niveles de estabilidad se decidió mezclar el EC con orina en vez de con agua ya que esta última tiene una buena capacidad buffer e igualmente estos dos residuos se mezclan en el sistema de alcantarillado de la granja. Cuando en el día 81 se añadió la orina a la EC la AP y el pH pudieron subir situándose alrededor de tal valor en tantos días. Como los reactores estaban operando en condiciones óptimas se decidió cambiar los parámetros de VCO y de TRH a los valores que se habían fijado para operar los reactores (3,0 gSV L_R^{-1} $día^{-1}$ and 20 días). Al bajar el TRH de R1 la PEM disminuyó de 0,22 a 0,16 LCH_4 gVS^{-1} day-1 posiblemente porque la celulosa necesitaba un tiempo de residencia más alto para degradarse (Z. Yue et al., 2013). Al final, los reactores se comportaron exactamente de la misma manera y disponían de la misma comunidad microbiana. No obstante la PEM no fue especialmente alta, posiblemente, porque las condiciones del reactor no eran las óptimas para el desarrollo del genero *Fibrobacter*, responsable de la hidrolisis en R.

Para asegurar la puesta en marcha de reactores anaeróbicos inoculados con R, el uso de un co-sustrato con una alta capacidad buffer es estrictamente necesario.

4.5 Co-digestión anaeróbica de sustratos ligno-celulósicos con estiércol de vaca y rumen como nuevo co-sustrato

Siguiendo los objetivos de la sección 4.4 en esta sección se usó el rumen como co-sustrato en vez de como inoculo para poder aportar de manera continua las poblaciones microbianas responsables de la actividad hidrolítica.

Para el experimento se usaron tres digestores en semi-continuo inoculados con inoculo MSU. Se operaron bajo temperaturas mesófilas y R1 se alimentó con PN y EV, R2 con PN, EV y R y R3 con PN y R.

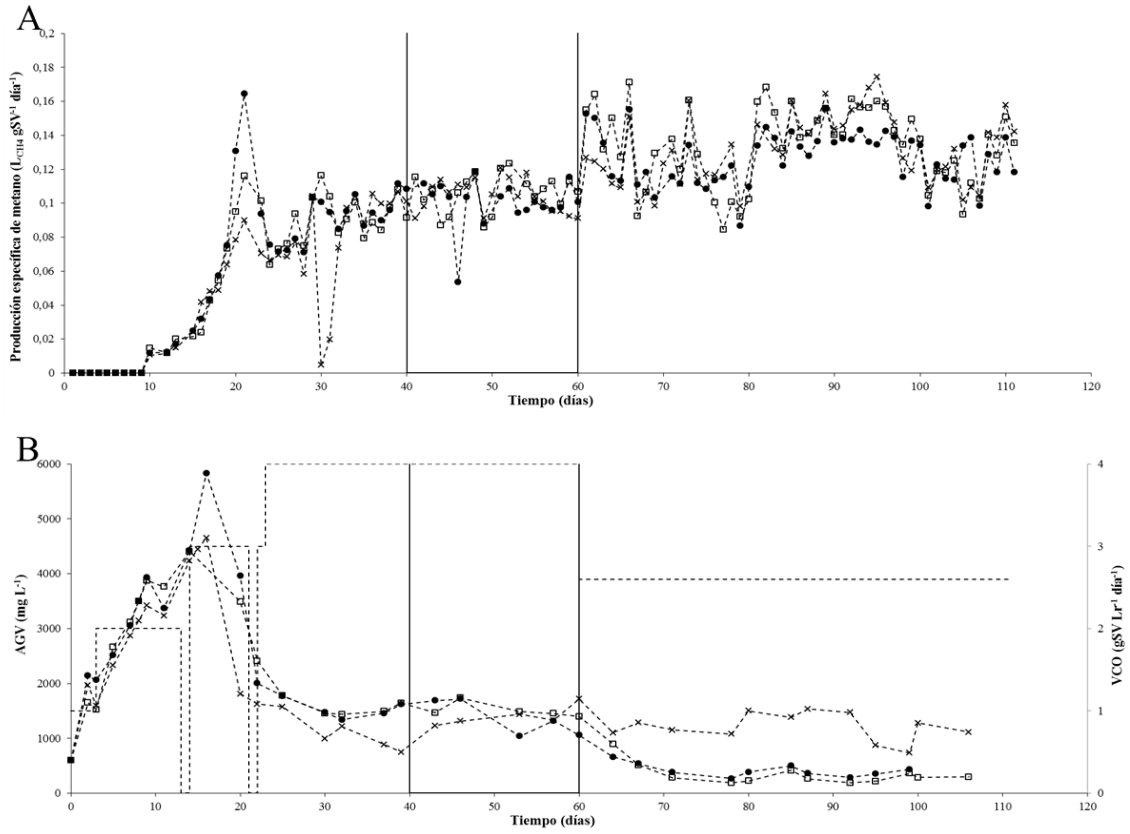

Figura 4.5 PEM y niveles de AGV en R1 (○), R2 (×) y R3 (■), R2y R3. VCO (--) y cambio realizados (-)

Esta vez se hizo una puesta en marcha más lenta donde el VCO fue subiendo de 1,0 a 4,0 gSV L^{-1} day^{-1} y el TRH fue bajando de 79 a 20 días. Aun así los niveles de AGV aumentaron hasta alcanzar valores entre los 4,5 y 6,0 g L^{-1} en el 16° día. Como en la sección 4.4, los niveles de acético disminuyeron más rápidamente quedándose constantes los niveles de propiónico durante 25 días más (0,7 1,0 g L^{-1}). Siendo el propiónico difícil de degradar en ambas secciones se confirma que las poblaciones bacterianas sintróficas del inoculo MSU estaban parcialmente inhibidas en el momento en que se recogió el inoculo. En este caso la AP y el pH se mantuvieron en buenos niveles en R1 y R2 (pH=7,4 y AP=8.5 gCaCO3 L^{-1}) debido a la adición de EV con orina, no siendo así para R3. Debido a que solo se usó rumen como co-sustrato en R3, se tuvo que empezar a añadir desde el día 43 alcalinidad en forma de NH_4HCO_3 continuamente para evitar que el pH bajara de 6,9. De nuevo, se vuelve a confirmar que el uso de un co-sustrato con alta capacidad buffer es esencial para llevar a cabo la CoDA de PN a TRH bajos.

Aun así, la PEM era igual en ambos reactores y muy baja (0.10 LCH_4 gVS^{-1} day^{-1}).

Además, la eliminación de SV fue tan solo de 25%. Se intentó agitar los reactores para promover la floculación de los microorganismos y luego se aumentó el valor del TRH para ver si compuestos más recalcitrantes no se degradaban debido al corto tiempo que ocupaban en el digestor. Aun así no hubo mejoras drásticas en la PEM ($0.13\ LCH_4\ gVS^{-1}\ day^{-1}$) o en la eliminación de SV (30%). Aparentemente, el R como co-sustrato no tuvo ningún efecto en la PEM sugiriendo que los digestores no estaban operando en las condiciones necesarias para activar los microorganismos hidrolíticos.

5 Conclusiones y recomendaciones

5.1 Conclusiones

Referente a la sección 4.1

- Solo cuando empezó la CoDA se vio un aumento de los ácidos debido al cambio drástico de la VCO. Cuando el co-sustrato se cambiaba o se dejó de añadir la estabilidad del digestor no se vio afectada
- Ya que algunos de los RF usados tenían una alta biodegradabilidad, la AP del sistema disminuyó con la CoDA. Es necesario que los lodos de depuradora presenten una alcalinidad alta para mantener el digestor estable.
- La producción de metano puede ser muy baja si el RF tiene un alto contenido en fibras. Para evitar balances económicos negativos durante la CoDA, los co-sustratos deben de ser bien caracterizados o una estrategia para hidrolizarlos debe de ser implementada.

Referente a la sección 4.2

- La separación del PR de la FORSU antes de entrar en el digestor aumento la biodegradabilidad del alimento
- La mitad o toda la separación del PR puede llegar a incrementar la PEM hasta un 17 y 27% respectivamente
- Debido al aumento de biodegradabilidad del alimento, el reactor se vuelve más frágil ante episodios de inestabilidad. En caso de que dicho episodios ocurran, el digestor tardara más tiempo en recuperarse que un digestor donde se alimenta el papel
- Debido al aumento de la biodegradabilidad por la separación del PR, la calidad del digerido puede disminuir si se trabaja a TRH muy bajos.

Referente a la sección 4.3

- Cuando se aplicó el pretratamiento TBT y el de US la PEM en los digestores aumento en un 26 y un 12% respectivamente.
- Según el balance de energía realizado cuando la PEM de los digestores aumentó debido al pretratamiento, solo se recomendaría la aplicación a gran escala del pretratamiento TBT.
- La PEM disminuyó al cabo de 30 días en los digestores donde se aplicaban los pretratamientos hasta ser igual reactor de referencia. No obstante, ningún otro

parámetro cambio en los digestores y no se pudo dar una razón coherente a la disminución de la PEM.

- El uso de un inoculo adaptado a los altos niveles de amonio puede permitir que la DA re realice a altas concentraciones de amonio sin problemas de toxicidad.

Referente a la sección 4.4

- Si el R se usa como inoculo para aportar poblaciones microbianas hidrolíticas, es necesario mezclarlo con un inoculo y utilizar un co-sustrato que tengan una alta capacidad buffer para evitar un periodo de puesta en marcha largo.
- La inoculación de los reactores con R pudo aumentar la producción de biogás tan solo al principio debido a las bacterias hidrolíticas del rumen
- El comportamiento de los reactores fue idéntico hacia el final del experimento sugiriendo que la comunidad microbiana dejo de depender del inoculo y paso a ser gobernado por el alimento.

Referente a la sección 4.5

- Cuando el R se usa como co-sustrato para aumentar la degradación de PN es necesario añadir un tercer co-sustrato con una alta alcalinidad o de lo contrario se deberá añadir productos químicos continuamente para evitar que el pH baje
- La adición continua de R no tuvo ningún efecto sobre la PEM posiblemente porque el reactor no disponía de las condiciones idóneas para la actividad de las poblaciones microbianas procedentes del R.

5.2 Recomendaciones

- Estudiar cómo afectaría el uso de un co-sustrato cercanos a las plantas de TMB, como las grasas de depuradora o los residuos de matadero, para suplir la producción de biogás que aportaba el PR al digestor de FORSU.
- Estudiar la inmovilización de las poblaciones hidrolíticas del R cuando este se utilice como inoculo.
- Estudiar la CoDA entre PN y R en un sistema de dos fases, donde, en el primer reactor se encuentren las condiciones idóneas para que dichas poblaciones puedan efectuar la hidrolisis.
- Realizar estudios de secuenciación del RNA para averiguar cuáles son las poblaciones microbianas activas durante los procesos.

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