ANAEROBIC DIGESTION OF CELLULOSE AND HEMICELLULOSE IN THE PRESENCE OF HUMIC ACIDS

Samet Azman 2016

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Propositions

1. Addition of hydrolytic enzymes helps to mitigate humic acid inhibition of anaerobic digestion of cellulose and hemicellulose.
   (this thesis)

2. The inhibitory concentration of humic acids depends on their chemical structure.
   (this thesis)

3. Unreproducible results in preclinical sciences reflect the perpetual struggle of researchers to find fair funding (Inspired by Collins FS, Tabak LA (2014) NIH plans to enhance reproducibility. Nature 505: 612–613).

4. Speculations about extra-terrestrial life on other planets challenge scientists to rethink the unexplored species on Earth.

5. Solitude is enhancing creativity in art, but not in science.

6. Equal career opportunity in science and life for all gender and race can only be obtained via persistent solidarity against otherisation.

7. Increased frequency of meetings with supervisors is a good indicator of mutual discontentment about on-going research.

8. Not all people need good weather to enjoy and explore life.

Propositions belonging to the PhD thesis entitled “Anaerobic digestion of cellulose and hemicellulose in the presence of humic acids”.

Samet Azman

Wageningen, 14 December 2016

Anaerobic digestion of cellulose and hemicellulose in the presence of humic acids

Samet Azman

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Anaerobic digestion of cellulose and hemicellulose in the presence of humic acids

Samet Azman

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Chapter 1

General Introduction

Samet Azman

Prologue

There is a desire to predict the future. Forecasters from the past generations have extensively tried to predict how future would be. Postcards made between 1899 and 1910 by Jean-Marc Côté and colleagues show optimistic and eerie artwork about how the world would look like in the year 2000. These predictions were more about how convenient the daily life of the humans would be in 100 years. However, our future may not be very optimistic in 100 years from now due to the increased human aggression, climate change and the depletion of the energy sources around the world (Hawking 2015; Sorrell et al., 2010; Swyngedouw, 2010), unless we change our way of living, implement renewable energy techniques and close resource cycles for compounds such as phosphorus, nitrogen and metals.

1.1 Energy consumption of the world

Fossil fuels (oil, biogas and coal) have been the primary energy sources since the industrial revolution. In the last 200 years, dependency on fossil fuels has increased (Sorrell et al., 2010). In recent years, energy consumption in the world was reported as 9301 Mtoe (Million Tonnes of Oil Equivalent) in 2014 (EIA, 2015). Emerging economies such as those of China and India are responsible for over half of the consumption. In contrast, oil consumption in OECD (The Organization for Economic Co-operation and Development) economies continues to decline. More than 65 % of the primary energy consumption is from fossil fuels such as oil (40%), natural gas (15%) and coal (12%) (IEA, 2015a). Additionally, 59% of the electricity produced in OECD countries was derived from fossil fuels in 2014 (IEA, 2015a). Fossil fuels are excellent energy sources and they will remain the backbone of the world's energy supply for a long time. However, their availability is limited in many locations around the world. Furthermore, extensive utilization of fossil fuels for energy production contributes to greenhouse gas emissions (about 70%) which are the primary reason for global warming (Höök and Tang, 2013). Therefore, renewable, environmentally friendly energy carriers should be used to supply energy for the rapidly growing human population.

1.2 Renewable energy around the world

Energy carriers can also be produced from wind, sunlight, water and biomass, known as renewables. In 2013, approximately, 1440 Mtoe energy (15 % of the primary energy

consumption of the world) was from renewable energy sources. Biofuels and biowaste are the dominant renewable energy sources. 12 % of the consumed energy originates from these renewable energy supplies while, the rest (3 %) is based on other renewables such as hydropower, wind, and tidal, solar and geothermal sources (IEA, 2015b).

In OECD countries, the total primary energy supply from renewables showed an annual growth of 2.5 % over the last 25 years (IEA,2015b). The increase of the renewable share in OECD Europe is the result of the implementation of strong policies supporting renewable energy, public support, education and given subsidies to the industries that are willing to use renewable energy (Apergis and Payne, 2010; IEA, 2015b).

The largest proportion of renewable primary energy supply in OECD countries comes from biofuels and biowaste, with a 55% share of the renewable energy supply (Figure 1.1). Biofuels and biowaste are mainly derived from solid biofuels such as wood, wood waste, charcoal and they constitute 38 % of the overall renewable energy supply. Liquid biofuels such as; bioethanol, biodiesel, butanol have a share of 10 % in biofuels and biowaste energy supply. The second-largest renewable energy source is hydroelectric power, providing 25% of the renewable energy (IEA, 2015b) (Figure 1.1).

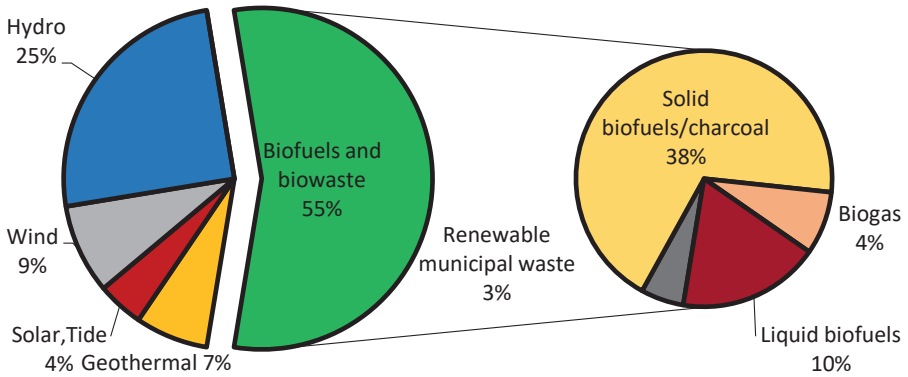

Figure 1.1 Renewable energy supply among the OECD countries in 2014. Adapted from IEA (2015b).

As can be seen from Figure 1.1, biowaste dominates the renewable energy supply. The term biowaste is often used to describe a variety of materials, from forestry and agricultural residues to organic waste by-products from various industries, purpose-grown energy crops, human household waste and wastewater and animal manure, to

woody weeds and municipal waste (Schuck, 2006). In Europe, more than 2.5 billion tons of waste are produced annually. Approximately 37 % of the produced waste is composed of carbon-based materials while the rest is composed of mineral materials which are produced via mining, construction and manufacturing activities (Eurostat, 2015). Even though the percentage of carbon-based biowaste is low compared to the overall waste production, there is still a high potential to generate renewable energy from biowaste. However, there is a need for technologies to process biowaste and recover renewable energy efficiently (Scarlat et al., 2015).

1.3 Available technologies for biowaste conversion to energy

Several types of technologies are available to convert biowaste into energy. These include thermochemical, physicochemical and biochemical processes as summarized in Figure 1.2. The main challenge in the application of these technologies is to overcome energy losses in conversion to electricity or heat (Deublein and Steinhauser, 2010; Fernandes, 2010; Turkenburg, 2000). Although the application of each process is case specific and dependent on the type of biowaste, gasification, pyrolysis, and anaerobic digestion technologies are the most frequently applied, cost effective processes for bioenergy production (Digman, 2009; Mc Kendry, 2002).

1.4 Anaerobic digestion processes for recovering energy

Anaerobic digestion is the conversion of organic material directly to biogas, which contains methane, carbon dioxide and some trace amount of gases such as hydrogen sulphide. Anaerobic digestion is a microbiological process in which complex organic matter is converted to carbon dioxide and methane via four sequential steps; hydrolysis, acidogenesis, acetogenesis and methanogenesis, as seen in Figure 1.3 (Angenent et al., 2004; Gujer and Zehnder, 1983; Plugge et al., 2009; Sanders, 2001; Weiland, 2010). During hydrolysis, carbohydrates, proteins and lipids are converted into monomeric compounds, such as sugars, amino acids and fatty acids and glycerol by hydrolytic bacteria. These monomeric compounds are converted to VFA (Volatile Fatty Acids), alcohols, NH_3, lactic acid, H_2, CO_2 and H_2S in the acidogenesis step. Consequently, the products from the acidogenesis step are degraded to acetate, formate, H_2 and CO_2 in the acetogenesis. Finally, methanogenic archaea reduce the CO_2 + H_2 and cleave the acetate molecule to produce methane and CO_2 (Figure 1.3).

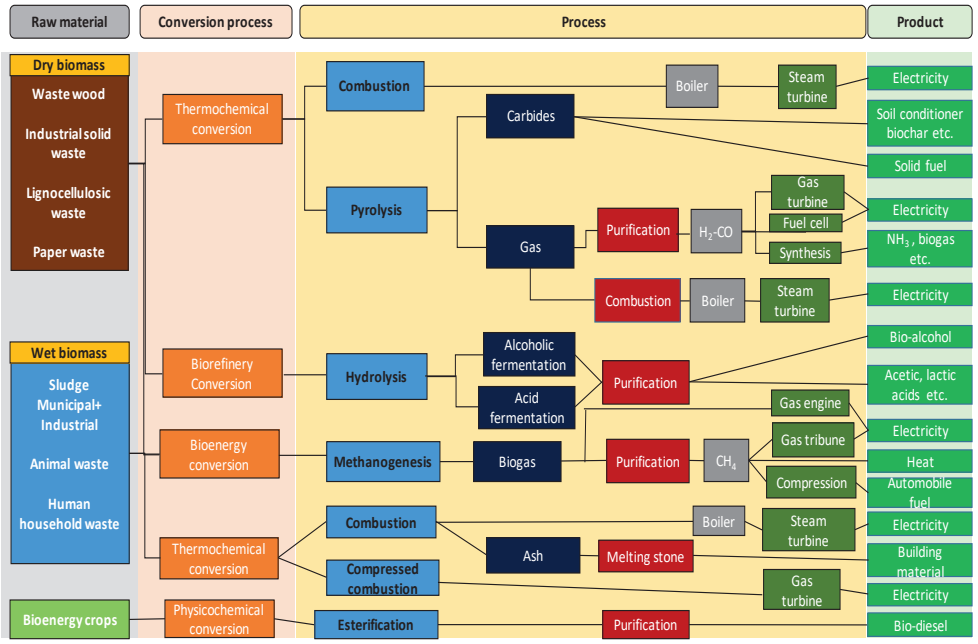

Figure 1.2: Available technologies for conversion of biowaste to energy. Adapted from Turkenburg et al. (2000) and http://www.tsk-g.co.jp/en/tech/industry/pop.html.

Hydrolysis is the first step of the anaerobic digestion. It is often considered as the rate limiting step for biowaste such as lignocellulosic biomass, primary sludge, industrial wastes and manure (Ma et al., 2013; Vavillin et al., 2008). Although extensive research has been performed to improve the understanding of the anaerobic digestion process, research on anaerobic hydrolysis and its microbiology is still poorly understood (Azman et al., 2015a). Since the biogas yield is depending on the extent of hydrolysis, improvement of the hydrolysis step is required to enhance the overall anaerobic digestion.

1.5 Available pre-treatment strategies to improve biomass hydrolysis and methane yields

Several pre-treatment technologies have been applied to complex substrates, particularly to lignocellulosic material. The mutual aim of these technologies is to increase the hydrolysis rate and yield, increase biogas yields, increase the bioavailability of the recalcitrant substrates and reduce the operational costs such as; energy requirement for mixing and removal of floating layer (Montgomery and Bochmann,

2014). Pre-treatments can be divided into four categories, i.e. physical, chemical, thermal and biological, also known as enzymatic, treatment methods (Monlau et al., 2013).

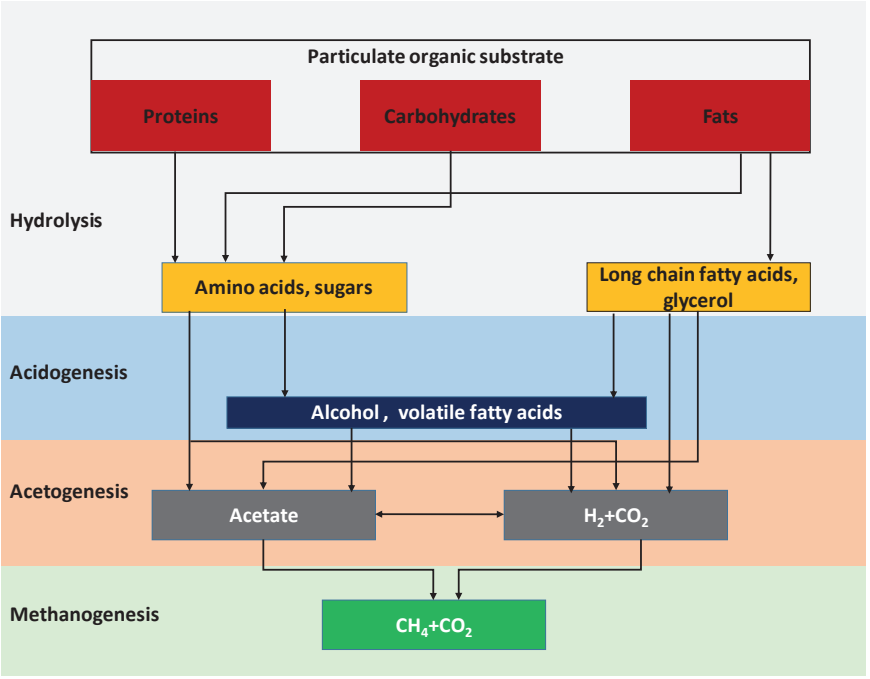

Figure 1.3 Anaerobic digestion process. (Adapted from Angenent et al. (2004) and Plugge et al. (2009)).

1.5.1 Physical pre-treatment

Physical pre-treatment usually refers to methods that do not use chemicals or microorganisms during the pre-treatment processes (Harmsen et al., 2010; Hendriks and Zeeman, 2009; Zheng et al., 2014). Amongst the numerous physical pre-treatment techniques, mechanical and ultrasound treatments are the most commonly applied (Montgomery and Bochmann, 2014).

Mechanical pre-treatment is carried out by mills and/or knives to reduce the particle size of the biomass. The method is used to break down the lignocellulosic structure and therefore increases the specific surface area of the biomass (Hendriks and Zeeman, 2009; Montgomery and Bochmann, 2014). Up to 30 % increase in methane yields and 23-59 % increase in hydrolysis rates are achieved when mechanical treatment

is used (Zheng et al., 2014). Even though positive effect of physical pre-treatment is observed, increased electricity consumption and sensitivity of the equipments are considered as drawbacks of this methodology (Montgomery and Bochmann, 2014).

Ultrasound pre-treatment, using ultrasound frequencies over 20 kHz, is mainly used for increasing biodegradability and hydrolysis rate of biomass. Depolymerization and separation of lignin can be achieved by the influence of ultrasound. (Bussemaker and Zhang, 2013; Rehman et al., 2013). Increased hydrolysis rates are only observed at batch wise incubations (Rehman et al., 2013). Pilot-scale studies are required to validate the promising results of batch experiments and demonstrate a scale up capability for ultrasound pre-treatment of lignocellulosic material (Bussemaker and Zhang, 2013; Onyeche et al., 2002; Rehman et al., 2013; Tyagi et al., 2014). However, ultrasound treatment is more suited to disintegrate microbiological biomass and it has efficiently been used for sludge disintegration (Braguglia et al., 2012; Tyagi et al., 2014; Westerholm et al., 2016).

1.5.2 Chemical pre-treatment

Chemical pre-treatment refers to the use of a wide range of chemicals, such as acids, bases, and ionic liquids, to alter the physical and chemical characteristics of lignocellulosic biomass. Acid, alkali and oxidative pre-treatments are the most frequently applied chemical pre-treatment methods.

Acid pre-treatment involves the addition of strong or diluted acids such as H_2SO_4, HCl, HNO_3, H_3PO_4, acetic acid, and maleic acid to break down hemicellulose that is linked to lignin (Hendriks and Zeeman, 2009; Humbird et al., 2011; Singh et al., 2015; Zheng et al., 2014). Alkali pre-treatment is carried out with the addition of different alkali solutions such as lime and sodium hydroxide. Unlike acid pre-treatment, solubilization of lignin can be achieved via alkali pre-treatment (Greg and Saddler, 1996). Oxidative pre-treatment with hydrogen peroxide or ozone has similar effects as alkali pre-treatment and it can also help to break down lignin (Montgomery and Bochmann, 2014). The effect and feasibility of chemical treatment on biomass pre-treatment have been reviewed (Chen et al., 2009; Hendriks and Zeeman, 2009; Kumar et al., 2009; Taharzadeh et al., 2008). Reported increase in methane yields usually varies with the type of biomass and utilised methods. Methane yields of chemically pre-

treated biomass may increase 2 folds, compared to the untreated biomass (Chen et al., 2009; Hendriks and Zeeman, 2009). Despite the positive results of the chemical treatment, no full-scale application for biogas production exists due to the economic reasons although, it is in widespread use for ethanol production (Montgomery and Bochmann, 2014).

1.5.3 Thermal pre-treatment

Thermal pre-treatment is a method in which lignocellulosic biomass is heated, generally above 150°C. In this way, hemicellulose and lignin start to solubilise via thermal hydrolysis reactions. Up to 40% increase in methane yields can be achieved by thermal pre-treatment. However, optimisation of the temperature for increased yields is case specific and hard to control (Ferreira et al., 2013; Howe et al., 2016; Yan et al., 2009). Furthermore, during heat treatment, there is a risk of formation of phenolic and heterocyclic compounds, like vanillin, vanillin alcohol, furfural, and hydroxymethylfurfural. These formed compounds may be inhibitory for anaerobic digestion or fermentation processes (Hendriks and Zeeman, 2009; Pereira-Ramos, 2003; Shoil Toor et al., 2013; van der Pol et al., 2014). Therefore, their application is limited in full-scale applications.

1.5.4 Biological pre-treatment

Biological pre-treatment methods include: fungal pre-treatment, aerobic/anaerobic pre-treatment by mixed cultures, and enzymatic pre-treatment (Sindhu et al., 2016; Zheng et al., 2014). Biological pre-treatment is usually preferred due to its lower operational costs and process suitability for lower temperatures (Kumar et al., 2009; Sindhu et al., 2016).

Fungal pre-treatment is generally applied to remove inhibitory compounds from the waste streams (Almeida et al., 2007; Zhang et al., 2010) and degrade lignin (Chen and Qui, 2010; Kumar et al., 2009) prior to anaerobic digestion or fermentation. Some fungi can also mineralize organic compounds that could have been converted to methane and consequently, methane yields can be reduced during anaerobic digestion (Chen and Qui, 2010; Sindhu et al., 2016; Tian et al., 2012). Moreover, extended pre-treatment time is a major and serious drawback for the application of fungal pre-treatment in full-scale applications (Wan and Li, 2012).

Aerobic/anaerobic pre-treatment by mixed cultures is usually applied to release cellulose and hemicellulose from the lignin and overcome the limitations of the hydrolysis step of anaerobic digestion. The best example of anaerobic pre-treatment is a two-stage anaerobic reactor system. In the first reactor, hydrolysis and acidification are achieved at pH between 4-6 in which methanogenesis is inhibited. Consequently, acetogenesis and methanogenesis proceed in the second tank around pH 6.8-7.2. This reactor set-up is used at full scale but is still not yet very common (Oles et al., 1997; Parawira et al., 2008; Schievano et al., 2014).

Enzymatic pre-treatment (enzyme addition) for AD has been investigated in many different studies (Alvira et al., 2010; Sindhu et al., 2016; van Dyk and Pletschke, 2012). Contradictory results have been reported. There are studies showing positive effects of enzyme addition in batch wise incubations. Gerhardt et al. (2007) showed an increased biogas production (15%) adding commercially available enzyme cocktails from *Trichoderma* species to 36 L batch incubations, degrading lignocellulosic biomass. Romano et al. (2009) observed increased solubilization of wheat grass by using cellulases while they observed no effect on overall VFA reduction or methane yields. On contrary, other studies suggest that the impact of the enzyme addition is minimal since the added enzymes are degraded very rapidly (Binner et al., 2011; Rintala and Ahring, 1994). Therefore, direct addition of hydrolytic enzymes to anaerobic reactors is not feasible when the costs of the enzymes are considered.

1.6 Application of pre-treatment technologies for anaerobic digestion

Application of pre-treatment technologies described above are usually case specific and their effect is not the same for all type of substrates. The combination of pre-treatments is possible to reach better methane yields in different reactors and processes. Combined processes are more effective than the processes, using only one mechanism and they are frequently used for anaerobic digestion (Carrere et al., 2016; Montgomery and Bochmann, 2014; Zheng et al., 2014) Steam explosion, extrusion and thermochemical pre-treatment methods are the most common combined technologies for biogas production.

Steam explosion is a process in which biomass is treated with hot steam (180 to 240 °C) under pressure (1 to 3.5 MPa), followed by an explosion that results in a rupture

of the rigid structure of biomass (Stelte, 2013). Steam explosion is effective to reduce the crystallinity of cellulose and enhance hydrolysis of the hemicelluloses by delignification (Liu et al., 2013; Singh et al., 2015). In this way, an increase in biogas yield by 40 % is achieved for agricultural biomass (Zheng et al., 2014).

In extrusion pre-treatment, raw materials are put into the extruder and then they are transported along the length of the vessel with a driving screw. During the transportation, raw materials are heated, mixed, and vigorously sheared upon pressure release at the finishing end (Zheng and Rehmann, 2014). The effect of extrusion is similar to the steam explosion and up to 70 % increase in methane yields is achieved for biomass (Zheng et al., 2014).

Thermochemical pre-treatment combines chemicals such as solvents, ammonia, acids and bases with temperature treatment. The addition of chemicals to thermal pre-treatment usually lowers the optimal pretreatment temperature and gives better enzymatic hydrolysable substrates. Up to 70 % increase in methane yields is reached via thermochemical pre-treatment (Hendriks and Zeeman, 2009; Montgomery and Bochmann, 2014). Table 1.1 shows the advantages and disadvantages of the different pre-treatment technologies.

Table 1.1 Advantage and disadvantage of pre-treatment technologies (Adapted from Hendriks and Zeeman, 2009; Montgomery and Bochmann, 2014)

Pre-treatment	Advantage	Disadvantage
Mechanical	• Increases surface area • Makes material storage easier	• Extensive energy demand • Sensitive equipment, high costs for maintenance
Thermal	• Increased solubilization of lignin and hemicellulose	• High heat demand • Hard to optimise the process • Production of inhibitory compounds
Ultrasound	• Easy to apply • Breaks down big flocs	• Not suitable for lignocellulosic material

Table 1.1 Advantage and disadvantage of pre-treatment technologies (Adapted from Hendriks and Zeeman, 2009; Montgomery and Bochmann, 2014) (Continued)

Acid	• Solubilises hemicellulose	• High costs • Corrosion • Production of inhibitory compounds
Alkali	• Lignin degradation	• High costs
Fungal	• Mitigates the possible inhibition of lignin by-products • Lignin degradation	• Potentially reduces methane yields • Not suitable for large application
Microbial	• Low cost and temperature	• Slow degradation of lignocellulose • No lignin degradation
Enzymes	• Easy to apply • Low energy requirements	• High costs • Effects are unpredictable

1.7 Scope and outline of the thesis

Much research has been done to find efficient ways to produce renewable energy carriers from biomass. From waste collection/separation to biomass conversion technologies, a broad range of applications are available to generate renewable energy. Anaerobic digestion is one of the prominent technologies to recover energy from biomass. However, pre-treatment of biomass is required to increase hydrolysis rates and methane yields.

Inhibitory, lignocellulosic biomass related, compounds might not only be produced during pre-treatment but can be present already in the raw material (Fernandes 2010; van der Pol et al., 2014). Humic compounds are one of the complex biomolecules that can be present in the raw materials and they are produced as a result of decaying processes. Fernandes (2010) extracted humic and fulvic compounds from cow manure and maize silage to test their inhibitory potential on anaerobic cellulose and tributyrin degradation. The inhibitory effect of these compounds on enzymatic hydrolysis has been shown in batch incubations (Fernandes et al., 2015). However, the

effect of these inhibitory compounds on anaerobic digestion, especially hydrolysis and methanogenesis, is vastly underestimated and often unknown. Therefore, more information is required about the effects of these inhibitory compounds derived from biomass or produced during pre-treatment to overcome the inhibition.

The major goal of the thesis is to increase knowledge about one class of the biomass derived inhibitory compounds; humic acids (HA). This thesis investigates HA inhibition, mitigation potential of the inhibition and the effect of the HA on microbial populations during anaerobic digestion of cellulose and hemicellulose. In this section, the importance of biomass for producing renewable energy carriers, the role of anaerobic digestion for energy recovery and main challenges for improved biogas production are discussed. Special emphasis is given to the hydrolysis step since it is considered as the rate limiting step for anaerobic digestion of lignocellulosic biomass. Despite the importance of the hydrolysis step, little information is available on hydrolytic microbes within anaerobic bioreactors. Therefore, in **Chapter 2** available scientific knowledge on hydrolysis, abundant hydrolytic microbes and their response to environmental/operational parameters, as well as to inhibitors, is reviewed. From this review, improvement of hydrolysis and biogas production is possible by getting more information about hydrolytic/fermentative microorganisms. **Chapter 3** describes the inhibitory effect of HA on anaerobic cellulose degradation and evaluates the mitigation potential of several cations (calcium, magnesium, iron, sodium and potassium) on HA inhibition. Results in chapter 3 suggest that not only hydrolysis but also methanogenesis is inhibited by HA. **Chapter 4** aims to find out which types of methanogens (hydrogenotrophic or acetoclastic methanogens) are the most susceptible to HA inhibition. In this scope, the HA inhibition on methanogenic activity of several pure cultures of methanogens and mixed cultures is investigated. Chapter 3 and Chapter 4 describe the effect of HA on hydrolysis and methanogenesis. However, it is not clear whether the HA inhibition persists during long-term reactor operation. Therefore, **Chapter 6** evaluates HA inhibition in long-term lab-scale CSTR reactor operation in the presence of increasing HA concentrations. Two inhibition mitigation strategies; calcium (**Chapter 3**) and hydrolytic enzyme addition (Fernandes et al .2015) are tested to reverse the inhibitory effect of HA in CSTR degrading cellulose and xylan. Additionally, microbial population dynamics is monitored in the presence and the absence of HAs

during the digestion. **Chapter 5** aims to monitor microbial community changes during the start-up period of 5 CSTRs that are used in chapter 6. In this chapter, a transient feeding strategy is used to acclimatise anaerobic sludge, which is not primarily degrading cellulosic compounds, to cellulose and xylan degradation. This chapter shows reactor performances and microbial community dynamics of the anaerobic reactors, operated in parallel at identical conditions. In **Chapter 7**, the conclusions and discussion from each chapter are integrated to propose possible solutions for the current challenges in HA inhibition. Future research opportunities are also discussed in that chapter.

Chapter 2

Presence and role of anaerobic hydrolytic microbes in conversion of lignocellulosic biomass for biogas production

This chapter is adapted from: Azman S*, Khadem AF*, van Lier JB, Zeeman G, Plugge CM (2015) Presence and role of anaerobic hydrolytic microbes in conversion of lignocellulosic biomass for biogas production. Crit Rev Environ Sci Technol 45:2523–2564. (*) Contributed equally

Abstract

In recent years, biogas production from complex biomass has received great interest. Therefore, many studies have been conducted to understand the anaerobic digestion process and to characterize responsible microbes for the biochemical conversions. Although the knowledge about biogas production in general is rapidly increasing, less information is available about hydrolytic microbes within anaerobic bioreactors. Here, the authors pinpoint the urgent need for solid fundamental knowledge about hydrolytic bacteria within biogas plants. In this review, current knowledge about anaerobic hydrolytic microbes is presented, including their abundance in biogas plants, and the factors impacting their activity.

2.1 Introduction

Biomass is a carbon rich material that is derived from agriculture, forestry, food-processing industries, industrial and animal residues and wastewater. treatment plants (Deublein and Steinhauser, 2010). Biomass residues are of quantitative interest to partly substitute our present fossil fuel requirements (UNEP, 2009). A recent study from the European Union (EU) showed that biomass was the major source (more than 60%) for renewable energy production in 2012 among EU countries (Eurostat, 2012). However, on global scale, biomass represents only 10% of the produced renewable energy (Mota et al., 2011).

Alcoholic fermentation and anaerobic digestion (AD) are the most prominent biochemical transformation technologies for bioenergy production from biomass worldwide (Deublein and Steinhauser, 2010). During alcoholic fermentation and AD, biomass can be converted to liquid biofuels (ethanol, butanol), chemical building blocks such as volatile fatty acids and biogas. After that, the produced energy carriers can be used for electricity generation, fueling engines, or heating. The complex AD process involves the activities of chemolithoautotrophic and chemoheterotrophic bacteria and methanogens. These microbial activities can be classified in different sequential reactions: hydrolysis, acidogenesis, acetogenesis, and methanogenesis. Throughout the entire food web, complex organic polymers are broken down to smaller molecules and consequently, biogas is produced as a final product. As in all other multistep reaction processes, the rate of each reaction in the AD process is different. Slow reactions determine the overall rate of the conversion process and they are usually termed the rate-limiting step. In the AD process, the type of substrate and operational conditions determine the rate-limiting step (Speece, 1983). For example, when simple organic matter, such as sugars and starch is converted to methane (CH_4), methanogenesis will be the rate-limiting step (Noike et al., 1985), as acidogenesis rates are higher than methanogenesis rates. However, during complex biomass digestion, due to the rigid structure of plant materials (e.g., straw, wood, corn stover), hydrolysis will be the rate-limiting step and directly affect CH_4 production (Appels et al., 2008; Lynd et al., 2002; Palonen and Viikari, 2004; Vidal, 2011). Methane production efficiencies from agricultural biomass digestion are estimated to be only about 50% due to inefficient hydrolysis of biomass within full-scale biogas reactors (Weiland, 2010). However,

theoretical yield calculations based on the cellulose content of the biomass predict that about 90% of the biomass energy can be recovered as CH_4. To increase the total CH_4 production from biomass, further fundamental studies are required to understand the hydrolytic microorganisms within biogas plants. Since the hydrolysis of biomass is the first and rate-limiting step in biogas production, improving the hydrolysis yields of the bioreactors will eventually help to increase methane production. A better understanding of hydrolytic microorganisms within an engineered mixed culture environment will contribute to uncover the rate-limiting phenomena of hydrolysis. The obtained knowledge will give insights how to improve hydrolysis efficiencies (therefore methane yields) without the necessity of relatively expensive and inefficient pretreatment methods. However, more knowledge is required about hydrolytic microorganisms to understand the nature of hydrolysis within engineered mixed culture environment. At the moment, the knowledge on microorganisms involved in hydrolysis and their physiology is still limited and is mostly based on pure culture studies. This review will highlight the current knowledge about anaerobic hydrolytic bacteria within biogas plants and their abundance, activity and role is discussed.

2.2 Directions and progress in biogas research

Biogas production from agricultural biomass, excess sewage sludge and domestic/municipal refuse sludge is a rapidly growing market in EU countries. The market growth has been accelerated by the directives of the EU Commission of Energy (Tilche and Galatola, 2008). In this directive, it is recommended that at least 35% of the manure, 40% of the suitable/available organic biomass and excess sludge of treatment plants should be used for biogas production by the year 2020. In this respect, research on biogas production has increased during the last two decades to reach the goal of EU Commission of Energy. Mutual aim of the ongoing biogas research is to enhance biogas production from agricultural biomass by reducing the costs. Although biogas research has broadened in recent years, it can be classified in three related categories. These are feedstock, process technology and microbiology related research. Feedstock related research focuses on characterization and evaluation of the different raw materials by means of biodegradability and methane potential. In recent years, evaluation of a large variety of raw material or feedstocks for biogas production has been performed.

Researchers have tested individual energy crops, agricultural residues, manure and as well as different combinations of feedstocks for biogas production. Feedstock characterization has been well documented since these characteristics influence the overall process efficiency and stability. Although good feedstocks for biogas production are known and used frequently, there are also large reservoirs of low methane yielding biomass. The current challenge is to increase methane yields from this low methane yielding biomass. Research focuses on pretreatment methods to increase the biodegradability of the biomass by removing specifically the lignin content. A lot of information about pretreatment methods of cellulose can be found in the literature (Chiaramonti et al., 2012; Fernandes et al., 2009; Hendriks and Zeeman 2009; Kumar et al., 2009; Taherzadeh et al., 2008). Process technology related research mainly deals with stability and performance of different reactor configurations. Researchers in this field are improving existing technologies or developing new technologies for mixing, process monitoring, process control and developing novel reactor configurations.

On the other hand, microbiology related research is still progressing to close the knowledge gaps on the activity of hydrolytic microbes within biogas plants. Microbiology related research mainly focuses on understanding which hydrolytic microorganisms are involved in biogas production, what are their metabolisms and how do they interact during anaerobic digestion. As fundamental studies are conducted to understand the nature of hydrolysis, the metabolic engineering approach is extensively studied to increase the hydrolytic activity. There are two strategies for that purpose: (a) native cellulolytic strategy, based on genetic modification of cellulolytic microorganisms and (b) the recombinant cellulolytic strategy, using non-cellulolytic microbes with high growth yield as expression host for cellulases or hemicellulases. The challenge of both strategies is to deliver microbial biomass with a high performance for industrial applications (Lynd et al., 2005; Olson et al., 2012). Production of hydrolytic enzymes by microbes and their applications are listed by Sukumaran et al. (2005). So far most industrial cellulases are produced by fungi, but there are studies also focusing on bacterial cellulases. The majority of the bacterial cellulases are produced through metabolic engineering (Olson et al., 2012; Sukumaran et al., 2005). Although little is known about cellulase activity improvements using genetic modification, some studies have shown that fermentation of cellulose can be greatly improved by using genetically

engineered *Clostridium cellulolyticum*. For example, at high carbon flux, lactate accumulation inhibits the growth of *C. cellulolyticum* on cellulose. Less lactate accumulation and more acetate/ethanol production was achieved by heterologous expression of a pyruvate decarboxylase and alcohol dehydrogenase from *Zymomonas mobilis* in *C. cellulolyticum* (Guedon et al., 2002). In addition, a recent study showed that metabolic engineering of *C. bescii*, by deleting the gene encoding for lactate dehydrogenase, resulted in increased acetate and hydrogen production (Cha et al., 2013).

Fundamental microbiology and bioengineering research will certainly lead us to further optimize the hydrolysis process to increase methane production. Although bioengineering studies may present improved results on hydrolysis efficiencies, their applications will still be limited due to the restrictions on the utilization of genetically modified microorganisms. On the other hand, research on hydrolysis microbiology within biogas plants has not received enough attention for years. Most of the hydrolysis related problems solved with pretreatment methods instead of taking a holistic approach (combining the knowledge of feedstock and technology knowledge with microbiological knowledge) to get in-depth analyses and answers. The fast developments in molecular microbial methods have led to increased knowledge, and to date we know more about which hydrolytic species are present within biogas plants. However, there are still questions about which species are more abundant or stable within bioreactors and how they are affected by environmental and operational conditions. In the following sections, we present information about the abundance of hydrolytic bacteria and the environmental factors that are most suitable for both hydrolytic bacteria and methanogens.

2.3 Anaerobic hydrolytic bacteria in biogas plants

The anaerobic hydrolytic bacteria can be found in soil, wood chip piles, sewage, hot springs, rumen of the animals, compost sites and biogas plants (Kublanov et al., 2009; Lynd et al., 2002; Wilson, 2011). In biogas plants, anaerobic cellulolytic bacteria have a crucial role in converting organic matter (e.g., polysaccharides, lipids, proteins), into low molecular weight intermediates that can be further used by other microbes in the anaerobic food web. Without their activity, the anaerobic digestion processes

cannot be naturally initiated, and every biogas plants should be started with an inoculum containing hydrolytic microorganisms. Therefore, it is important to know which hydrolytic microorganisms can survive in bioreactor environments and what is known about their metabolism.

Although many hydrolytic microbes already have been isolated and described, little is known about their role in bioreactors. Table 2.1 overviews research on representative examples of hydrolytic bacteria that have been detected in, or isolated from various biogas plants. In the following section, abundance and known features of hydrolytic bacteria within biogas plants will be discussed in more detail.

From a microbiological point of view, anaerobic hydrolytic bacteria can be found within the phylum *Firmicutes* (genus: *Clostridium*, *Ruminococcus*, *Caldicellulosiruptor*, *Caldanaerobacter*, *Butyrivibrio*, *Acetivibrio*, *Halocella*, and *Eubacterium*), *Bacteroidetes*, *Fibrobacter*, *Spirochaetes* (*Spirochaeta*), and *Thermotogae* (genus: *Fervidobacterium* and *Thermotoga*). Although the abundance of each hydrolytic bacterial species depends on the inoculum type of the bioreactors, members of the *Firmicutes* and *Bacteroidetes* are the most commonly found in biogas plants, whereas the abundance of *Fibrobacter*, *Spirochaetes*, and *Thermotogae* is less frequent. Figure 2.1 shows the abundance of the hydrolytic bacteria at phylum level. Data sets were collected from several literature sources that used molecular methods to detect their relative abundance.

2.3.1 Phylum *Firmicutes*

2.3.1.1 Genus *Clostridium*

The *Clostridium* genus harbours Gram-positive, rod-shaped and endospore forming microbes that are obligate anaerobes. Biotechnologically important species of *Clostridium* typically belong to the cluster III and IV. These clusters are known for their ability to degrade cellulosic biomass. These microbes are assigned to the new genera *Ruminiclostridium* (Yutin and Galperin, 2013). The known cellulolytic clostridia species are: *Clostridium thermocellum*, *C. aldrichii*, *C. alkalicellulosi*, *C. caenicola*, *C. cellobioparum*, *C. cellulolyticum*, *C. cellulosi*, *C. clariflavum*, *C. hungatei*, *C. josui*, *C. leptum*, *C. methylpentosum*, *C. papyrosolvens*, *C. sporosphaeroides*, *C. stercorarium*, *C. straminisolvens*, *C. sufflavum*, *C. termitidis*, *C. thermosuccinogenes*, *C. viride*, *Bacteroides cellulosolvens* (*Pseudobacteroides cellulosolvens*), *Eubacterium siraeum*, and

Clostridium sp. BNL1100 (Collins et al., 1994; Ludwig et al., 2009; Yutin and Galperin, 2013). The members of the genus *Clostridium*, usually dominate the microbial population in the biogas plants (Burrell et al., 2004; Horino et al., 2014; Liu et al., 2009; Lucas et al., 2015; Nishiyama et al., 2009a; Shiratori et al., 2006; Shiratori et al., 2009; Sundberg et al., 2013; Syutsubo et al., 2005; Wirth et al., 2012; Zverlov et al., 2010). Examples of *Clostridium* species that are present in biogas plants can be found in Table 2.1.

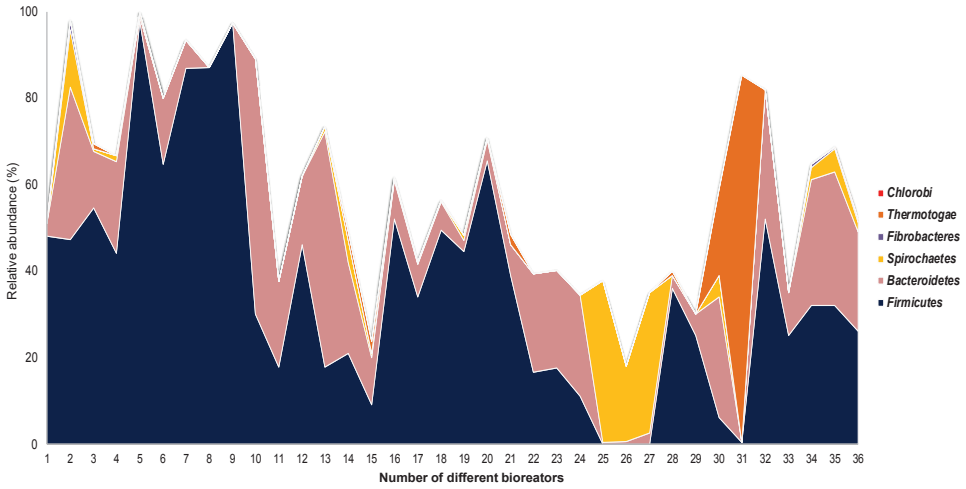

Figure 2.1 Stacked area graph showing the abundance of different phyla within anaerobic biogas reactors. Data were extracted from 36 different biogas bioreactors.Data were extracted from following publications; Wirth et al., 2012; Liu et al., 2009; Schlüter et al., 2008; Klocke et al., 2007; Jaenicke et al., 2011; Weiss et al., 2008; Goberna et al., 2009; Tang et al., 2011; Kampmann et al., 2012; Lee et al., 2012; Hanreich et al., 2013; St-Pierre et al., 2013; Wu and He, 2013; Li et al., 2013; Wong et al., 2013; Rademacher et al., 2012; Guo et al., 2013; Jensen et al, 2014; De Francisci et al., 2015; Lucas et al., 2015; Luo et al., 2015.

Table 2.1 Anaerobic hydrolytic microbes in bioreactors

Anaerobic hydrolytic microbes	Feed	pH	Temperature	Type of the reactor	Location	Isolated or detected	Details	References
Genus *Clostridium* *Clostridium stercorarium* *Clostridium thermocellum* *Clostridium* sp.	Microcrystalline cellulose and sterile landfill leachate	n.r.	n.r.	1.25-litre anaerobic landfill leachate bioreactor	n.a.	Detected by using cloning and 16S rRNA sequencing techniques	Similarity of the clones were 95.5% (*C. thermocellum*), 90% (*C.stercorarium*) and 97.6% (*C.* sp. strain XB90) to cultured species	Burrell et al., 2004
Clostridium sp.	Cellulose powder	7	55	9.5- litre lab-scale Baffled reactor	n.a.	Previously isolated by the same team (Unpublished data)	Reactors were operated to assess the abundance of the strain JC3	Syutsubo et al., 2005
Clostridium clariflavum *Clostridium caenicola*	Synthetic wastewater includes Photocopy paper and a traditional food ingredient	n.r.	55	4 litre methanogenic bioreactor	n.a.	Isolated from lab scale reactor	—	Shiratori et al., 2006 and 2009
Clostridium sufflavum sp.	Cattle waste	n.r.	Mesophilic range	Methanogenic full scale reactor (1500 m^3	Hokkaido, Japan	Isolated from digested rice straw residues that is taken from the reactor	—	Nishiyama et al., 2009a

Clostridium cellulosi *Clostridium thermocellum*	Decomposing plant material and cattle manure	5.2-8	37,55, 60	Mesophilic and thermophilic biogas plants	Bavaria state, Germany	Detected by using cloning and 16S rRNA sequencing	Similarity of the clones were 91-99% to cultured species	Zverlov et al., 2010
Clostridium cellulolyticum *Clostridium saccharolyticum* *Clostridium acetobutylicum* *Clostridium thermocellum*	Maize silage added to pig manure slurry	7.9-8.4	37	6 litre lab. scale reactor	n.a.	Detected by metagenomic approach 454-pyrosequencing	—	Wirth et al., 2012
Clostridium straminisolvens *Clostridium chartatabidum* *Clostridium thermocellum*	Pig manure	n.r.	n.r.	Full-scale biogas plant (600 m^3)	Shanghai, China	Detected by using cloning and 16S rRNA sequencing	Similarity of the clones were 86% (*C.thermocellum*), 88% (*C. straminisolvens*) and 99% (*C. chartatabidum*) to cultured species	Liu et al., 2009
Genus *Ruminococcus* *Ruminococcus obeum* *Ruminococcus gnavum* *Ruminococcus torques*	Mixture of maize silage (63%), green rye (35%), chicken manure (2%)	7.7	41	Full-scale biogas plant	Bielefeld-Jöllenbeck, Germany	Detected by metagenomic approach 454-pyrosequencing	—	Schlüter et al., 2008

Table 2.1 Anaerobic hydrolytic microbes in bioreactors (Continued)

Anaerobic hydrolytic microbes	Feed	pH	Temperature	Type of the reactor	Location	Isolated or detected	Details	References
Ruminococcus albus	Maize silage added to pig manure slurry	7.9 - 8.4	37	6 litre lab. scale reactor	n.a.	Detected by using metagenomic approach applied by 454-pyrosequencing	—	Wirth et al., 2012
Ruminofilibacter xylanolyticum	n.r.	n.r.	n.r.	Full-scale biogas plant	n.a.	Detected based on sequencing of 16S rDNA clone libraries sequence data obtained by 454-pyrosequencing	Similarity of the clone was 99% to cultured species	Kröber et al., 2009
Genus *Acetivibrio* *Acetivibrio cellulolyticus*	Fodder beet silage	7.8	35	8 litre lab scale reactor	n.a.	Detected by using cloning and 16S rRNA sequencing	Only 3 OTUs were found related with *A. cellulolyticus*	Klocke et al., 2007
Acetivibrio sp.	Landfill leachate	n.r.	Mesophilic range	1 litre batch digester	n.a.	Detected by flourescent in situ hybridization	—	Li et al., 2009a

Genus *Butyrivibrio* *Butyrivibrio fibrisolvens*	Napier grass	6.8 -7	35	Anaerobic CSTR	n.a	Isolated	—	Sewell, 1988
Butyrivibrio sp.	Agricultural waste	n.r.	55	Full-scale biogas plant (6600 m^3)	Fürstenwalde /Spree, Germany	Detected by using 16S rRNA sequencing	—	Weiss et al., 2008
Genus *Halocella* *Halocella cellulosilytica*	Biowaste (separated organic household waste and garden	n.r.	50-55	Full scale biogas plant (750 m^3)	Tirol, Austria	Detected by using cloning and 16S rRNA sequencing	Similarity of the clones were 91-92% to cultured species	Goberna et al., 2009
Halocella cellulosilytica	paper based waste and dog food	n.r.	Thermophilic range	5 litre flasks used for dry fermentation	n.a.	Detected by using cloning and 16S rRNA sequencing	Similarity of the clones were 99% to cultured species	Tang et al., 2011
Genus *Eubacterium* *Eubacterium ventriosum*	Mixture of maize silage (63%), green rye (35%), chicken manure (2%)	7.7	41	Full-scale biogas plant	Bielefeld-Jöllenbeck, Germany	Detected by using metagenomic approach applied by 454-pyrosequencing	—	Schlüter et al., 2008

Table 2.1 Anaerobic hydrolytic microbes in bioreactors (Continued)

Anaerobic hydrolytic	Feed	pH	Temperature	Type of the reactor	Location	Isolated or detected	Details	References
Uncultured *Eubacterium*	Agricultural waste	n.r.	55	Full-scale biogas plant (6600 m^3)	Fürstenwalde /Spree, Germany	Detected by using 16S rRNA sequencing	—	Weiss et al., 2008
Genus *Fibrobacter* uncultured *Fibrobacteres*	Pig manure	n.r.	n.r.	Full-scale biogas plant (600 m^3)	Shanghai, China	Detected by using cloning and 16S rRNA sequencing	Similarity of the clone was 90% to the closest cultured species	Liu et al., 2009
Genus *Bacteroides* *Bacteriodes graminisolvens*	Cattle farm waste	n.r.	Mesophilic range	Methanogenic full scale reactor (1500 m^3)	Hokkaido, Japan	Isolated from digested rice straw residues that is taken from the reactor	—	Nishiyama et al., 2009b
Bacteriodites cellulosolvens	Cellulose	7	35	Municipal wastewater treatment plant	Canada	Isolated from sewage sludge	—	Murray et al., 1984

Genus *Spirochaeta* *Spirochaeta xylanolyticus*	Seaweed	7.3	37	1.1 litre batch digesters	n.a	Detected by using cloning and 16S rRNA sequencing	Similarity of the clone was 85% to cultured species	Pope et al., 2012
Genus *Thermotoga* *Thermotoga lettingae*	Mixture of maize silage (63%), green rye (35%), chicken manure (2%)	7.7	41	Full-scale biogas plant	Bielefeld-Jöllenbeck, Germany	Detected by using metagenomic approach applied by 454-pyrosequencing	—	Schlüter et al., 2008
Petrotoga mobilis	Agricultural waste	n.r.	55	Full-scale biogas plant (6600 m3)	Fürstenwalde /Spree, Germany	Detected by using 16S rRNA sequencing	—	Weiss et al., 2008

n.r= not reported , n.a.=not available

2.3.1.2 Genus *Ruminococcus*

Ruminococcus is another genus of the *Firmicutes* and its members are non-sporulating, coccoid shaped microbes. *Ruminococcus flavefaciens*, *R. albus*, *R. callidus*, and *R. bromii* are the members of the clostridial cluster IV, which is known for their effective ability to degrade cellulosic biomass. In biogas plants, *Ruminococcus* species are less frequently reported (Morrison et al., 2009). In parallel to this knowledge, reported sequences are generally derived from manure-treating biogas plants. Schlüter et al. (2008) and Kröber et al. (2009) reported *R. obeum*, *R. gnavum*, and *R. torques* from biogas plant, treating maize silage, green rye, and small amount of chicken manure. Recently, by using short read next generation DNA sequencing technique, *R. albus* was reported as one of the dominant hydrolytic microbes in lab-scale anaerobic digesters that were treating maize silage and pig manure (Wirth et al., 2012).

2.3.1.3 Genus *Caldicellulosiruptor*

Within the phylum *Firmicutes*, the genus *Caldicellulosiruptor* contains Gram-positive, non–spore-forming bacteria. The known crystalline cellulose degraders are *C. saccharolyticus*, *C. bescii*, *C. obsidiansis*, and *C. hydrogenoformans*. The hemicellulolytic members are *C. hydrothermalis*, *C. kristjanssonii*, *C. kronotskyensis*, *C. owensensis*, and *C. lactoaceticus* (Blumer- Schuette et al., 2011). This genus is also known as the cellulose degrader with the highest temperature optimum (70–78 °C). From a biotechnological point of view, interest in *Caldicellulosiruptor* has increased due to its potential utilization in biogas and hydrogen production (Bielen, 2013). It is known that *C. saccharolyticus* can produce hydrogen from a wide variety of carbon sources with high yields (Bielen, 2013; van Niel et al., 2002). The high yield of hydrogen production can be beneficial for hydrogenotrophic methanogens in biogas plants. Bagi et al. (2007) showed this possibility in lab-scale experiments by adding *C. saccharolyticus* to natural biogas producing consortia that stabilized excess sewage sludge, pig manure and plant biomass. As a result of the bioaugmentation, biogas formation increased up to 70% compared to a control without *C. saccharolyticus*. In 2010, the same research group showed the exploitation of the same microorganisms within biogas plants. However, bioaugmentation with *C. saccharolyticus* in full-scale biogas plants did not lead to increased biogas production due to wash out of the bacteria from the system at long

term reactor operation. For this reason, performance and persistence of *C. saccharolyticus* in full-scale biogas plants needs to be investigated in more detail (Herbel et al., 2010). Table 2.2 shows the *Caldicellulosiruptor* species that have a potential utilization for bioaugmentation within biogas plants.

2.3.1.4 Genus *Acetivibrio* and *Butyrivibrio*

The genus *Acetivibrio* includes non-motile, Gram-negative rods that produce acetic acid and ethanol from fermentation of carbohydrates. Known cellulolytic *Acetivibrio* species are *A. cellulolyticus* (Patel and MacKenzie, 1982; Patel et al., 1980) and *A. cellulosolvens* (Khan et al., 1984). In comparison with other hydrolytic members of the *Firmicutes*, the relative abundance of the genus *Acetivibrio* in biogas plants is less clear. Klocke et al. (2007) detected *Acetivibrio* sequences in lab-scale CSTRs, treating fodder beet silage. Another study by Krause et al. (2008) showed that *Acetivibrio* were present in the fermenters of agricultural biogas plants, but they were less abundant when compared to other hydrolytic microbes. A recent study by Jaenicke et al. (2011) identified *Acetivibrio* as one of the dominant genera in the samples from Krause et al. (2008) by using different metagenomic analyses.

Like *Acetivibrio*, the role of the genus *Butyrivibrio* in biogas plants is not clearly understood. *Butyrivibrio* are anaerobic, xylanolytic, amylolytic curved rod-shaped bacteria that can produce butyric acid. They are abundant in the gastrointestinal tract of domestic and wild ruminants (Orpin et al., 1985). Known xylanolytic strains of the *Butyrivibrio* species are listed by Mc-Sweeney et al. (1998); including the fastest xylan degrader known to date, strain H17c, and *Butyrivibrio fibrisolvens* from an anaerobic digester that was fed with napier grass (Cotta and Zeltwanger, 1995; Sewell et al., 1988). *Butyrivibrio* is not frequently reported in mesophilic anaerobic digesters. In 2008, Weiss et al. (2008) detected relatively small amounts of *Butyrivibrio* sequences (relative abundance was 2.5%), in biogas plants, treating municipal waste and agricultural residues. Although both *Butyrivibrio* and *Acetivibrio* are detected less frequently, their abundance within biogas plants might be underestimated due to technical limitations in DNA technologies and also sampling techniques. As *Butyrivibrio* and *Acetivibrio* have cellulolytic and proteolytic activity, biogas plant environments may provide a perfect habitat for them.

Table 2.1 Anaerobic extremely thermophilic *Caldicellulosiruptor* species that can have bioaugmentation potential for biogas

Microorganisms	Substrate	pH	Temperature (°C)	Habitat of isolation	Country	References
Caldicellulosiruptor bescii DSM 6725	Lignocellulose	7.2	78-80	Hot spring	Kamchatka, Russia	Yang et al., 2010
Caldicellulosiruptor obsidiansis sp. nov. strain OB47	Lignocellulose	6-8	78	Hot spring	Yellow stone national park, The	Hamilton-Brehm et, al., 2010
Caldicellulosiruptor hydrothermalis sp. nov. DSM 18901	Lignocellulose	7	65	Hot spring	Kamchatka, Russia	Miroshnichenko et al., 2008
Caldicellulosiruptor kronotskyensis sp. nov. DSM 18902	Lignocellulose	6-8	70	Hot spring	Kamchatka, Russia	Miroshnichenko et al., 2008
Caldicellulosiruptor owensensis OLATCC 7001 67	Xylan	7.5	75	Sediment	Owens Lake, California, The USA	Huang et al., 1998
Caldicellulosiruptor kristjanssonii sp. nov. DSM 12137	Lignocellulose	7	78	Hot spring	Iceland	Bredholt, 1999
Caldicellulosiruptor saccharolyticus DSM 8903	Lignocellulose	7	70	Hot spring	New Zeeland	Rainey, 1994
Caldicellulosiruptor lactoaceticus sp. nov. DSM 9545	Lignocellulose	7	68	Hot spring	Iceland	Mladenovska, 1995

Their relationship with fermenters and methanogens is also not fully understood. Further research is required to assess the function and abundance of these groups within biogas plants.

2.3.1.5 Genus *Halocella*

Halocella are straight or slightly curved nonsporulating motile rod-shaped bacteria. They hydrolyze and ferment cellulose to acetate, ethanol, lactate, hydrogen and CO_2. Within this genus, *H. cellulolytica* is the only cellulolytic bacterium reported, which was isolated from hyper saline lagoons of Lake Sivash (Simankova et al., 1993). *H. cellulosilytica* was also detected in thermophilic biogas plants, at lower abundance (Goberna et al., 2009; Tang et al., 2011).

2.3.1.6 Genus *Eubacterium*

Eubacterium species are characterized by a rigid cell wall. The fiber degrading species are *E. cellulosolvens*, *E. uniforme*, *E. ventriosum*, and *E. xylanophilum* and they were isolated from mammalian gut (Wade et al., 2006). However, very little is known about their role and abundance in biogas plants (Wildenauer and Winter, 1985; Zellner and Winter, 1987). Recent analyses at DNA and proteome level in mesophilic and thermophilic biogas plants revealed the presence of *Eubacterium* (Schlüter et al., 2008; Weiss et al., 2008).

2.3.2 Phylum *Fibrobacteres*

2.3.2.1 Genus *Fibrobacter*

Cellulose degraders are also known to belong to the phylum *Fibrobacteres*. This phylum consists of a single genus *Fibrobacter*, with so far only two cultured representatives, *Fibrobacter succinogenes and F. intestinalis. F. succinogenes* has received attention mostly because of high fibrolytic activity and being a true cellulolytic specialist (Lissens et al., 2004; Suen et al., 2011). *Fibrobacteres* are also found in biogas plants; however, they are less abundant than *Firmicutes* and *Bacteroidetes* (Liu et al., 2009).

2.3.3 Phylum *Bacteroidetes*

2.3.3.1 Genus *Bacteroides*

Bacteroides are Gram-negative, non–endospore-forming bacteria. In general, *Bacteroides* are abundant in biogas plants together with the *Firmicutes* (Hanreich et al.,

2013; Kampmann et al., 2012; Lee et al., 2012; Li et al., 2013: Lucas et al., 2015; Schlüter et al., 2008; St-Pierre et al., 2013; Sundberg et al., 2013; Wu and He, 2013). Within this genus, *B. cellulosolvens* and *B. xylanolyticus* are able to degrade cellulose and xylan, respectively (Chassard et al., 2008; Giuliano and Khan, 1984; Murray, 1986; Robert et al., 2007; Scholten-Koerselman et al., 1986). Another xylan degrader, *B. graminisolvens*, was isolated from a methanogenic reactor treating cattle waste (Nishiyama et al., 2009b).

2.3.4 Phylum *Spirochaetes*

2.3.4.1 Genus *Spirochaeta*

Members of the *Spirochaetes* are chemoorganotrophic, spiral-shaped bacteria that can inhabit anaerobic to microaerophilic niches. *Spirochaeta* can ferment several carbohydrates and amino acids. The main carbohydrate fermentation products are ethanol, acetate, CO_2, and H_2. The only known cellulose degrading *Spirochaeta* species was isolated from a hot spring by Rainey et al. (1991). Although the *Spirochaetes* cannot degrade cellulose, they may dominate anaerobic digester environments (Briones et al., 2007; Klocke et al., 2007; Lee et al., 2012; Lee et al., 2013; Liu et al., 2009; Pope et al., 2012; Wong et al., 2013). The high abundance of *Spirochaetes*, without having the ability of cellulose degradation, within anaerobic digesters suggests that they may interact with cellulose degraders. Stanton and Parola (1980) and Kudo et al. (1987) showed the potential interactions between *Spirochaeta* and cellulose degraders, by using cocultures of saccharolytic *Treponema bryantii* and the ruminal cellulose degraders: *F. succinogens* and *R. albus*. Both papers showed the increase in cellulose degradation in the presence of *Spirochetes*. Pohlschroeder et al. (1995) also tested this hypothesis in thermophilic conditions by using *Spirochaeta caldaria* and *C. thermocellum.* They also reported increased cellulose degradation rates in co-cultures of the two microorganisms compared to monocultures of *C. thermocellum*. Although cellulose degradation rates increased, it is still not clear how the interaction occurs between the *Spirochaetes* and cellulose degraders. Leschine (1995) suggested that *Spirochaeta* may prevent cellobiose dependent cellulase system inhibition of the cellulose degraders, by lowering cellobiose concentrations, which is produced by cellulose degradation. However, more research should be done to disclose the role of *Spirochaeta* in cellulose degradation.

2.3.5 Phylum *Thermotogae*

2.3.5.1 Genus *Thermotoga*

The members of the genus *Thermotoga* are hyperthermophilic and the known species are anaerobic, with oxygen tolerance. Based on metagenomics analyses, these microbes were also detected in mesophilic and thermophilic biogas plants (Guo et al., 2013; Krause et al., 2008; Li et al., 2013; Rademacher et al., 2012; Schlüter et al., 2008). *Thermotogales* are known to metabolize carbohydrates with production of hydrogen (Eriksen et al., 2008). Known hydrolytic species are *T. hypogea*, *T. maritime*, and *T. neapolitana* (Evans et al., 2000; Fardeau et al., 1997; Kim et al., 2011; Nguyen et al., 2008a; Nguyen et al., 2008b; Weiss et al., 2008).

2.3.6 Phylum *Chlorobi*

Recently the facultative anaerobic thermophilic *Melioribacter roseus* was isolated from an oil field in Russia. This microorganism belongs to the phylum *Chlorobi*, which shares a common root with *Bacteroidetes* in the tree of life. This microorganism is able to grow on avicel and xylan. Genome analyses of *Chlorobi* confirmed the presence of genes coding for hydrolytic enzymes (Kadnikov et al., 2013; Podosokorskaya et al., 2013). In biogas plants, members of the Chlorobi were less frequently detected (Kampmann et al., 2012; Li et al., 2013).

In this section, hydrolytic bacteria within biogas bioreactors were introduced to emphasize their known functions and their abundance. As it can be seen from Figure 2.1, anaerobic bioreactors are low in richness in terms of hydrolytic bacteria. Members of the *Firmicutes* and *Bacteroidetes* are the generalist microorganisms that are mostly dominating the bioreactors whereas the other hydrolytic bacteria are less abundant, even though they have high cellulolytic activities in pure cultures. The reason for this phenomenon is not well known and open for research opportunities. On the other hand, functions of the individual hydrolytic species are known from pure culture studies, but their interaction with other microorganisms within biogas reactors is still unclear. With the wide spread utilization of next generation sequencing methods, the microbial profile of many biogas plants has been identified. The new challenge for the researcher will be linking microbial profiles to metabolic functions. By this way, detailed information about interaction between hydrolytic bacteria and other microorganisms

can be gathered to understand 'who does what, where, when, and together with whom.' Revealing the exact metabolic interaction between microorganisms within bioreactor will surely help to inspire bioengineers to design new type of processes and reactors in the near future.

After presenting information about the abundant hydrolytic bacteria within bioreactors, in the following section, the hydrolytic activity of anaerobic microorganisms will be discussed.

2.4 Anaerobic hydrolytic activity

Hydrolytic anaerobic microbes (excluding extremely thermophilic, anaerobic conditions) have developed a special multienzyme complex, known as the cellulosome (Doi et al., 2004; Felix and Ljungdahl, 1993). Anaerobic bacteria cannot produce the required enzymes to degrade lignocellulosic biomass without cellulosomes. Thus, cellulosomes have an important role in building a bridge between anaerobic bacteria, enzymes and substrates. Cellulosomes were first described in 1983 by Lamed et al. (1983), in the anaerobic, Gram-positive, thermophilic *Clostridium thermocellum*. After this discovery, research on cellulosome structure and function had increased rapidly, which led to the discovery of cellulosomes in mesophilic anaerobic bacteria within the genera *Clostridium*, *Acetivibrio*, *Bacteroides*, and *Ruminococcus* (Dassa et al., 2012; Doi et al., 2003; Xu et al., 2004). Progress in cellulosome research is reviewed by Schwarz (2001), Bayer et al. (2007), and Fontes and Gilbert (2010). In anaerobic extremely thermophilic conditions, microbial groups such as *Caldicellulosiruptor* have distinct hydrolytic enzyme systems. Unlike other anaerobic microorganisms, species of *Caldicellulosiruptor* do not have cellulosomes (Blumer-Schuette et al., 2008). They secrete many free hydrolases that contain multiple catalytic domains. Secretion of free hydrolases with multiple catalytic domains, allows *Caldicellulosiruptor* to hydrolyze even un-pretreated biomass with higher yields.

Overall, anaerobic microorganisms secrete different types of enzymes during lignocellulosic biomass hydrolysis. Their complex enzyme systems produce glucanases, hemicellulases, chitinases, and lichanases, which will be introduced shortly in the following subsections.

2.4.1 Glucanase activity

In general, glucanases perform the breakdown of glucan polymers to glucose moieties. As they hydrolyze glucosidic bonds, they can be clustered as glycoside hydrolases (GH). GH are known as a very diverse enzyme family, with 133 subgroups, which are classified based on amino acid sequence similarities (Henrissat and Davies, 1997). However, cellulose-degrading glucanases can be distinguished from other GH by their ability to hydrolyze β-1, 4-glucosidic bonds between glucose residues (Henrissat and Davies, 1997). In addition, these glucanases show both endoglucanase and exoglucanase activity. Endoglucanases cleave the cellulose chain randomly and generate new oligosaccharides, whereas exoglucanases are able to cut the cellulose chains into smaller molecules, releasing either glucose (glucanohydrolase activity) or cellobiose (cellobiohydrolase activity) (Lynd et al., 2002).

2.4.2 Hemicellulase activity

Hemicellulases are a diverse group of enzymes that hydrolyze hemicellulose polymers to several different oligomers and monomers. Hemicellulases may belong to either GH family or carbohydrate esterases, which embrace many diverse enzymes that have different functions in hemicellulose degradation. The hemicellulolytic enzymes that belong to the GH family are xylanases, α-d-glucuronidases, β-xylosidases, β-mannanases, and α-l-arabinofuranosidases (Shallom and Shoham, 2003). Independent of hemicellulose composition, xylanases (EC 3.2.1.8) hydrolyze the β-1,4 bond in the xylan backbone and liberate short-chain xylooligomers. Other xylan related enzymes are the α-d-glucuronidases, able to cleave the α-1,2-glycosidic bond of the 4-O-methyl-d-glucuronic acid side chain of xylans. Consequently, the liberated short-chain xylooligomers can be hydrolyzed by β- xylosidases (EC 3.2.1.37) to single xylose units. On the other hand, different hemicellulose compositions require different types of enzymes to degrade the complex structures. Mannan-based hemicellulose structures can be hydrolysed by β-mannanases (EC 3.2.1.78) to short β-1,4-manno-oligomers, which can be further hydrolyzed to mannose by β-mannosidases (EC 3.2.1.25). On the other hand, arabinose-based hemicellulose structures need α-l-arabinofuranosidases (EC 3.2.1.55) and α-l-arabinanases (EC 3.2.1.99) to substitute complex arabinose based-hemicellulose to xylans, xylooligomers, and arabinans.

Carbohydrate esterases related hemicellulases include acetyl xylan esterases (EC 3.1.1.72), that hydrolyze the acetyl substitutions on xylose moieties, and feruloyl esterases (EC 3.1.1.73), which hydrolyze the ester bond between the arabinose substitutions and ferulic acid (Shallom and Shoham, 2003).

2.4.3 Chitinase and lichanase activity

Chitins are cellulose-like molecules that are abundant in the exoskeleton of the insects and cell wall of several fungi. Chitinases (EC 3.2.1.14) degrade complex chitin polymers to linear, insoluble β-1,4-linked polymer of N-acetyl glucosamine (Bhattacharya et al., 2007).

Lichenins are complex glucans, produced by some lichen species. Lichenin is composed of glucose molecules that are linked with β-1,3 and β- 1,4 glycosidic bonds. Anaerobic hydrolytic microorganisms produce licheninases (EC 3.2.1.73) to hydrolyze glucosidic linkages in β-1,3 and β-1,4 glucan molecules.

The present knowledge on hydrolytic enzyme activity was mainly obtained from pure culture studies. Hydrolytic enzyme activity studies from bioreactors is limited in terms of enzyme measurement methodologies. Reported enzyme activities for membrane bound enzymes can be questionable due to the distribution of these enzymes within solid media. Indeed, it was reported that abundance of hydrolytic enzymes within bioreactors were related with their type of secretion (Morgenroth et al., 2002; Parawira et al., 2005). Therefore, traditional enzyme measurement methods may result in biased activity measurements. Because of that, molecular screening methods have been applied to get more in depth information about the abundance and diversity of hydrolytic enzymes. Li et al. (2009b) summarized those approaches that are used to discover novel enzymes. More recently, quantitative polymerase chain reaction (Q-PCR), and sequencing methods were applied to determine the abundance of genes that are coding for GH families (Pereyra et al., 2010; Sun et al., 2013). Although these approaches showed the diversity of GH 48 families, the interpretation of these results should be carefully considered since the amplicon size of the targeted genes are relatively small and the diversity of GH 48 enzyme family is very large for designing successful primers. Because of those reasons, hydrolytic activity within anaerobic sludge should be further investigated with combined metagenomic studies and enzyme activity

assays to get more in depth knowledge about endogenous hydrolytic activity of biogas reactors. To predict and understand the enzyme system of the anaerobic hydrolytic activity, we have to understand the complex microbial communities, metabolic pathways involved in the AD process and factors that affect hydrolytic activity in biogas plants.

2.5 Factors affecting the hydrolytic activity in biogas plants: a brief overview

2.5.1 Temperature

Biogas plants are usually operated either at mesophilic (35–45 °C) or thermophilic (45–60 °C) conditions. Fluctuating and changing temperatures within stable bioreactors may cause lower biogas production or temporarily disturbance in methanogenic activity (Chae, 2008; Cioabla, 2012). Sudden changes in temperature have more impact on thermophilic processes than mesophilic processes, since thermophilic microorganisms are more susceptible to temperature changes. Thus, temperature fluctuations in thermophilic range should not exceed ±1°C for optimal biogas production. However, this range can be more flexible (±3 °C) in the mesophilic range (Ahring et al., 2001; Ange lidaki et al., 2003; El-Mashad et al., 2004; Navickas, 2013; Van Lier et al., 1993). Hydrolytic microorganisms have an optimum temperature between 30°C and 60°C. Hydrolytic enzymes can be still active beyond this temperature range (Jonke and Michal, 2004). The relation between the anaerobic hydrolytic activity and temperature is generally expressed by the Arrhenius equation. According to that equation hydrolytic activity increases until an optimum temperature, after which the hydrolytic activity rapidly decreases (Veeken and Hamelers, 1999).

2.5.2 pH

pH is another important environmental factor that affects the stability of biogas plants. Each microbial group within the AD process has a different optimal pH for growth. Therefore, selection of operational pH is rather complex. Most hydrolytic microorganisms have an optimum pH between 5 and 7, whereas the pH optimum for methanogens varies between 6.5 and 8.5. Unexpected fluctuations in pH, affect methanogenesis rather than hydrolysis, since methanogenic communities are more susceptible to pH fluctuations (Ferry, 1992; Kim, 2003). For this reason, separated two phase reactor systems are also used to achieve better hydrolysis rates at lower pH levels

and consequently, neutral pH can be applied to maintain a high methanogenic activity in the second phase reactor. Although many studies focus on the effects of pH on anaerobic hydrolysis, there is no solid proof for improved hydrolysis efficiencies below pH 7 (Dinamarca, 2003; Lu et al., 2006). Apparently, pH could be set at neutral levels, to maintain both the hydrolytic and methanogenic activities in bioreactors.

2.5.3 Substrate related factors

2.5.3.1 Particle size

Particle size is one of the important factors that affects hydrolysis. Many studies showed increased hydrolysis rates with the particle size reduction (Dionisi, 2013; Pabón-Pereira et al., 2012; Sanders, 2001; Zhang and Lynd, 2004; Yeh et al., 2010). Zhang and Lynd (2004) proposed that the increase in hydrolysis efficiency is not only related with reducing particle size, but also with reduced mass transfer resistance that is related with enzyme adsorption. Another hypothesis on increased hydrolysis rates when particles size is low, is explained by the physical increase in the surface area of the substrates that leads to higher binding possibilities of the substrates with the enzymes (Sanders et al., 2000; Sun and Cheng, 2002; Wen et al., 2004). On the other hand, Hidayat et al. (2012) discussed the effects of dislocations (special, irregular regions within plant cell walls), on hydrolysis. During hydrolysis, plant cells break at dislocations, which implies that cellulose within dislocations is more accessible to enzymes. The number of dislocations can be increased by reducing particle size. Thus, the number of the dislocations is important for efficient hydrolysis. To date it is not yet clear whether the cellulases prefer to attack dislocations or carbohydrate binding modules play a role in the attachment. Particle size reduction is also recommended to get higher biogas production. Particle size reduction yields better hydrolysis efficiencies and consequently, the overall digestion process can be improved (Deublein and Steinhauser, 2010; Gunaseelan, 1997; Sreekrishnan et al., 2004).

2.5.3.2 Substrate accessibility and substrate structure

Lignocellulosic materials have a complex chemical and physical structure and are often described as recalcitrant compounds (Harris and Stone, 2009; Vidal et al., 2011). Lignocellulose is composed of three basic components: cellulose, noncellulosic carbohydrates (predominantly hemicellulose), and lignin. The extensive covalent

crosslinking of the lignin with other carbohydrates comprises a rigid structure that limits the accessibility of hydrolases (Vidal et al., 2011). Therefore, removal of lignin and hemicellulose is required for more efficient cellulose hydrolysis (Hendriks and Zeeman, 2009).

Crystallinity is another structure related factor that affects hydrolysis. Although there are discussions about evaluation of the methodologies that were used in the analyses (Lynd et al., 2002), increasing crystallinity reduces the hydrolysis rate of cellulose (Hall et al., 2010). A recent study from Igarashi et al. (2011) described why crystallinity can be a problem for cellulose hydrolysis. According to this study, the roughness of the crystalline cellulose surface leads to the formation of a crowded surface structure. Thus, flattening the structure by the means of pretreatment should reduce the molecular congestion, improving the mobility of the enzymes and increasing the efficiency of cellulose hydrolysis.

2.5.4 Feeding regime

Feeding regime is a controversial substrate related factor that is affecting the biogas plants. Many studies discussed the feasibility of different feeding regimes for optimal hydrolysis and biogas production. Different types of biomass and their residues have different biodegradability. Utilization of biomass for energy production depends on several factors, which are influencing the performance of the anaerobic degradation of lignocellulosic biomass. These are (a) abundance of lignin in biomass and its association with hemicellulose and cellulose; (b) abundance of structural and non-structural carbohydrates and lipids within biomass; (c) structure of cellulose (e.g., amorphous, crystalline); and (d) presence of inhibitory substances such as humic and fulvic acids (Ferreira-Leitao, 2006; Fernandes, 2015; Gunaseelan, 1997; Jimenez et al., 1990). As biodegradability rates are usually reciprocally proportional with complexity of the biomass, extensive characterization of the biomass should be done before selection of feeding regime for the biogas plants. There is useful information about biodegradability of agricultural substrates, manure and wastewater treatment sludge, that is reviewed by Lehtomaki et al. (2008), Labatut et al. (2011), Pabón-Pereira et al. (2012), and Raposo et al. (2011 and 2012).

2.5.5 Inhibitors

Hydrolysis inhibition is a result of activity loss and/or reduction of hydrolases. Hydrolase inhibitors mainly bind to the enzymes, hence influencing the activity of hydrolases. Inhibitors can bind to the enzymes, either reversibly or irreversibly. Reversible inhibitors bind to enzymes or enzyme substrate complexes with noncovalent bonds whereas irreversible inhibitors change the chemical structure of the enzymes. The effect of irreversible inhibition is more difficult to eliminate than reversible inhibition. Effective elimination of irreversible inhibition can only be achieved when the inhibitors are removed from the environment. Increasing the substrate concentrations or addition of components binding to the inhibitor can be a solution for reversible inhibition. Volatile fatty acids (VFA), ammonia, humic and fulvic acids (HA), and presence of different compounds can be the inhibitors of hydrolytic activity.

2.5.5.1 Volatile fatty acids

The inhibitory effect of VFA on hydrolysis is difficult to evaluate due to interactions between VFA concentrations and pH. Veeken et al. (2000) showed the effect of pH and VFA inhibition on organic solid waste hydrolysis. This study showed that hydrolysis inhibition was statistically more related to pH than VFA at concentrations up to 30 g L^{-1} COD (chemical oxygen demand) within pH range 5–7. Siegert and Banks (2005) showed that a VFA mixture, consisting of 18% acetic acid, 50% propionic acid, 5% *n*-butyric acid, 12% *iso*-butyric acid, 5% *n*-valeric acid, 5% *iso*-valeric acid, 2% caproic acid, and 3% heptanoic acid, inhibited the anaerobic cellulose and glucose digestion in batch reactors. They reported that independently from pH, VFA concentrations higher than 2 g L^{-1} caused up to 75% inhibition on cellulolytic activity. Another study by Romsaiyud et al. (2009) showed the VFA inhibition on enzymatically catalyzed cellulose hydrolysis. This study reported that acetic acid concentrations higher than 1.8 g L^{-1} inhibited hydrolysis at different pH values (pH 5–8).

In biogas plants, acetic acid, butyric acid and propionic acid are the common VFAs that accumulate in the reactor systems. Although VFAs have inhibitory effects on hydrolytic microorganisms, they also have a toxic effect on methanogens. The metabolism of methanogens is not affected by short chain fatty acid ≥ 10 g L^{-1}. However, they can be more sensitive to some VFAs, like propionic and butyric acid, of which the

concentration should not exceed 2 g L^{-1} (Inanc, 1999; van Lier et al., 1993). Therefore, VFA concentration in biogas plants is an important parameter, not only for hydrolytic microorganisms but also for methanogens.

2.5.5.2 Ammonia

Ammonia inhibition is mainly reported in manure digesters. According to our knowledge, van Velsen et al. (1979) was the first author that described ammonia inhibition on anaerobic digestion of pig manure within the mesophilic range. Later, Zeeman (1991) showed the ammonia inhibition on hydrolysis and methanogenesis during anaerobic cattle manure digestion within the mesophilic and psychrophilic range. Zeeman indicated that also other components, equally diluted with ammonia, like humic acids, could cause the observed hydrolysis inhibition. In 1993, Angelidaki and Ahring showed ammonia inhibition, when digesting manure with high ammonia content. They reported ammonia inhibition as the primary process controlling factor (Angelidaki and Ahring, 1993). Hansen et al. (1998) reported the inhibiting concentrations of ammonia on methanogenesis as 1.1 g N L^{-1} free ammonia for swine manure digestion within mesophilic and thermophilic range. El- Mashad (2004) showed the linear relationship between first order hydrolysis rate constant of liquid cow manure at 50°C and free ammonia concentrations in batch tests.

Recent work from Fernandes et al. (2012) reported contradictory results about anaerobic cellulose and tributyrin hydrolysis. In this research, no ammonia inhibition was found at mesophilic conditions, with free ammonia concentrations between 2.4–7.8 g N L^{-1}.

Although, the research, discussed in this section, showed the ammonia inhibition on hydrolysis and/or methanogenesis, inhibition mechanisms, effect of temperature, pH and VFA concentrations on ammonia inhibition are still unclear.

2.5.5.3 Humic and fulvic acids

HA are complex biomolecules that are produced as a result of decaying plant material. HA contain organic acids, mono/oligo/polysaccharides, proteins, peptides, amino acids, lipids, waxes, aromatic compounds, and lignin fragments (Saiz-Jimenez, 1993). HA are soluble at almost every pH and they behave like weak polyelectrolytes. Thus their removal from environments is hard to achieve (Tipping, 2002). Although, intensive

research is going on HA, there is still a lack of information on HA formation, composition and activity. Brons et al. (1985) described the inhibitory effects of HA on the hydrolysis of potato proteins. Almost 10 years later, Jahnel and Frimmer (1994) showed the inhibitory effects of different HAs on Pronase E activity on enzyme level. More detailed study from Fernandes et al. (2015) showed that HA extracted from cow manure and maize, inhibited the hydrolysis of cellulose and tributyrin. The results indicated that HA concentration as low as 0.5 g L^{-1} was enough for strong inhibition. Although inhibition mechanisms are not fully understood, binding properties of HA to enzymes were proposed for such an inhibition (Fernandes et al., 2015).

2.5.5.4 Other inhibitors

Microbiologically catalyzed hydrolysis acts based on the feedback mechanisms. Thus, hydrolysis end products, such as reducing sugars and alcohols can have inhibitory effects on hydrolysis (Gallert and Winter, 2005; Wu and Lee, 1997). It is known that absence of glucose can trigger hydrolytic activity, whereas high concentrations (10 g L^{-1}) repress hydrolytic activity (Gallert and Winter, 2005). Xiao et al. (2004) showed that glucose was the only reducing sugar that inhibited both cellulase and β-glucosidase activity, while other sugars, such as mannose, xylose, and galactose only repressed β-glucosidase activity. A recent study from Teugjas and Väljamäe (2013) concluded that cellobiose and glucose inhibition is mainly affecting cellobiohydrolases, GH 7 and 6. On the other hand, there are some lignin by-products that may act as inhibitors of microorganisms rather than the enzymatic hydrolytic activity. These by-products include phenolic and aromatic compounds, aliphatic acids, furan aldehydes, and inorganic ions (Jonsson et al., 2013). Additionally, iron and oxidative metal ion complexes are also reported as cellulase inhibitors, but the mechanisms underlying the inhibition are not clear (Liu et al., 2010; Tejirian and Xu, 2010).

In this section, environmental factors that affect the hydrolytic activity within biogas reactors were discussed. Figure 2.2 summarizes the effect of environmental conditions on hydrolytic activity in biogas plants, which are discussed in detail in different subsections. Although environmental factors were intensively studied, general knowledge about the observed effects was mainly obtained from bioreactor performance. For many years, microbial community changes with different

environmental and operational conditions remained unclear. There are few articles that discuss the microbial shifts during environmental disturbances and they do not contain conclusive discussions.

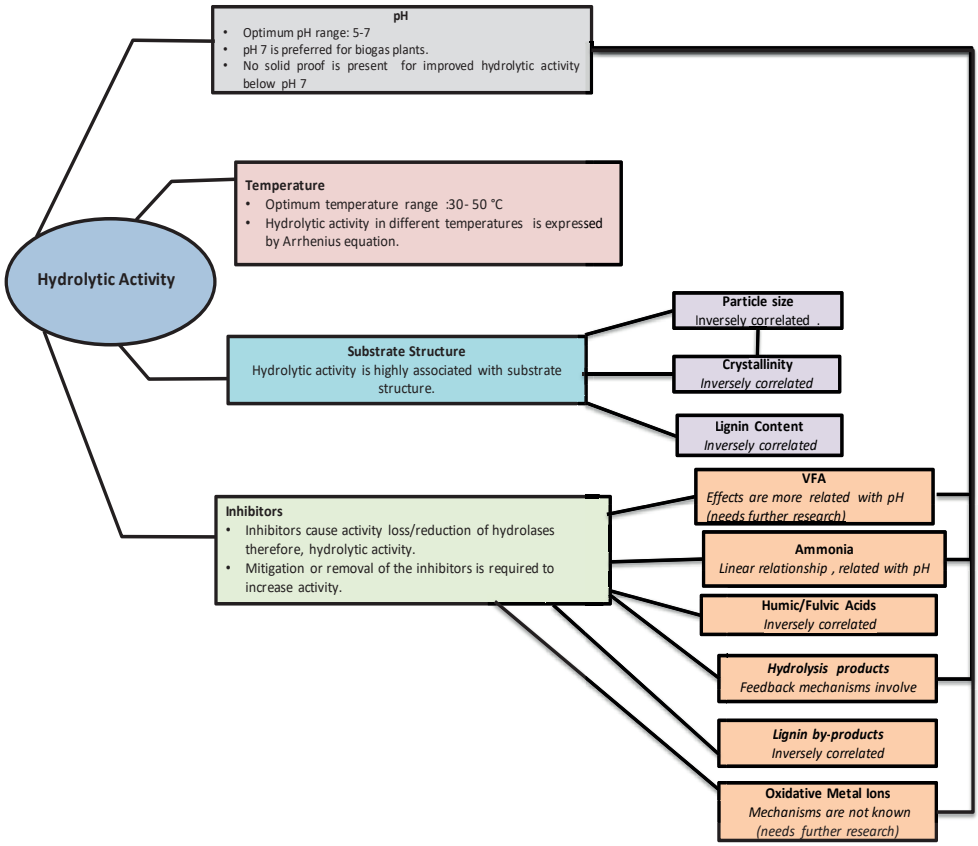

Figure 2.2 Effect of environmental parameters on hydrolytic activity in biogas plants.

Most of the authors focused on the deterministic factors of the anaerobic digestion such as, temperature, hydraulic retention time, organic loading rates on microbial community structures (Carballa et al., 2011; Nielsen et al., 2004; Rincón et al., 2008). Nielsen et al. (2004) proposed that hydraulic retention time and feedstock type had a major role in bacterial composition rather than temperature changes. However, Carballa et al. (2011) showed the correlations between bacterial and archaeal communities and changing environmental/operational conditions and they reported that changing temperature had a major role on microbial community shifts. On the

other hand, Rincón et al. (2008) showed that having lower organic loading rates can favour the growth of *Firmicutes*. Although these publications contributed valuable information, their sensitivity on detection of microbial communities can be questionable since these publications only used fingerprinting methods. Therefore, high throughput monitoring techniques should be used to reach more solid knowledge about the effect of environmental factors on hydrolytic bacteria. A recent publication from Luo et al. (2015) demonstrated that instead of stochastic factors, the temperature disturbances played an important role in shaping the *Firmicutes* and *Bacteriodetes* communities by using next generation sequencing. Another recent study from De Francisci et al. (2015) showed the effect of feedstock disturbance on the microbial ecology of biogas reactors. This study presented valuable information about to what extent change in feed composition affected the abundance of certain bacterial groups.

To conclude the effects of environmental factors on hydrolytic microorganisms, there is an emerging need to investigate how hydrolytic bacteria respond to environmental changes. New molecular methodologies should be used, in combination with reactor performance analyses, to obtain solid information to determine how environmental changes shape the microbial communities within biogas plants.

2.6 Conclusions

Biogas is becoming an important renewable energy source for the near future, not only because it provides low-cost green energy, but also because it is a substitute for fossil fuels. Current molecular microbiology techniques provide useful tools to study the abundance of the different hydrolytic microbes within the biogas plants. However, detailed knowledge on the physiology/ biochemistry of hydrolytic bacteria and their interactions with other microbes, involved in the anaerobic digestion processes are needed. Integration of this knowledge is important for efficient hydrolysis of lignocellulosic biomass and more biogas production. In addition, there are still many uncultured hydrolytic microorganisms, waiting to be explored for their potential utilization in efficient biomass conversion.

Chapter 3

Mitigation of humic acid inhibition on anaerobic digestion of cellulose by addition of various cations

This chapter is adapted from: Azman S, Khadem AF, Zeeman G, van Lier JB Plugge CM (2015) Mitigation of humic acid inhibition in anaerobic digestion of cellulose by addition of various salts. Bioengineering 2:54–65.

Abstract

Humic compounds are inhibitory to the anaerobic hydrolysis of cellulosic biomass. In this study, the impact of salt addition to mitigate the inhibitory effects of humic compounds was investigated. The experiment was conducted using batch tests to monitor the anaerobic hydrolysis of cellulose in the presence of humic acid. Sodium, potassium, calcium, magnesium and iron salts were tested separately for their efficiency to mitigate humic acid inhibition. All experiments were done under mesophilic conditions (30 °C) and at pH 7. Methane production was monitored online, using the Automatic Methane Potential Test System. Methane production, soluble chemical oxygen demand and volatile fatty acid content of the samples were measured to calculate the hydrolysis efficiencies. Addition of magnesium, calcium and iron salts clearly mitigated the inhibitory effects of humic acid and hydrolysis efficiencies reached up to 75%, 65% and 72%, respectively, which were similar to control experiments. Conversely, potassium and sodium salts addition did not mitigate the inhibition and hydrolysis efficiencies were found to be less than 40%. Mitigation of humic acid inhibition via salt addition was also validated by inductively coupled plasma atomic emission spectroscopy analyses, which showed the binding capacity of different cations to humic acid.

3.1 Introduction

Lignocellulosic biomass has been thoroughly studied for its energy potential, since there is an extensive effort to replace fossil fuels with renewable energy sources (Johnstone et al., 2010). Although, renewable energy from biomass can be produced by several processes, anaerobic digestion is one of the widely used processes to convert the chemically enclosed energy in the biomass to biogas (Appels et al., 2011). However, currently available technologies for anaerobic biomass digestion are not always efficient in converting biomass into biogas. Low biogas production within biomass digesters is mainly related to low hydrolysis rates and limited substrate biodegradability (Angelidaki and Ellegaard, 2003; Ma et al., 2013; Raven and Gregersen, 2007). Hydrolysis is the first step of anaerobic digestion in which complex molecules are converted to soluble monomers or/and oligomers. Hydrolysis is often considered as the rate limiting step in anaerobic digestion of biomass (Vavillin et al., 2008). This rate limitation phenomenon can be explained by the encrustation of biomass by lignin, and the presence of humic compounds (Fernandes, 2010; Gunaseelan, 1997; Jimenez et al., 1990; Pabón-Pereira et al., 2014)

Humic acids (HA) are complex mixtures of different organic molecules that are produced during decay and transformation of organic matter. HA are resistant to biodegradation but they can react physically and chemically with several compounds due to their weak polyelectrolyte behaviour at the same time. Because of the weak polyelectrolyte behaviour, HA can dissociate in aqueous solutions, and make several compounds partially charged. These properties make HA important components of soil, lake/sea sediments and anaerobic digester environments in which they affect the physicochemical properties such as; bio-availability of enzymes, metals and macro/micro nutrients, and biological processes (Saiz-Jimenez et al., 1993; Tipping, 2002). In an anaerobic digester environment, abundance and composition of HA mainly depend on the type of feed (Fernandes, 2010). Although, HA content within anaerobic digesters are not well defined in the literature, HA concentrations can reach up to 1.5% w/w of total solids in the treatment sludge, manure and maize (Fernandes, 2010; Li et al., 2014a; Rolando et al., 2011).

Fractions of HA can affect the biodegradation of biomass during anaerobic digestion since they strongly inhibit cellulose hydrolysis (Brons et al., 1985; Fernandes

et al., 2015). Although the exact mechanism of HA inhibition on hydrolysis is not known, binding properties of HA to hydrolytic enzymes are proposed for such an inhibition (Fernandes et al., 2015). Fernandes et al. (2015) hypothesized that binding of hydrolytic enzymes to HA lower the availability of enzymatic activity for cellulose hydrolysis. They observed strong inhibitory effects of HA on cellulose hydrolysis in batch tests. Thus, there is a need to reverse the inhibitory effects of HA on hydrolysis to improve cellulolytic biomass digestion. Consequently, to eliminate HA inhibition on cellulolytic biomass digestion, two approaches can be followed: (i) removal of the HA from the related environment and (ii) mitigation of the inhibitory effects by adding compounds that can reduce the binding capacity of HA. Removal of HA from aquatic environments has been successfully achieved by adding coagulants and flocculants to contaminated sites (Matilainen et al., 2010; Renou et al., 2008). Utilization of ion exchange resins was also reported as a successful method to remove HA from groundwater (Song et al., 2013). However, the increased solids content of biomass hampers the application of the aforementioned methods in anaerobic digesters. Alternatively, removal of HA by extraction methods can be considered as a solution in anaerobic digesters. A recent study showed that the extraction of HA via alkali pre-treatment methods from primary sludge increased the total methane yield by 50% (Li et al., 2014a). Although removal methods can be successful in lab-scale applications, their economic and practical feasibility for large scale applications is highly questionable. Therefore, mitigation strategies seem to have higher potential to overcome HA inhibition on hydrolysis during organic matter digestion, as mentioned in a few literature studies. In 1985, Brons et al. (1985) showed that the addition of Ca^{2+} cations reversed the inhibitory effects of humate on potato protein hydrolysis. More recently, Fernandes et al. (2015) proposed that the addition of excess amounts of hydrolytic enzymes may help to overcome HA inhibition. Although some methods were suggested to mitigate hydrolysis inhibition, detailed information was not available about mitigation of HA inhibition on anaerobic cellulo:In our present study, we aim to show the mitigation of HA inhibition on anaerobic cellulose digestion by adding several cations in salt form. Following the discussions of Fernandes et al. (2015), we hypothesized that reducing active enzyme binding sites of HA with cations may reverse the hydrolysis inhibition and subsequently

increase the methane production. In this scope, batch tests were set-up to monitor anaerobic digestion of cellulose in the presence of HA and salt addition was tested to find successful candidates to mitigate HA inhibition. During the experiment, hydrolysis efficiencies, methane yields and corresponding methane production rates were monitored with chemical oxygen demand (COD) and volatile fatty acids (VFA) analyses, to evaluate the utilization potential of Na^+, K^+, Ca^{2+}, Mg^{2+} and Fe^{3+} salts in mitigation of HA inhibition.

3.2 Materials and methods

3.2.1 Experimental set-up

Batch incubations were performed in 500 mL glass bottles, which contained 400 mL liquid anaerobic medium with the following composition in μM: 5000 Na_2HPO_4, 5000 KH_2PO_4, 5600 NH_4Cl, 680 $CaCl_2$, 600 $MgCl_2$, 5000 NaCl, 7.5 $FeCl_2$, 1 H_3BO_3, 50 HCl, 0.5 $ZnCl_2$, 0.5 $MnCl_2$, 0.5 $CoCl_2$, 0.1 $NiCl_2$, 0.1 Na_2SeO_3, 0.1 Na_2WO_4, 0.1 Na_2MoO_4 and vitamins ($\mu g \cdot L^{-1}$); 0.02 biotin, 0.2 nicotinic acid, 0.5 pyridoxine, 0.1 riboflavin, 0.2 thiamin, 0.1 cyanocobalamin, 0.1 p-aminobenzoic acid, 0.1 pantothenic acid. The bottles were inoculated with granular, methanogenic sludge from a full-scale Up-flow Anaerobic Sludge Blanket reactor (Eerbeek, the Netherlands). The reactor is treating pulp and paper industry waste water. Avicel PH-101 (Fluka) was chosen as model substrate at 1 $g \cdot L^{-1}$ COD (unless otherwise stated) and the ratio between substrate and microorganisms was set to 0.8 (g VS g VSS^{-1}) to obtain enough carbon for the inoculum (Angelidaki et al., 2007). All bottles were flushed with nitrogen gas prior to the start of the experiment.

The experiment bottles were set up in duplicates, including blank controls, positive controls, inhibition groups and salt addition groups. To the blank control, no carbon source was added, to determine the endogenous activity of the inoculum. In the positive controls, Avicel was added as a carbon source to determine net Avicel biodegradation. In the inhibition groups, 5 g L^{-1} HA (Sigma-Aldrich; CAS Number: 68131-04-4) was added to create an inhibitory environment for Avicel biodegradation (Fernandes et al.,2015). In salt addition controls, 5 mM of NaCl, KCl, $CaCl_2$, $MgCl_2$ and $FeCl_3$ were separately added to HA and Avicel containing experimental bottles to determine the effect of cation addition on HA inhibition and Avicel biodegradation.

Table 3.1 summarizes the experimental groups that were used in the whole experiment. The experiment was carried out for 14 days, at 30 °C. pH was set to 7 at the beginning of the experiment and all the experiments were conducted in between pH 6.8 and pH 7. Intermittent stirring was applied (60 s on and 60 s off at 90 rpm) to obtain efficient mixing in the experimental bottles. The first sampling was done immediately after all bottles were prepared. Then, sampling was done at four different times after 0, 96, 168 and 288 h. During the experiment, biogas production was monitored online by using Automatic Methane Potential Test System II (AMPTS II, Lund, Sweden). Soluble carbon content of the samples was determined by Chemical Oxygen Demand (COD) and Volatile Fatty Acid (VFA) analyses. Salt addition experiment bottles, were also analysed by ion chromatography, to determine the binding capacity of Na^{+}, K^{+}, Ca^{2+}, Mg^{2+} and Fe^{3+} to HA.

3.2.2 Monitoring methane production

Biogas production of the experimental groups, was measured online by the AMPTS II (Bioprocess Control, Lund, Sweden), and according to the protocol described by Badshah and co-workers (Badshah et al., 2012). Hourly recorded results were used to determine methane production and methane production rates. All measured gas volumes were normalized to standard temperature and pressure conditions (273 K, 1 atmospheric pressure and zero moisture content) and the results were corrected for the recorded values in the negative controls.

3.2.3 Analytical methods

Liquid samples were centrifuged (13,000× rpm, room temperature, 10 min), and the obtained supernatant was filtered through a polypropylene filter (Ø 0.45 µm). Supernatants that contained HA were first acidified with 1 M H_2SO_4 to pH 3 and subsequently centrifuged to remove HA. Acidified samples were neutralized to pH 7, prior to filtration. Samples without HA and VFA standards were treated using the same procedure. The filtered supernatant, was then analysed for VFA using a High Liquid Pressure Chromatography (Thermo Scientific Spectra System, HPLC), equipped with a Varian MetaCarb 67H column (300 mm × 6.5 mm), which was connected to a UV and refractive index detector (Middelburg, The Netherlands). The mobile phase and internal standard were 10 mM sulfuric acid and arabinose, respectively. The eluent had a flow of

0.8 mL min^{-1}. Data analyses, were performed with the ChromQuest (Thermo Scientific, Waltham, MA, USA) and Chromeleon software (Thermo Scientific, Waltham, MA, USA).

Table 3.1 Summary of the experimental set-up and abbreviations that are used in the text.

Experimental group	Abbreviation	Avicel g L^{-1} COD	HA g L^{-1}	Added salt (mM)
Blank (negative control)	_	0	0	0
Avicel (positive control)	**C**	1	0	0
Avicel+ HA (inhibition Control)	**I**	1	5	0
Avicel+HA+ KCL (Salt addition control)	**K**	1	5	5
Avicel+HA+NaCl (Salt addition control)	**Na**	1	5	5
Avicel+HA+ $CaCl_2$ (Salt addition control)	**Ca**	1	5	5
Avicel+HA+$MgCl_2$ (Salt addition control)	**Mg**	1	5	5
Avicel+HA+ $FeCl_3$ (Salt addition control)	**Fe**	1	5	5

Soluble COD analyses were done with COD cell kits (Spectroquant, 14-541) from Merck (Darmstadt, Germany), according to the manufacturer's instructions. The efficiency of hydrolysis, acidogenesis and methanogenesis was calculated using Equations (1–3), in which H is the hydrolysis efficiency (%) corrected for the soluble COD fraction at the start of the experiment, A is the acidogenesis efficiency (%) and M is the methanogenesis efficiency (%); $COD_{m,t=x}$ is methane expressed as COD (t = time; x = sampling time). $COD_{s,\ t=x}$ is the soluble COD at t = x, $COD_{v,\ t=x}$ is the VFAs at t = x and $COD_{total,\ t=0}$ is the total COD added at the beginning of each experiment.

$$H(\%) = \frac{CODm\ ,t=x\ + CODs,t=x - CODs,t=0}{COD\ total,t=0} * 100 \quad (1)$$

$$A\ (\%)\ = \frac{CODm\ ,t=x + CODv,t=x}{COD\ total,t=0} * 100 \quad (2)$$

$$M\ (\%)\ = \frac{CODm\ ,t=x}{COD\ total,t=0} * 100 \quad (3)$$

Solubilized substrate, at t = 0 was removed from the equation to assess specifically the hydrolysis of the particulate matter. Equation (1), which calculates the actual hydrolysis efficiency (H), was used for this assessment.

Volatile solid content of the substrates (VS), Volatile Suspended Solids (VSS) content of the inoculum and pH values were determined, using standard methods (APHA, 2005).

For the ion chromatography analyses (Na^+, K^+, Ca^{2+}, Mg^{2+} and Fe^{3+} were measured), liquid samples were centrifuged (10,000× g, RT, 5 min), and were subsequently measured (with technical triplicates of duplicate samples) by Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES) using a Vista MPX ICP-AES instrument.

3.3 Results and discussion

3.3.1 Hydrolysis, acidogenesis and methanogenesis efficiencies

Hydrolysis, acidogenesis and methanogenesis efficiencies of the experimental groups (Figure 3.1) were calculated with the formulas that were given in analytical methods section. During the experiments, VFA production was detected in most of the samples. The amount of detected VFAs was relatively low, i.e., less than 20% of the overall COD or VFAs were not detected at all, likely due to the rapid conversion of VFAs to methane. Acetate and propionate were found as the dominant VFAs. Results from the positive controls showed that hydrolysis was almost completed within the first 170 h of the experiment. Hydrolysis and methanogenesis efficiencies were calculated as 78% which are commonly found for crystalline cellulose (Avicel) with the selected inoculum concentrations (O'Sullivan et al., 2008). Complete degradation of VFAs accompanied by hydrolysis in positive controls indicated efficient digestion profiles for the cellulose (Figure 3.1 a). In the inhibition experimental group, hydrolysis efficiency was reduced by 50%, compared to the positive controls which showed the HA inhibition (Figure 3.1 b). The hydrolysis efficiency for the inhibition groups was 40% higher than previously reported (Fernandes et al.,2015). The main reason for the higher hydrolysis efficiency might be related to the type of HA (HA extracted from maize and manure) that was used. It is known that different types of HA have different effects due to the source of HA source and the extraction methods used (Fernandes, 2010). On the other hand, VFA

accumulation was observed in the inhibition groups, which indicated that HA possibly inhibited methanogenesis. The observed negative effects of HA on methanogenesis was previously reported by Brons and co-workers. They observed a significant delay in the methane production during potato protein digestion, in the presence of humate (Brons et al., 1985).

In the salt addition groups, calcium, magnesium and iron salts mitigated the inhibitory effect of HA on hydrolysis. In the Ca, Mg and Fe salt addition groups, hydrolysis efficiencies were 75%, 65% and 72%, respectively after 300 h incubation (Figure 3.1 c–e). In addition to hydrolysis efficiencies, acidogenesis efficiencies were slightly higher than methanogenesis efficiencies that indicated a delay in methanogenesis which was recovered at the end of the experiment. Recovered hydrolysis, acidogenesis and methanogenesis, compared to positive controls, at the end of the experiment revealed the positive effect of addition of Ca, Mg and Fe salts. The overall results showed that addition of calcium, magnesium and iron salts mitigated hydrolysis inhibition, most probably, by shielding or attachment to the active binding sites of the HA. Apparently, reducing the number of active binding sites prevented scavenging of hydrolytic enzymes from the liquid media that consequently improved the cellulose hydrolysis and therefore methanogenesis (Fernandes et al., 2015).

On the other hand, sodium and potassium salts did not mitigate the hydrolysis inhibition (Figure 3.1 f,g, respectively). In these salt addition groups, hydrolysis efficiency was 30% which was slightly lower than the hydrolysis efficiency of inhibition groups even though they were expected to show similar results. This may indicate the possible inhibition caused by the high concentration of monovalent sodium and potassium cations (Kugelman and Mc Carty, 1965). Thus, sodium and potassium salts were not effective to diminish the inhibitory effect of HA.

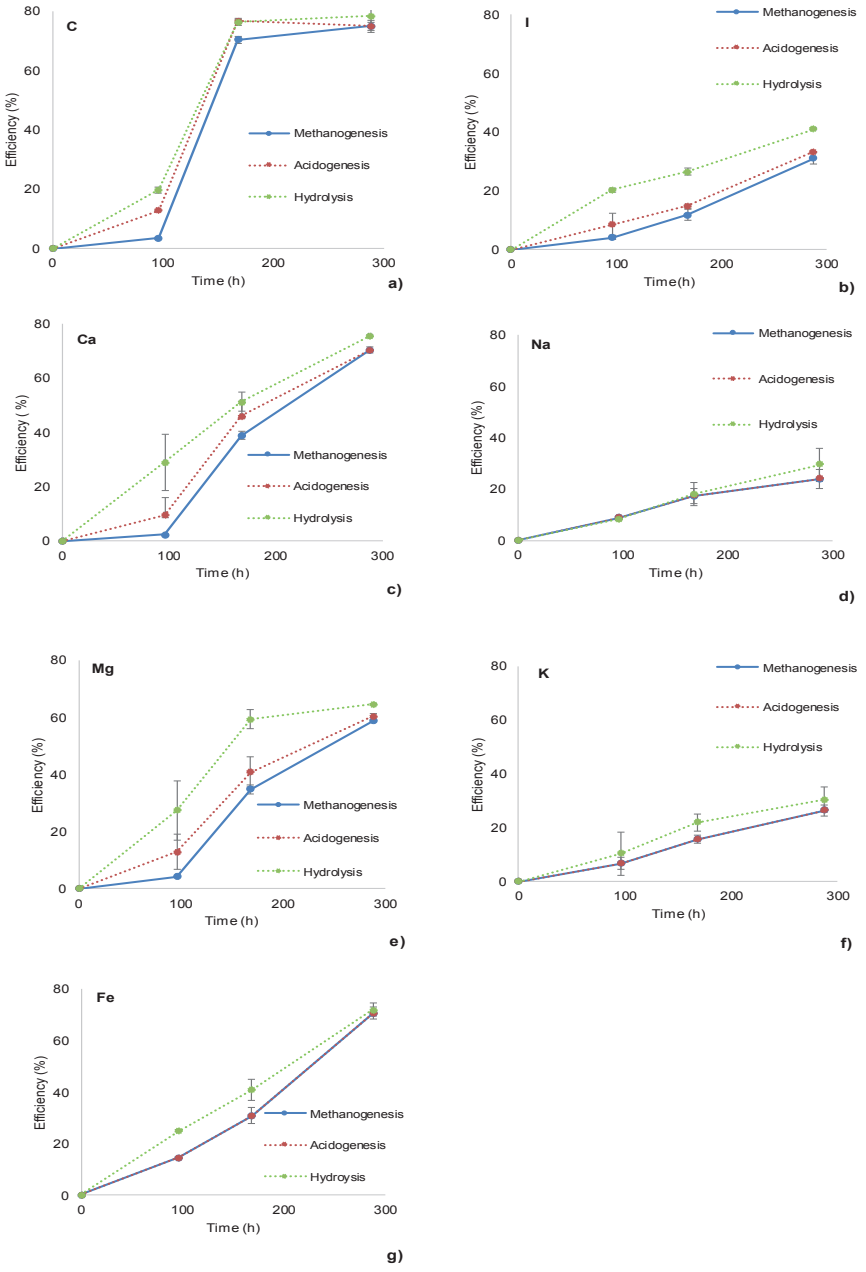

Figure 3.1 Salt addition experiments, namely efficiency (%) of methanogenesis, acidogenesis and hydrolysis over time. Results of each experimental group is illustrated with; (a) positive control (C), (b) inhibition control (I), salt addition experiment group of; (c) calcium (Ca), (d) sodium (Na), (e) magnesium (Mg), (f) potassium (K) and (g) iron (Fe) salts. (Error bars show the standard deviation between measurements, n = 2).

3.3.2 Methane yield and methane production rates

The effects of HA on anaerobic digestion of cellulose (Avicel) and strategies to overcome HA inhibition were evaluated in terms of methane yield and methane production rates. In Figure 3.2, the overall methane yield, achieved for the cellulose digestion within the experimental groups is shown. Avicel degradation yielded 310 mL CH_4 g VS^{-1} at the end of the experiment which was previously found for this type of cellulose (Raposo et al., 2011). However, HA addition decreased the methane yield three folds, which showed the strong inhibitory effect of HA on the anaerobic digestion of cellulose. Since HA inhibition was mitigated with the addition of calcium, magnesium and iron salts, consequently, methane yield was recovered to 295 mL, 273 mL, and 294 mL CH_4 g VS^{-1}, respectively. The sodium and potassium salt additions did not improve the methane yield.

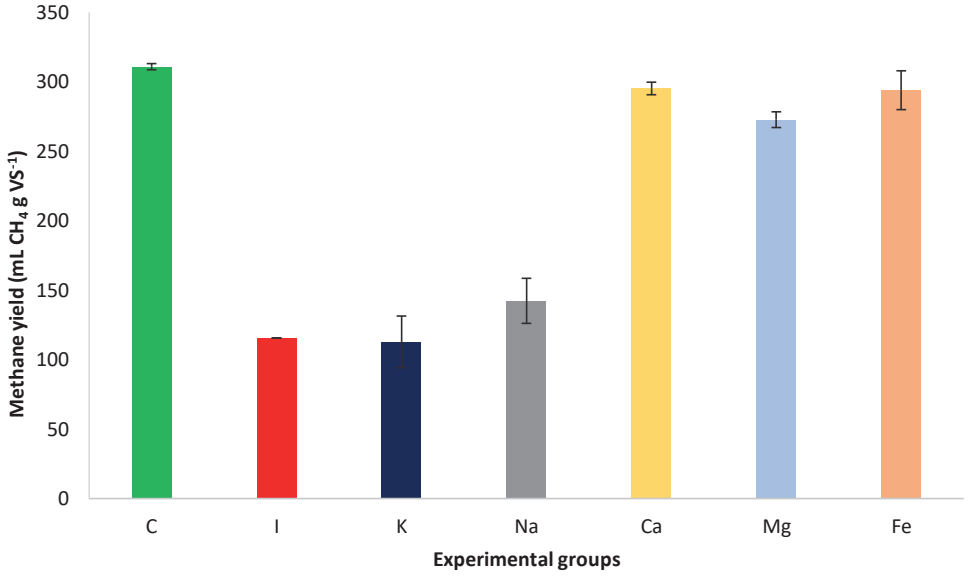

Figure 3.2 Methane yield (mL CH_4 g VS^{-1}) of the positive control (C), inhibition control (I) and salt addition controls; with calcium (Ca), magnesium (Mg), iron (Fe), sodium (Na) and potassium (K). Yields were calculated at the end of the anaerobic degradation tests (14 days). (Error bars show the standard deviation between measurements, n = 2).

Methane production rates were assessed in the positive controls, inhibition groups and salt addition groups to determine the corresponding maximum methane production rates (Figure 3.3). The first activity peaks were observed in the first 50 h, which can be attributed to the conversion of easily degradable substrates, such as residual glucose in Avicel powder (Figure 3.3). The second activity peaks were observed between 100 and 300 h, and were related with cellulose hydrolysis (Figure 3.2). No clear second peaks were found in the Na and K salt addition groups, indicative of low hydrolysis activity. The positive controls showed the highest methane production rate (1 mL CH_4 g VSS^{-1} h^{-1}, on hour 168). In the presence of HA, maximum methane production rate was 0.3 mL CH_4 g $VSS^{-1}h^{-1}$, showing once more the inhibitory effect of HA. In addition to methane yields, Ca, Mg and Fe salts increased the maximum methane production rates to 0.92 mL, 0.85 mL and 0.75 mL CH_4 g $VSS^{-1}h^{-1}$, respectively. However, maximum production rates in salt addition groups were still lower than in the positive controls, and it took a longer amount of time to reach the maximum activity.

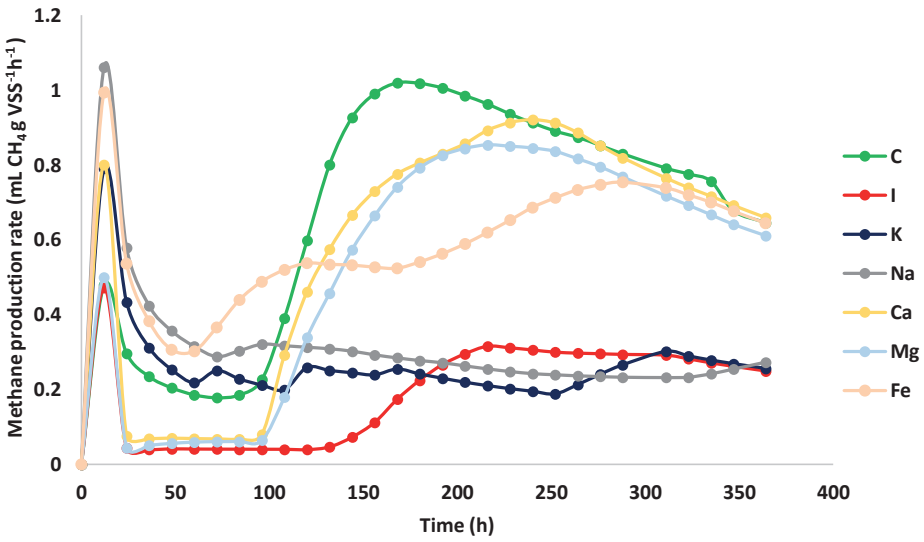

Figure 3.3 Maximum methane production rates of positive control (C), inhibition group (I) and salt addition groups; of calcium (Ca), magnesium (Mg), iron (Fe), sodium (Na) and potassium (K).

3.3.3 Effects of salt addition

The liquid phase of all experimental groups was analyzed by ICP-AES for Na, K, Ca, Mg and Fe to validate the interaction of HA with the respective cations. The cation concentrations were analysed at the beginning (initial = i) and at the end (final = f) of the experiment (Table 3.2). HA addition to the inhibition groups introduced a significant amount of sodium (approximately 300 mg L^{-1}) and a small amount of calcium and iron to the anaerobic media, when compared with the positive controls (Table 3.2). Although an excess of sodium could potentially inhibit anaerobic digestion, sodium in HA was still 10-folds lower than inhibitory sodium concentrations that were previously reported (Chen et al., 2008; Kugelman and Mc Carty, 1965; Liu et al., 1991). Moreover, the presence of the other cations such as potassium, magnesium and calcium, are likely to show antagonistic effects to sodium inhibition (Kugelman and Mc Carty, 1965).

The removal of cations from the liquid phase seems to be directly related with the interaction of cations with HA. The proposed interaction is more likely in terms of ionic binding with the formation of HA-cation complexes.

Table 3.2 Cation concentrations of the experimental groups in the beginning (initial = i) and at the end (final = f) of the experiment. Results of each experimental group abbreviated with; positive control (C), inhibition groups (I), salt addition groups of calcium (Ca), magnesium (Mg), iron (Fe), sodium (Na) and potassium (K).

	Cations (mg L^{-1})				
Samples	**K^{+}**	**Na^{+}**	**Ca^{++}**	**Mg^{++}**	**Fe^{++} + Fe^{+++}**
C-i	116.03	217.4	24.81	12.947	0.001<
C-f	85.6±0.44	160.71±0.26	14.51±0.38	10.42±0.01	0.001<
I-i	166.11	525.97	85.33	13.54	32.05
I-f	137.47±4.03	523.22±17.8	91.58±1.35	13.47±0.55	32.85±1.71
K-i	306.515	514.01	60.165	12.168	23.437
K-f	298.67±0.29	494.31±0.28	61.83±3.2	12.80±0.78	31.31±2.9
Na-i	141.59	717.24	80.57	13.1	25.46
Na-f	142.1±0.26	679.24±11	82.81±0.12	14.44±0.4	37.35±0.4
Ca-i	128.63	501.2	**285.08**	6.48	**32.05**
Ca-f	123.71±1.71	470.98±9.16	**32.63±0.93**	4.89± 0.11	**0.27±0.01**
Mg-i	127.87	517.42	**99.331**	134.958	**32.047**
Mg-fi	107.3±2.24	424.56±8.56	**9.51±0.24**	117.31±0.3	**0.001<**
Fe-i	124.95	688.05	68.40	10.84	**347.38**
Fe-f	145.39±0.52	643.30±3.69	89.59±0.04	15.35±0.21	**55.44±1.50**

The cation binding and complexation with HA can be explained by the model that was described by Tipping et al. (2011). According to this model, binding of cations to HA takes place at discrete sites of binding domains of HA by electrostatic attraction. Also, counter ion accumulation in the environment promotes non-specific binding of cations to HA. Therefore, the strength of the binding depends on the valence of the cations. In this respect, divalent or trivalent cations have more affinity for HA (Tipping et al., 2011). The results from this study also validated the aforementioned theory (Tipping, 2002; Tipping et al., 2011). HA formed precipitates with calcium and magnesium cations whereas sodium and potassium did not form precipitates. In the Fe groups, the precipitation was not observed even though iron has a higher valence than the other cations. Loose binding between iron and HA can be explained by the chemical reduction of Fe^{3+} to Fe^{2+} in anaerobic environments (Weber et al., 2006). Because of this reduction, strong Fe^{3+}-HA bonds might be converted to weaker Fe^{2+}-HA binding (Tipping, 2002). The other weak binding, observed with sodium and potassium, was not very surprising, since these cations are recognized as deflocculating agents (Kara et al., 2008). Therefore, they probably prevented binding via increasing the repulsion between the HA and cations.

3.4 Conclusions

Methane potential and hydrolysis efficiencies of cellulose are noticeably decreased in the presence of HA. In the present study, it was demonstrated that it was possible to reduce the active sites of HA with salt addition, mitigating the inhibitory effects of HA on the hydrolysis and consequently on the methane yields. Compared to HA inhibited groups, calcium, magnesium and iron salt addition increased the methane yields by 60% and increased the hydrolysis efficiencies by 30%, whereas sodium and potassium salts addition did not mitigate HA inhibition. Even though calcium and magnesium mitigated the HA inhibition, the affinity of each cation to HA was not the same. Binding capacity of the cations to HA increased in order $K^+ = Na^+ < Mg^{2+} < Fe^{3+}$ (Fe^{2+}) $< Ca^{2+}$. Although the proposed method was successful in batch tests, it is worth testing the effects of calcium or magnesium salts to continuously fed anaerobic bioreactors, treating high solid content residues.

Chapter 4

Effect of humic acids on the activity of pure and mixed methanogenic cultures

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Submitted for publication (*) Contributed equally

Abstract

The impact of humic acid (HA) on methanogenic activity was investigated. Methanogenic, crushed granular sludge and pure cultures of mesophilic methanogens were incubated in batch cultures with HA. Initial methane production rates and substrate consumption rates were quantified. In the presence of 1 g L^{-1} HA, the methane production rate of all hydrogenotrophic methanogens was inhibited by more than 75%, except *Methanospirillum hungatei* that was not inhibited up to 5 g L^{-1} HA. The acetoclastic *Methanosarcina barkeri* was completely inhibited by HA ≥1 g L^{-1}. However, *Methanothrix concilii* was only slightly affected by HA up to 3 g L^{-1}. When methanogenic granular sludge was incubated with HA, the specific methanogenic activity (SMA) tests showed less inhibition, when compared to the pure cultures of methanogens. The SMA test with H_2/CO_2, formate and acetate showed inhibition in initial methane production rate of 42%, 23% and 40%, respectively. Differences in HA susceptibility were explained by differences in cell wall structure.

4.1 Introduction

Anaerobic digestion (AD) is one of the most promising technologies for biogas and green chemical production. The AD process involves four steps, i.e. hydrolysis, acidogenesis, acetogenesis and methanogenesis that are mediated by specialized groups of microorganisms. During AD of biomass, complex molecules are broken down to simple molecules (hydrolysis) and consequently volatile fatty acids and hydrogen are produced (acidogenesis and acetogenesis). In the last step, methanogens metabolize the produced hydrogen, carbon dioxide/formate, acetate and some methylated compounds to methane (Mao et al., 2015).

Methanogens are strictly anaerobic archaea that have diverse morphology and phylogeny. Their ecological niches are widely distributed. They can be found in aquatic sediments (marshes and swamps), stagnant soil (peat bogs and rice fields), marine geothermal vents, the digestive tract of animals (ruminants and termites) and in engineered anaerobic digesters. Physiologically, methanogens are divided in acetoclastic, hydrogenotrophic and methylotrophic groups (Liu and Whitman, 2008). In anaerobic digesters, abundance and activity of methanogens is crucial for optimal biogas production. Therefore, it is important to establish environmental and operational conditions in biogas reactors that support optimal methanogenic activity and growth. However, methanogens are often considered the microorganisms most vulnerable to inhibition within the AD processes. A wide range of organic compounds, such as long chain fatty acids, aromatic compounds, xenobiotics, and inorganic compounds such as ammonia and heavy metals have been found to inhibit the methanogenic activity (Chen et al., 2008; Azman et al., 2015a)

Lignin derived compounds, such as resin acids, volatile terpenes, triterpenes, triterpenols, apolar phenols and humic acids-like and fulvic acids-like molecules are reported to inhibit methanogenic activity (Sierra-Alvarez and Lettinga, 1990). Humic acids (HA) are charged polyelectrolyte complexes due to the presence of carboxylic, phenolic, ketonic, aromatic and aliphatic groups and interact with both living and non-living matter (Steinberg et al., 2008). They can function as electron shuttles in anaerobic environments for fermenting-, iron reducing- and sulphate reducing bacteria, as well as for methanogenic archaea (Benz et al., 1998; Cervantes et al.,2008; Klüpfel et al., 2014; Minderlein and Blodau, 2010; Zhou et al., 2014). In an anaerobic digester

environment, abundance and composition of HA mainly depend on the type of the feed (Klüpfel et al., 2014). Although, HA content within anaerobic digesters are not well defined in the literature, HA concentrations can reach up to mass fraction of 1.5% of total solids in the treatment sludge, manure and maize (Fernandes et al.,2015; Klüpfel et al., 2014). Inhibitory effects of HA on hydrolysis was reported by Brons et al. (1985), Fernandes et al. (2015) and Azman et al. (2015b). The mechanism of inhibition of methanogenesis is still not clear, but it has been hypothesised that the redox active HA can act as a terminal electron acceptor and may competitively suppress the electron transfer to carbon dioxide, thereby reducing the methane production (Klüpfel et al., 2014). Recently, Azman et al. (2015b) observed a decrease in methanogenic activity in the presence of HA, confirming observations by Brons et al. (1985). From these experiments it was not evident whether the methanogens were affected and if so, which physiological group/phylotype of methanogens were most vulnerable to HA inhibition.

In this study, the effect of HA on methanogenic activity was investigated. Batch tests were performed with pure cultures of methanogens and with anaerobic crushed methanogenic granular sludge from a full scale Upflow Anaerobic Sludge Blanket (UASB) reactor treating paper mill wastewater.

4.2 Materials and methods

4.2.1 Experimental set-up

The effect of humic acid (CAS Number 68131-04-4, Sigma-Aldrich, Zwijndrecht, The Netherlands) on mesophilic methanogens was investigated in batch tests. Crushed mesophilic anaerobic granular sludge and pure cultures of methanogens were tested. Batch incubations were performed in 120 mL bottles with 50 mL bicarbonate buffered mineral salts medium, supplemented with cysteine (4 mM), trace elements and a vitamin mixture. Additionally, 2 mM acetate was added to the hydrogenotrophic cultures (also when grown on formate) as additional carbon source (Plugge, 2005; Stams et al., 1993). The bottles were inoculated with 10% (v/v) of a culture pre-grown on the same substrate. Depending on the metabolic property of the strain, the growth substrates were H_2/CO_2 (80%/20%, v/v at 1.5 bar), 1 mmol formate (final concentration: 20 mM) or 1 mmol acetate (final concentration: 20 mM), the latter two having a headspace of N_2/CO_2; 80%/20%, v/v at 1.5 bar. In the assays 0, 1, 3 and 5 g L^{-1} humic acid were tested, unless stated otherwise. The batch incubations were performed in

duplicate and in the dark at 37°C, pH 7. Methane (CH_4) production and hydrogen (H_2) consumption were monitored by gas chromatography. Liquid samples were collected to measure changes in acetate and formate concentrations.

4.2.2 Growth conditions of methanogenic cultures and anaerobic sludge

In this study, *Methanothrix concilii* (DSM 2139), *Methanosarcina barkeri* (DSM 800), *Methanobacterium formicicum* (DSM 1535), *Methanospirillum hungatei* (DSM 864) and *Methanobrevibacter arboriphilicus* (DSM 744) were used as pure cultures. All cultures were routinely grown at 37°C in an anaerobic bicarbonate buffered medium (Plugge,2005; Stams et al., 1993). Three subsequent transfers of each strain were made to ensure optimum growth conditions in the defined medium. After successful transfers, the microorganisms were used in the batch activity tests.

Granular methanogenic sludge was obtained from a UASB reactor treating pulp and paper industry effluents (Industriewater Eerbeek, The Netherlands). Sludge samples were collected on 10th of April, 2014. Immediately after collection, granules were crushed under nitrogen gas flow in a 500-mL serum bottle that contained 250 mL phosphate saline buffer solution (0.1 M, pH 7). The slurry obtained was transferred to a 500-mL serum bottle and flushed with nitrogen gas. About 5 mL of the prepared slurry (1g L^{-1} volatile solids) was used for the batch activity tests.

4.2.3 Analytical methods

4.2.3.1 Gas measurements

CH_4 and H_2 content of the gas phase was analyzed with a Shimadzu GC-14B gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a 2 m long, 3 mm internal diameter and 60–80 mesh packed column (Molsieve 13X) (Varian, Middelburg, The Netherlands). The column had a thermal conductivity detector that was operated at 70 mA, 150°C. Argon was the carrier gas at a flow rate of 30 ml.min^{-1}. Gas samples (0.2 ml) were taken by syringe and the gas content was expanded to 1 ml while the needles were in the rubber stopper, and injected to the column. All measurements were performed in duplicate and data was analysed using ChromQuest software (Thermo Scientific, Waltham, MA).

4.2.3.2 Organic acid measurements

Liquid samples were collected to determine acetate and formate concentrations. Liquid samples were centrifuged (10,000 × *g*, room temperature, 10 min) and filtered through a polypropylene filter (0.45 µm). The obtained supernatants were analysed by Thermo Scientific Spectrasystem HPLC system, equipped with a Varian Metacarb 67H 300 × 6.5 mm column kept at 45°C, running with 0.005 M sulphuric acid as eluent. The eluent had a flow rate of 0.8 mL min^{-1}. The detector was a refractive index detector. Data was analysed using ChromQuest (Thermo Scientific, Waltham, MA).

4.3 Results and discussion

4.3.1 Effect of humic acid on methanogenic cultures

For all methanogenic pure cultures used in this study, the recovery of reducing equivalents in the form of CH_4, produced from H_2/CO_2, acetate and formate, was always higher than 85% (Figure 4.1).

4.3.1.1 Hydrogenotrophic methanogenesis

When *Methanobacterium formicicum* was grown on formate in the absence of HA, the maximum total amount of methane (0.2 mmol) was produced within one day (Figure 4.2 a). In the presence of HA, methane was also produced, but after a long lag phase of 20 days (Figure 4.2 b). The duration of the lag phase was similar for the cultures grown with different HA concentrations. During the lag phase, accumulation of trace amounts of H_2 was observed (around 0.027-0.035 mmol, Table S1). After day 20, the trace amounts of H_2 started to be consumed, which coincided with methane production, reaching the same level as the control (Figure 4.2 b). The observed initial CH_4 production rate was lower at higher HA concentrations (Table S1). The occurrence of H_2 production from formate may be a physiological response of *M. formicicum* to the presence of HA. It is known that H_2 formation by some methanogens is enhanced when the ambient H_2 concentration becomes low (Schauer and Ferry, 1980; Valentine et al., 2000; Wu et al., 1993). In this respect, the presence of HA can create a stress condition that inhibits the methanogenic process after formate cleavage.

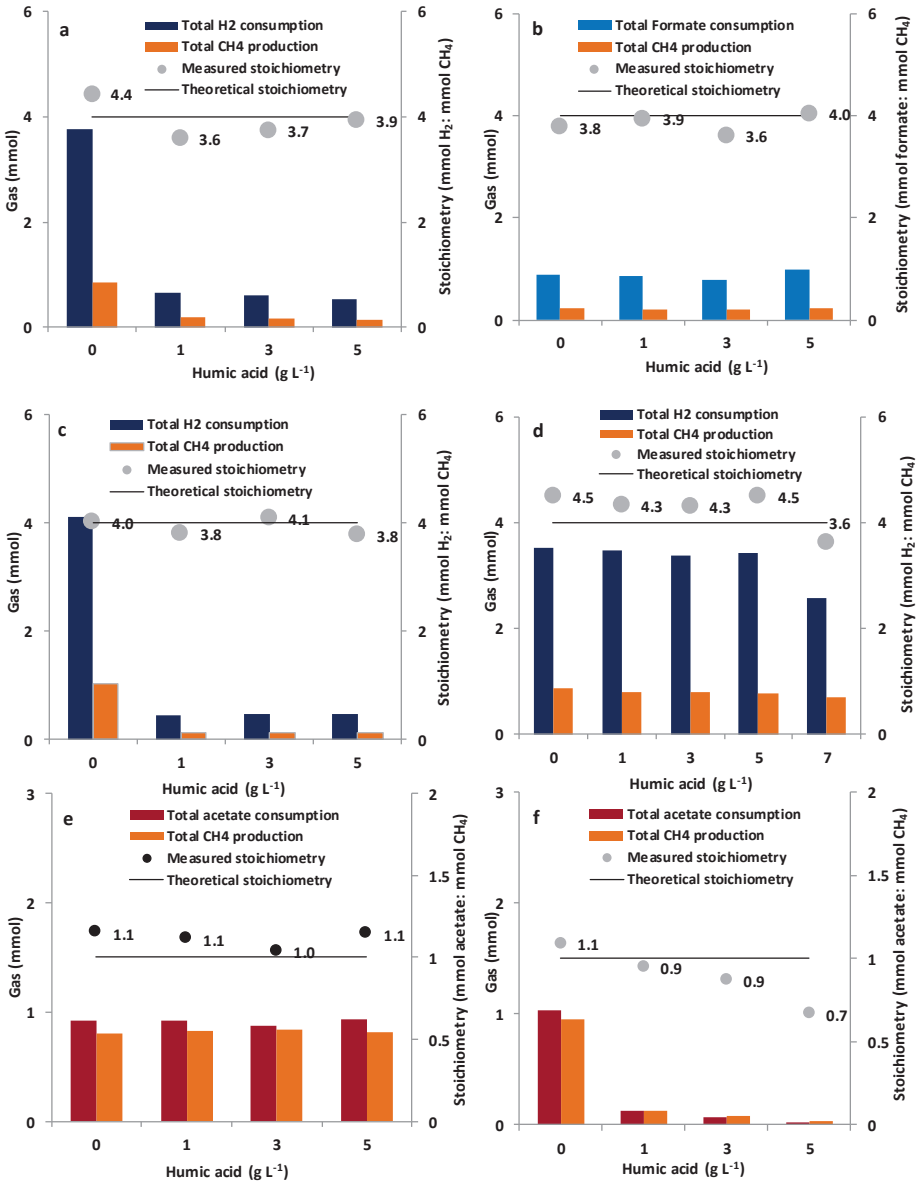

Figure 4.1 The observed averaged stoichiometry of methanogenesis in the presence and absence of humic acid. The observed stoichiometry of *Methanobacterium formicicum* incubated with H_2/CO_2 (a) and with formate (b). The observed stoichiometry of *Methanobrevibacter arboriphilicus* (c) and *Methanospirillum hungatei* (d), both incubated with H_2/CO_2. The observed stoichiometry of *Methanothrix concilii* (e) and *Methanosarcina barkeri* (f), both fed with acetate.

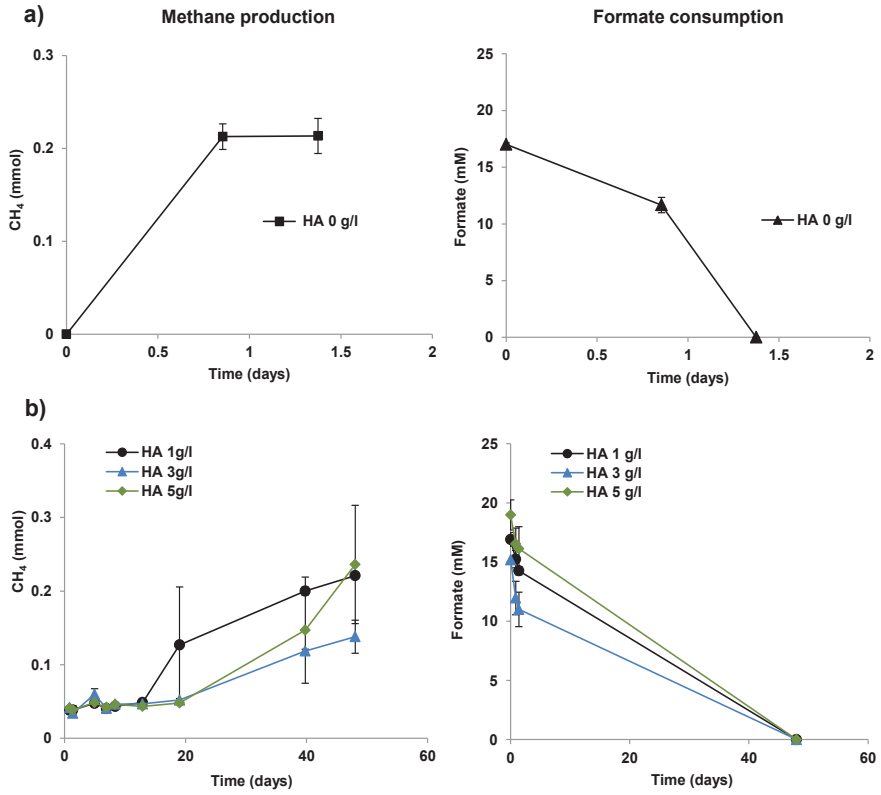

Figure 4.2 Effect of humic acid (HA) on the methanogenic activity of *Methanobacterium formicicum*, fed with formate. Left panel: CH_4 production over time in the absence (a) and in the presence (b) of HA. Right panel: the corresponding formate consumption.

Methane production started after a relatively long lag phase when apparently sufficient excess reducing equivalent in the form of H_2 was obtained.

In batch incubations of *M. formicicum* with H_2/CO_2, methanogenic activity was inhibited at HA concentrations ≥ 1 g L^{-1}. Addition of 1, 3 or 5 g L^{-1} HA to the bottles resulted in a slow linear methane production (Table S1). In the absence of HA, this culture produced 0.85 mmol methane within 18 days (Figure 4.3 a). The total amount of produced methane at the end of the experiment was reduced by 79, 81 and 84% at 1, 3 and 5 g L^{-1}, respectively (Table S1).

HA was also inhibitory to *Methanobrevibacter arboriphilicus* that was grown on H_2/CO_2. In the absence of HA, methane was produced at a linear rate and 1.02 mmol of methane was produced at the end of the experiment (Figure 4.3 b, Table S1). In the presence of HA, the total amount of methane produced was reduced by 89% and reduced methane production rates were observed for all tested HA concentrations (Table S1). As was the case for *M. formicicum*, HA was already inhibitory at 1 g L^{-1} for *M. arboriphilicus*.

By contrast, *Methanospirillum hungatei* was not much affected by the presence of HA (Figure 4.3 c). In the absence of HA, 0.81 mmol methane was produced within 8 days. In the presence of 1 g L^{-1} of HA, the total amount of produced methane was reduced by 9% and even at HA concentration of 7 g L^{-1} the total amount of methane produced was only reduced by 13% (Table S1). The overall results showed that activity of *M. hungatei* was not much affected by the presence of HA.

An explanation for this lack of inhibition might be the complex, proteinaceous impermeable envelope layer (the sheath) of *M. hungatei*, which might prevent HA to penetrate inside the cells (Albers and Meyer, 2011). In contrast, *M. formicicum* has a much thinner pseudomurein surface envelope. Experiments by Prokhotskaya and Steinberg (2007) on the effect of HA on cyanobacteria and eukaryotic algae support this hypothesis. Cyanobacteria were more susceptible to HA inhibition than eukaryotic algae, because of their difference in cell wall structure. The observed inhibitory effects of HA on *M. formicicum* and *M. arboriphilicus* may be explained by the accumulation of HA inside the cells. Once HA is concentrated inside the cells, electron transport system of the methanogens might be altered due to the negative charge and the electron shuttling properties of the HA. Alternatively, reducing equivalents inside the cells might be transported through the cell membrane to the exterior of the cells, where HA acts as an electron acceptor. Such potential losses of reducing equivalents will suppress microbial growth. However, at present it is not clear which reactions or enzymes in the cell are affected by HA.

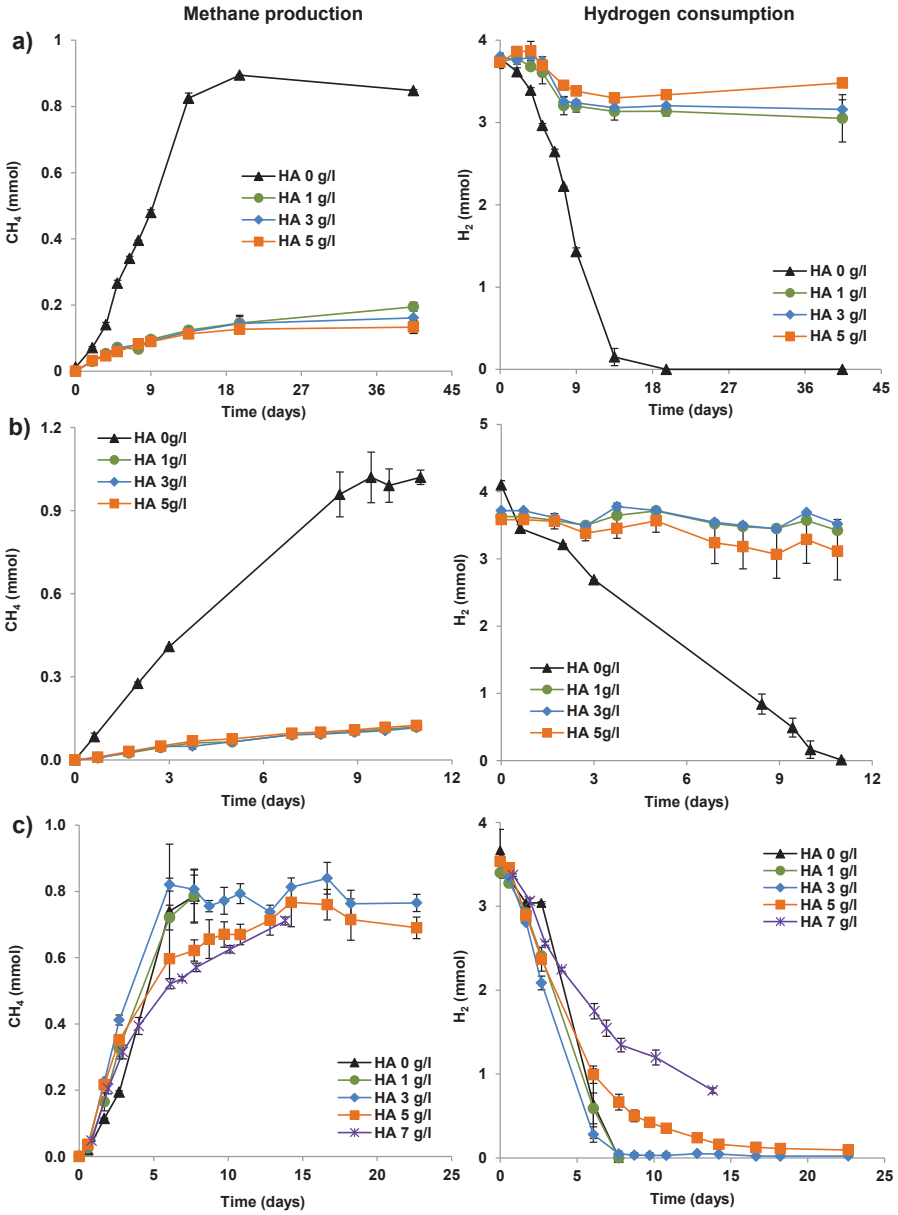

Figure 4.3 Effect of humic acid on methane production of a) *Methanobacterium formicicum*, b) *Methanobrevibacter arboriphilicus* and c) *Methanospirillum hungatei*. Left panel: CH_4 production over time. Right panel: the corresponding H_2 consumption.

4.3.1.2 Acetoclastic methanogenesis

Methanothrix concilii grown on acetate was not much affected by HA. With all tested conditions, methane production reached 0.8 mmol after 20 days and acetate was completely converted (Figure 4.4 a). Furthermore, the CH_4 production rate was not strongly affected by HA (Table Sl). In contrast, the acetoclastic activity of *Methanosarcina barkeri* was strongly affected by the presence of HA (Figure 4.4 b and Table Sl). At HA concentrations of 1, 3 and 5 g L^{-1}, the total amount of methane decreased with 86, 92 and 96%, respectively (Table Sl). Inhibition of methane production from acetate by *M. barkeri* in the presence of the anthraquinone-2, 6-disulfonate (AQDS) was also observed by Bond and Lovley (2002). However, in that study Fe (III)-containing growth media were used and it was not clear whether AQDS was indeed the inhibitory compound (Bond and Lovley, 2002).

The differences in HA sensitivity between the two acetoclastic methanogens can also be due to the proteinaceous cell wall of *M. concilii*, which may prevent HA to enter the cells as described above for *M. hungatei*. *M. barkeri* lacks such a thick cell wall (Albers and Meyer, 2011).

4.3.2 Methanogenic activity of crushed granular sludge

Batch tests were performed using crushed granular sludge in the presence and absence of HA and with either H_2, formate or acetate as growth substrates. When H_2 was used as an electron donor, HA had only a small inhibitory effect compared to the results with the pure cultures of hydrogenotrophic methanogens (Figure 4.5 a and Figure 4.3). HA concentrations of 1, 3 and 5 g L^{-1} had similar inhibitory effects on the methane production rates, but total methane produced at the end of the experiment was only slightly affected by the HA concentration (Table S2).

Results of the formate fed batch tests showed a very rapid conversion of formate to methane (Table S2 and Figure 4.5 b). In one day, all added formate was converted to methane. Overall, the total methane produced at the end of the experiment was hardly affected by the presence of HA. A maximum reduction of 23% in total methane production was observed at 5 $g.l^{-1}$ of HA (Table S2). As observed in the *M. formicicum* incubations, trace amounts of H_2 were formed in all the incubations, but the H_2 was consumed at the end of the experiments (Table S2).

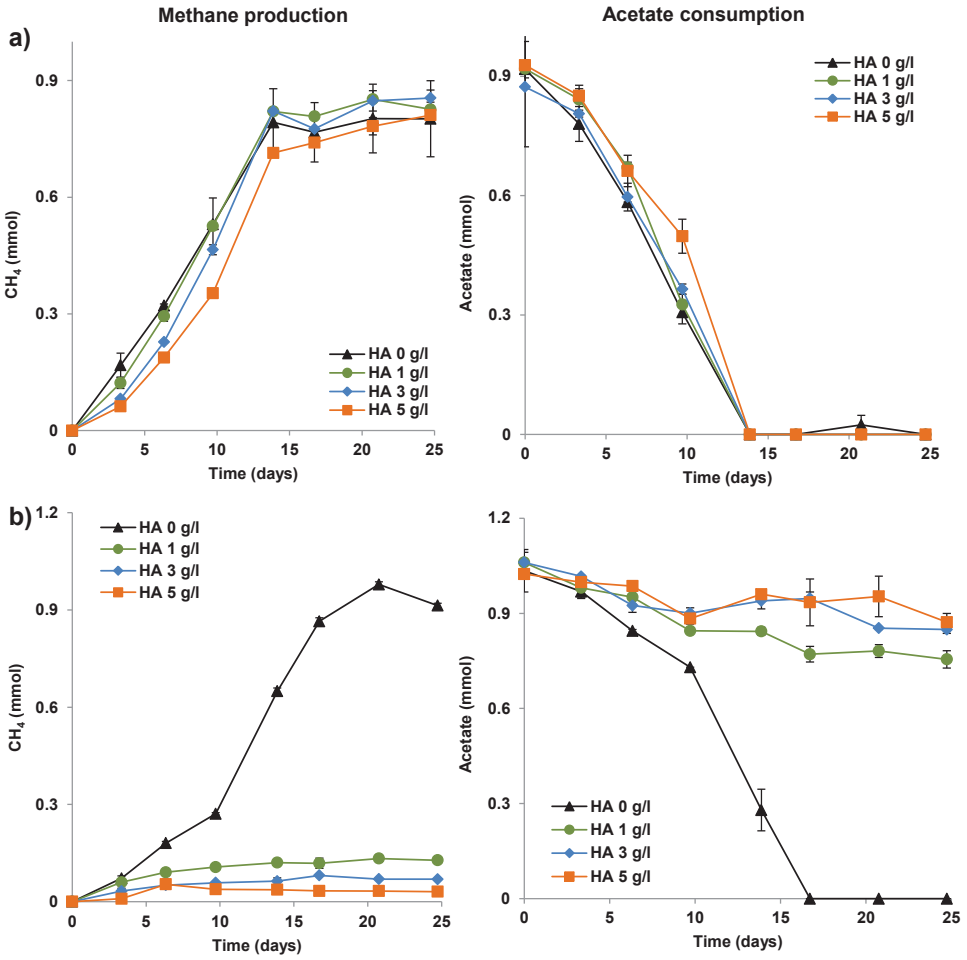

Figure 4.4 Effect of humic acid on methanogenic activity of a) *Methanothrix concilii*, b) *Methanosarcina barkeri*. Left panel: CH_4 production over time. Right panel: the corresponding acetate consumption.

In the acetate fed batch incubations, the total amount of methane produced in the control bottle reached 0.97 mmol within 5 days (Figure 4.5 c). Addition of 1 g L^{-1} HA, did not affect the total methane produced at the end of the experiment nor the rate of production (Figure 4.5 c and Table S2). However, addition of 3 g L^{-1} of HA reduced the CH_4 production rate, whereas the total methane produced was not strongly affected (Table S2). Addition of 5 g.L^{-1} HA resulted in 24% reduction in total amount of methane produced and the methane production rate was reduced by 40% (Table S2).

The results from the batch activity tests with H_2, formate and acetate showed that methanogenesis with anaerobic crushed granular sludge was not strongly affected by the presence of HA. When the crushed granular sludge was fed with formate or acetate, maximum observed reduction in the total amount of CH_4 produced was 24% at 5 g L^{-1} of HA (Table S2). Apparently, the mixed methanogenic population present in the crushed granular sludge is sufficiently diverse to sustain methane production regardless of HA presence. However, results show a clearly increasing gap in the stoichiometry of substrate conversion to methane production with increasing amounts of added HA (Figure 4.1 f). At 3 g L^{-1} of HA, the gap in reducing equivalents balance was about 30%, when acetate was added as the substrate. Likely, in the presence of HA, reducing equivalents are diffusing or transported out of the archaeal cell leading to reduced methane formation and reduction of oxidised HA moieties.

The latter would mean a drop in the biomethane production potential of a substrate when HA concentrations in the medium are high. Methanogenic populations in the used inoculum sludge were previously characterized by Roest et al. (2005) and Worm et al. (2009). These authors found that *M. concilii* was the main acetoclastic methanogen, whereas, *M. formicicum* and *M. hungatei* were both found as the main hydrogenotrophic methanogens. Therefore, obtained results were consistent with the results for each of the pure methanogenic cultures. The methanogenic activity of crushed granular sludge, fed with H_2, was inhibited less strongly by HA than the inhibition observed when *M. formicicum* was grown in pure culture. When crushed granular sludge and the pure culture of *M. concilii* were incubated separately with acetate, HA was inhibiting at concentrations higher than 3 g L^{-1}. In addition, these results can be used to explain lower methane yields in manure digesters, which are characterised by high levels of HA and ammonia concentrations, which are limiting factors for the overall anaerobic digestion process. Because of this high ammonia content in manure digesters, the operating pH may reach above the neutral range. Shifts in pH can result in changes of dominant methanogenic populations from *Methanosaeatacea* (*Methanotrichaceae*) to *Methanosarcinaceae* (Karakashev et al., 2005). As we show here that *Methanosarcinaceae* may be more inhibited by HA than *Methanosaeatacea* (*Methanotrichaceae*), a shift in population due to pH changes can result in decreasing methane yields when HA are present in the digester environment.

To test this hypothesis, the effect of HA on methane production by anaerobic sludge from manure digesters should be investigated.

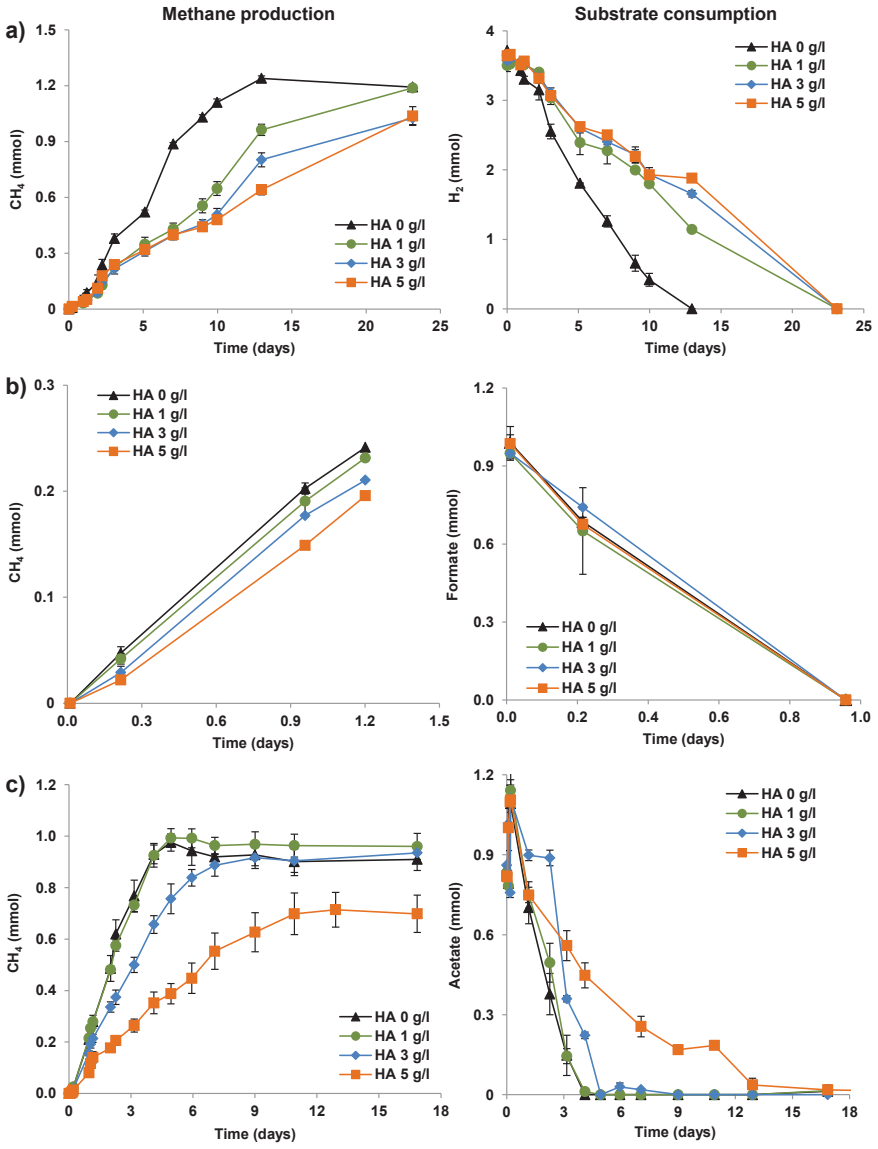

Figure 5.5 Effect of humic acids on methanogenic activity of crushed granular sludge, incubated with H_2/CO_2 (a), with formate (b) and with acetate (c). Left panel: CH_4 production over time. Right panel: the corresponding substrate consumption.

4.4 Conclusions

The effect of HA on methanogenic activity was demonstrated using pure cultures and mixed cultures. Except for *Methanospirillum hungatei*, all pure cultures of hydrogenotrophic methanogens tested were severely affected by addition of HA. Of the acetoclastic methanogens tested, *Methanothrix concilii* was not affected by HA, whereas *Methanosarcina barkeri* was severely affected by HA. Anaerobic sludge was less affected by the addition of HA. However, a clear gap in the reducing equivalent balance was observed, probably due to HA acting as an alternative electron acceptor and resulting in reduced methane production in the presence of HA. Due to their insensitivity to HA inhibition, *M. hungatei* and *M. concilii* can be candidates of interest to bio-augmentation studies in anaerobic reactors that are suffering from HA dependent losses in methane yields.

4.5 Supplementary Material

Table S1 Initial methane production rates and total amounts of methane produced at the end of the experiment, by the different methanogenic pure cultures, in the absence and presence of humic acid. The presented values in the Table are the average of representative duplicate measurements.

Microorganisms Humic acid ($g.L^{-1}$)	Production rate ($mmol\ day^{-1}$)		Consumption rate ($mmol\ day^{-1}$)			Inhibition percentage of CH_4 production rate [a]	Inhibition percentage of total amount CH_4 [b]
	H_2	CH_4	H_2	Acetate	Formate		
M. formicicum **fed with H_2/CO_2**							
0	-	0.06	0.32	-	-	0	0
1	-	0.02	0.13	-	-	73	79
3	-	0.01	0.15	-	-	75	81
5	-	0.01	0.11	-	-	77	84
M. formicicum **fed with formate**							
0	-	0.25	-	-	6.26	0	0
1	0.01	0.00	-	-	1.92	99	5
3	0.01	0.00	-	-	3.78	99	6
5	0.01	0.00	-	-	2.87	99	0
M. arboriphilicus **fed with H_2/CO_2**							
0	-	0.12	0.36	-	-	0	0
1	-	0.01	0.04	-	-	87	88
3	-	0.02	0.04	-	-	87	89
5	-	0.02	0.05	-	-	85	88

Table S1 Initial methane production rates and total amounts of methane produced at the end of the experiment, by the different methanogenic pure cultures, in the absence and presence of humic acid. The presented values in the Table are the average of representative duplicate measurements. (Continued)

M. hungatei **fed with H_2/CO_2**							
0	-	0.11	0.49	-	-	0	0
1	-	0.11	0.48	-	-	0	9
3	-	0.14	0.55	-	-	0	9
5	-	0.13	0.43	-	-	0	12
7	-	0.10	0.31	-	-	8	18
M. concilii **fed with acetate**							
0	-	0.06	-	0.07	-	0	0
1	-	0.06	-	0.07	-	0	0
3	-	0.05	-	0.06	-	6	0
5	-	0.04	-	0.07	-	21	0
M. barkeri **fed with acetate**							
0	-	0.03	-	0.03	-	0	0
1	-	0.02	-	0.01	-	45	86
3	-	0.01	-	0.01	-	69	92
5	-	0.01	-	0.00	-	74	97

[a] The total amount of methane refers to the average amount of methane produced from duplicate experiments at the end of the experiment. To calculate the percentage inhibition of the methane production rates and the total amount of methane in the presence of HA, the methanogenic activity in the control bottles (without HA) was considered 100%.

[b] Methane production rates were calculated as initial production rates relative to the initial methane production rate in the control bottles without humic acid.

Table S2 Initial methane production rates and total amounts of methane produced at the end of the experiment, by sludge from the Eerbeek paper mill digester, in absence and presence of humic acid. The presented values in the Table are the average of representative duplicate measurements.

Humic acid ($g\ L^{-1}$)	Production rate ($mmol\ day^{-1}$)		Consumption rate ($mmol\ day^{-1}$)			Inhibition percentage of CH_4 production rate[a]	Inhibition percentage of total amount CH_4[b]
	H_2	CH_4	H_2	Acetate	Formate		
Fed with H_2/CO_2							
0	-	0.07	0.35	-	-	0	0
1	-	0.04	0.14	-	-	42	0
3	-	0.04	0.15	-	-	43	13
5	-	0.04	0.19	-	-	42	12
Fed with formate							
0	0.00	0.21	-	-	1.01	0	0
1	0.00	0.20	-	-	0.97	5	2
3	0.00	0.18	-	-	1.00	13	12
5	0.00	0.16	-	-	1.01	23	24
Fed with acetate							
0	-	0.24	-	0.32	-	0	0
1	-	0.23	-	0.32	-	2	0
3	-	0.16	-	0.24	-	33	1
5	-	0.14	-	0.15	-	40	24

[a] The total amount of methane refers to the average amount of methane produced from duplicate experiments at the end of the experiment. To calculate the percentage inhibition of the methane production rates and the total amount of methane in the presence of HA, the methanogenic activity in the control bottles (without HA) was considered 100%.

[b] Methane production rates were calculated as initial production rates relative to the initial methane production rate in the control bottles without humic acid.

Chapter 5

Microbial community dynamics in five replicate CSTRs during start-up period with transient feeding strategy to achieve anaerobic digestion of cellulose and xylan

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-Manuscript in preparation for publication

Abstract

The aim of this study is to evaluate a transient feeding strategy to develop a microbial community that efficiently degrades cellulose and xylan and to monitor how the microbial community structure changes during the start-up of five replicate mesophilic anaerobic reactors inoculated with crushed granular anaerobic sludge. Transient feeding strategy can be used to obtain efficient conversion of cellulose and xylan with providing sufficient iron supplement. During the start-up period, microbial populations that perform hydrolysis, fermentation and methane formation were detected; *Bacteriodales*, *Clostridiales* and *Anaerolineales* were dominant within the reactors. Archaeal communities mainly consisted of *Methanobacteriaceae* and *Methanospirillaceae*. 16S rRNA next generation amplicon sequencing results showed that the shift in microbial community was related to the feeding regime and not to reactor performance parameters, such as biogas production, and effluent volatile fatty acid concentration. Bacterial richness, diversity and evenness decreased at the end of the start-up period. This decrease indicated the selection of adapted bacterial communities involved in anaerobic conversion of cellulose and xylan.

5.1 Introduction

Anaerobic digestion (AD) of waste materials is widely used as a method for renewable energy production. Many waste streams such as industrial wastewater, domestic wastewater, plant residues and manure can be used to generate biogas and green chemicals (Appels et al., 2011; Clark and Deswarte, 2015). The AD process is divided into four phases (hydrolysis, acidogenesis, acetogenesis and methanogenesis), which are driven by complex microbial communities (Weiland, 2010). The performance of AD is linked to structure and functionality of the microbial communities involved (Carballa et al., 2015; Lucas et al., 2015; Shin et al., 2016).

Start-up procedure of the anaerobic digesters is important for stable and efficient biogas production (Kim et al., 2002; Escudié et al., 2011). Start-up is usually considered as the required time period in which, a dedicated microbial community for anaerobic digestion of a specific waste stream can grow, develop and become stable. AD without a start-up period may lead to inefficient organic matter conversion, consequently to inefficient biogas production, extended acclimation time to the selected compounds and unexpected process failures during the reactor operation (Griffin et al., 1998; Liu et al., 2002). Therefore, establishing a dedicated microbial community requires a start-up period (Escudié et al., 2011). Several strategies have been reported to start-up anaerobic bioreactors. These strategies involved the evaluation of selecting different seed sludge (Fang and Lau, 1996), organic loading rates (Bolzonella et al., 2003), inoculum/substrate ratios (Fernandez et al., 2001; Angelidaki et al., 2006), temperature (van Lier et al., 1992) and different type of reactors (Zeeman et al., 1988). All these strategies helped to avoid accumulation of intermediate products such as volatile fatty acids which can potentially inhibit methanogenesis and limit biogas yield during the reactor operation.

In current practices, start-up periods are usually monitored in terms of biogas production, metabolite measurements and effluent quality (Kim et al., 2002). Monitoring of the microbial populations during start-up is not routinely performed. There are several studies describing microbial community changes during start-up period and usually these results are based on 16S rRNA clone libraries, Sanger sequencing and/or conventional microbiological techniques (Angenent et al., 2002; Colins et al., 2003; Ike et al., 2010; Kim et al., 2013; Li et al., 2014b; Goberna et al., 2015).

Developments in next generation sequencing (NGS) technologies allow researchers to monitor the microbial communities cheaper and more accurately (Shendure and Ji, 2008) in lab-scale and full-scale anaerobic digesters (Azman et al., 2015a). Although NGS helps to identify microbial community dynamics during start- up (Yu et al., 2014; Tian et al., 2015; Goux et al., 2016), limited knowledge is available on microbial community changes in relation to transient feeding regimes.

In this study, a transient feeding strategy for the start-up of five identical lab-scale mesophilic CSTRs was evaluated. We aimed to increase the cellulose and xylan degradation capacity of the anaerobic seed sludge which was obtained from a bioreactor that was mainly fed with soluble short chain fatty acids and carbohydrates originating from pulp and paper. A four-stage feeding strategy was applied to obtain dedicated biomass for cellulose and xylan degradation. In the first stage, the reactors were fed with a synthetic feed that was similar to the influent composition of the anaerobic reactors from which the inoculum was taken. In the second stage, cellulose was introduced to the feed and short chain fatty acids and glucose were omitted from the feed. In the third stage, cellulose was used as a sole carbon source and in the final stage xylan was also added to the feed. We investigated the microbial community dynamics of the reactors during the whole start-up period. We used 16S rRNA-based next generation sequencing to monitor both bacterial and archaeal population dynamics. We also performed statistical analysis to correlate reactor performances to microbial community composition.

5.2 Material and methods

5.2.1 Operation of fed-batch reactors

Five lab-scale CSTRs, fed once a day (total volume 6L; working volume 5L), were operated in parallel (R1-R5). All five reactors were inoculated with crushed anaerobic granular sludge (10g VS L^{-1}) which was taken from a full-scale UASB reactor, treating pulp and paper industry effluents (Industriewater Eerbeek, Eerbeek, The Netherlands). The UASB reactor was maintained at a constant HRT of 4.6 h, and a temperature of 30°C (winter) to 37°C (summer). The paper mill wastewater, fed to the UASB reactor, mainly contains starch, acetate, propionate, butyrate and formate (Oude Elferink et al., 1998).

Constant temperature was assured for each individual reactor at 30±0.5 °C and operational pH was kept between 6.8 and 7.2 by addition of 5 M NaOH, when necessary. Continuous stirring of the reactors was obtained by anchor type propellers at 100 rpm. Following the inoculation of the reactors, a starch, glucose and VFA (acetate, propionate and butyrate) mixture (55:15:30 w:w) (Sigma- Aldrich, Darmstadt) was fed for 55 days; after that, the feeding continued with cellulose (avicel; PH-101, Fluka, Darmstadt) and xylan (Roth, Karlsruhe, Germany). The composition of the feeding is given in Table 5.1. The feed was mixed with mineral based medium which was previously described, omitting the reducing solutions (Plugge, 2005; Stams et al., 1998). Organic loading rates varied during the experiment but the final OLR was 2.3 g COD $L^{-1}day^{-1}$. The hydraulic retention time (HRT) was kept at 20 days.

Table 5.1 Operational conditions during a 190 days acclimation period of five identical lab-scale reactors operated at 30°C and an HRT of 20 days.

Stage	Operation time (days)	Substrate	OLR (g COD $L^{-1}d^{-1}$)	Notes
I	0-41	Starch+glucose+VFA mixture (55:15:30 w:w)	2.2	Between day 30-41 only starch was fed at 1.4 g COD $L^{-1}d^{-1}$
II	42-55	Cellulose+starch (50:50 w:w)	2.1	day 48 to 55 R3 and R5 were not fed
III	56-96	Cellulose	0.5-2.3	Step wise increase of OLR to reach the final COD concentrations
IV	96-190	Cellulose+xylan (75:25 w:w)	2.3	_

Biogas production was monitored by a gas flow measurement device (µflow, Bioprocess Control, Sweden). Cumulative biogas production values were recorded daily and expressed in mL at normal atmospheric pressure and ambient temperature. Biogas composition was measured biweekly via gas chromatography (Interscience GC 8000 series) equipped with a thermal conductivity detector and two columns (Molsieve 5A 50 m × 0.53 mm for nitrogen and methane and Porabond Q 50 m × 0.53 mm for CO_2). Temperature of the injector, detector and oven were 110, 99 and 50 °C, respectively. Organic acids were quantified using a Thermo Scientific Spectra system HPLC system, equipped with a Varian Metacarb 67H 300 × 6.5 mm column kept at 45°C, running with

0.005 M sulphuric acid as eluent. The eluent had a flow rate of 0.8 mL min^{-1}. The detector was a refractive index detector. Data analyses were performed with the ChromQuest (Thermo Scientific, Waltham, MA). The total organic acid concentrations were expressed as their COD equivalents (mg COD L^{-1}) of measured acetate and propionate concentrations. Hydrolysis and methanogenesis efficiencies were calculated as described by Azman et al., (2015b), with the assumption that acidogenesis is not limiting.

For the iron measurements, liquid samples were centrifuged (10,000× g, RT, 5 min), and were subsequently measured by Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES) using a Vista MPX ICP-AES instrument.

5.2.2 Microbial Community Analyses

Genomic DNA was extracted from 50 mL sludge samples taken at eight sampling days (day 0,40,55,82,96,120, 160 and 190) using the Fast DNA® SPIN kit for soil (MP Biomedicals, OH) following the manufacturer's protocol. The DNA concentrations were measured with a Nano drop® (ND-1000) spectrophotometer (Nano drop Technologies, Wilmington, DE).

Extracted DNA from selected samples was used for bacterial and archaeal community analysis. The amplification of bacterial and archaeal gene fragments was done using a 2-step PCR. The first amplification of bacterial 16S rRNA gene fragments was done using the 27 F- DegS (5′-GTT[TC]GAT[TC][AC]TGGCTCAG-3′) (van den Bogert et al., 2011 and 2013) and equimolar mix of two reverse primers; 338R-I and 338-R-II (5′-GC[AT]GCC[AT]CCCGTAGG[TA]GT-3′) (Daims et al., 1991) and the first amplification of archaeal 16S rRNA gene fragments was done using primers 518F (5′-CAGC[AC]GCCGCGGTAA-3′) (Wang and Qian, 2009) and 905R (5′-CCCGCCAATTCCTTTAAGTTTC-3′) (Kvist et al., 2007). PCR amplifications were carried out in technical duplicates in a total volume of 50 µl containing 500 nM of each forward and reverse primer (Biolegio BV, Nijmegen, The Netherlands), 1 unit of Phusion DNA polymerase (Thermo Scientific, MA), 10 µl of HF-buffer, 200 µM dNTP mix, 1 µl DNA template (20 ng μl^{-1}), made to a total volume of 50 µl with nuclease free sterile water. The PCR program was as follows: denaturing at 98°C for 30 s, followed by 25 cycles of denaturing at 98°C for 10 s, annealing at 56°C for bacterial and 60 °C for

archaeal for 20 s, extension at 72°C for 20 s, followed by a final extension step at 72°C for 10 min. After positive amplifications, technical duplicates were pooled and prepared for the second step PCR amplification. A second amplification was performed to extend 8 nt barcodes to the amplicons, as described previously (Hamady et al., 2008). Barcoded amplification was performed in a total volume of 100 µl containing 5 µl of the first PCR product, 500 nM of each forward and reverse primer (Biolegio BV, Nijmegen, The Netherlands), 2 units of Phusion DNA polymerase (Thermo Scientific, Waltham, MA), 20 µl of HF-buffer, 200 µM dNTP mix, made to a total volume of 100 µl with nuclease free water. The PCR program was as follows: denaturing at 98°C for 30 s, followed by five cycles of denaturing at 98°C for 10 s, annealing at 52°C for 20 s, extension at 72°C for 20 s, followed by a final extension at 72°C for 10 min. Barcoded PCR products were cleaned using the HighPrep PCR clean-up system (MagBio Genomics Inc., Gaithersburg, MD). DNA was quantified using Qubit (Invitrogen, Bleiswijk, The Netherlands). After the second PCR, barcoded samples were pooled in equimolar quantities to create a library. The libraries were purified again by using the same purification protocol Prepared libraries were sent to GATC company (Konstanz, Germany) for Hiseq sequencing on the Illumina platform.

5.2.3 Sequencing data analysis

16S rRNA gene sequencing data was analysed using NG-Tax, an in-house pipeline (Ramiro-Garcia et al., 2016). Paired-end libraries were filtered to contain only read pairs with perfectly matching barcodes, and those barcodes were used to demultiplex reads by the sample. Resulting reads were separated by sample using the affiliated barcodes. Taxonomy affiliation was done with the SILVA 16S rRNA reference database by using an open reference approach as described by Quast et al. (2013). Quantitative Insights into Microbial Ecology (QIIME) v1.2 were used to define microbial compositions based on the described pipeline. Based on the sequencing results, evenness (J), Shannon-Weaver (H) and Simpson (D) diversity indices were calculated to evaluate the microbial diversity through the acclimation period. The sequence data was deposited to NCBI's SRA archive under the project number PRJNA340335.

The microbial community composition was analysed using Non-metric multidimensional scaling (NMDS) analyses with the CANOCO software (version 5)

(Šmilauer and Jan, 2014). Ranked Spearman correlation was applied to determine the correlation between microbial groups and operational conditions. All statistical and correlation analyses were performed by IBM SPSS Statistics 23.

5.3 Results and discussion

5.3.1 Reactor performance during the transient feeding regime

During the start-up period reactor performances were evaluated with monitoring metabolite measurements, hydrolysis and methanogenesis efficiencies.

Phase I (Day 0-41, feed mixture)

During this phase, all reactors were fed with a glucose, starch and VFA mixture, mimicking the conditions of the reactor where the inoculum originated from. This was done to keep the microbial community active and to prevent washout of biomass. OLR was kept constant at 2.2 g COD L^{-1} d^{-1} for 30 days (Figure S1).

Maximum biodegradability of the feed mixture was calculated as 90% by considering the maximum biodegradability of starch as 80 ± 19% (Raposo et al., 2011) in this period. In the influent, approximately 40% of the COD was already hydrolysed. During this period, hydrolysis efficiencies varied from 45 to 99 % between reactors.

Average total hydrolysis efficiencies (including influent VFAs) during this phase were calculated as 75±8, 67±6, 58±14, 68±8, 77 ±16 % for R1-R5, respectively. Methanogenesis efficiencies, at the end of phase I, coincided with hydrolysis efficiencies except for R3 and R5. In R5, methanogenesis was lower than the hydrolysis (Figure 5.1) and methane production decreased from 1.6 L to 1L at the end of Phase 1 (Figure S1 b). On the other hand, hydrolysis efficiency was relatively low in R3. Total VFA concentration in R3 reached up to 1 g COD L^{-1} while, it was 3.5 g CODL^{-1} in R5. Increased levels of acetate, propionate and C4-C5 acids (Figure S1 d-f) indicate that activity of microorganisms in the inoculum was insufficient to completely convert the residual COD. Despite the relatively high levels of VFAs (3.5 g COD L^{-1}), pH remained in the neutral range due to the buffering capacity of the medium.

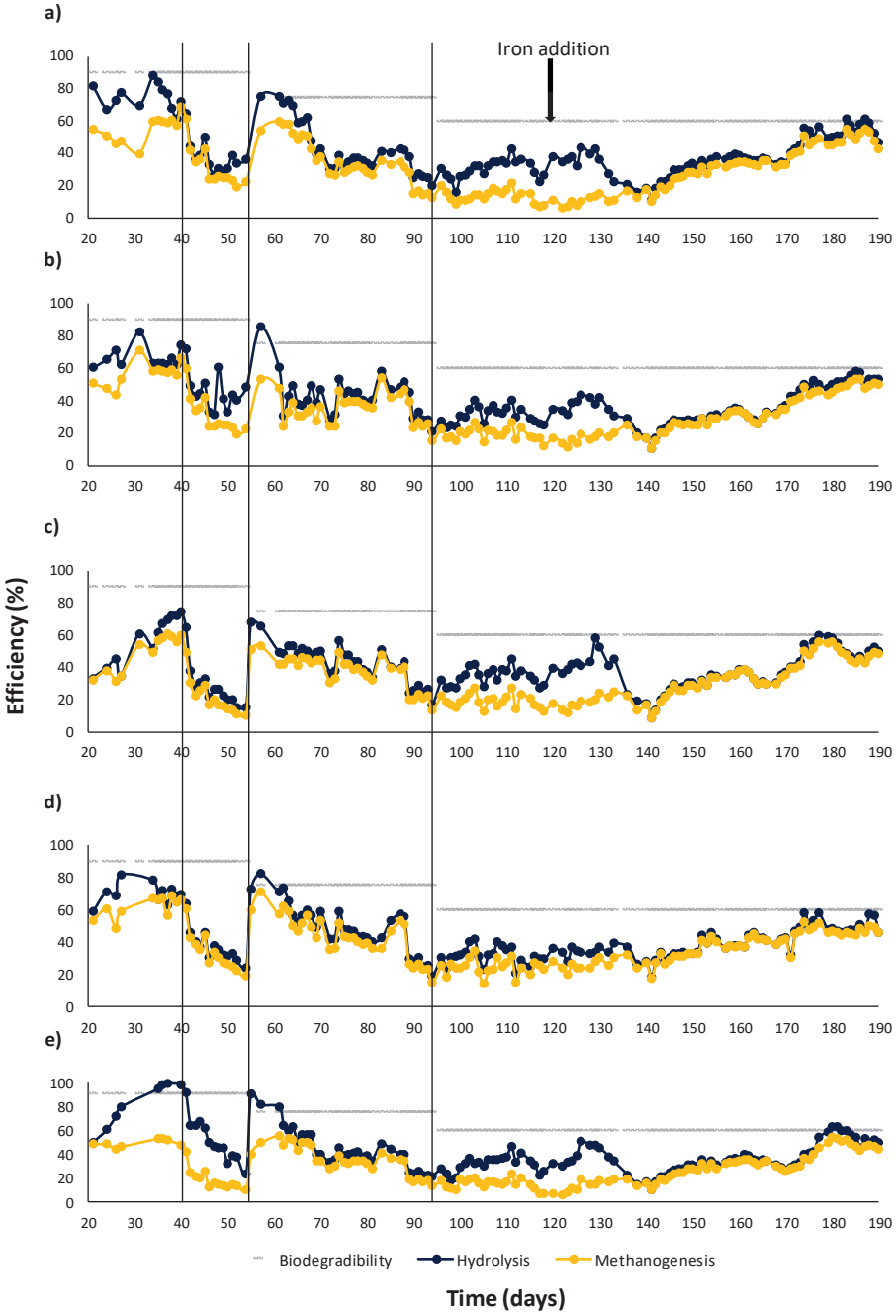


Figure 5.1 Hydrolysis and methanogenesis efficiencies of the reactors a) R1, b) R2, c) R3, d) R4 and e) R5. Grey dots indicate the calculated biodegradability of the feed

Despite the stable pH, reactor performances did not improve in R3 and R5. Inefficient conversion in these reactors are likely not due to VFA inhibition since measured VFA concentrations were far below the reported inhibitory concentrations (Veeken et al., 2000). Inefficiency of performance in R3 and R5 could be related to the disruption of syntrophic communities, caused by crushing of granular sludge prior to reactor inoculation and stirring of the reactors (Schmidt and Ahring, 1995; Stams and Plugge, 2009). To achieve stable reactor performance, the VFA mixture was omitted from the feed and only starch was fed to the reactors at a decreased loading rate of 1.4g COD L^{-1} d^{-1} for 10 days, between day 30 and 41. During the 10 days total effluent VFA's decreased to approximately 0.35 g COD L^{-1} except in R3 (0.82 g COD L^{-1}) and R5 (0.55 g COD L^{-1}).

Phase II (Day 42-55, cellulose and starch as a feed)

During this phase, half of the feeding (w:w) was replaced with cellulose to start acclimation for this substrate. OLR was kept constant at 2.1 g COD L^{-1} d^{-1} (Figure S1 a). Hydrolysis and methanogenesis efficiencies showed similar decreasing trends in all reactors. Hydrolysis efficiencies reduced to 15-40% at the end of this period (Figure 5.1). Reduction of hydrolysis and methanogenesis efficiencies could be related to addition of cellulose as feed. In R3 and R5, VFA remained accumulated. Therefore, these two reactors were not fed between the day 42 and 55 to reduce VFA concentrations. At the end of this period, total VFA concentration was <0.7 g COD L^{-1} in R1, R2 and R4, whereas in R3 and R5 the total VFA concentration varied from 1 g COD L^{-1} to 4 g COD L^{-1} (Figure S1 d-f).

Phase III (Day 56-96, cellulose as a feed)

During this phase, all reactors were fed with cellulose as a sole carbon source. Stepwise acclimation was used to allow the microbial population to adapt to cellulose. In the beginning of phase III, OLR was decreased to 0.5 g COD L^{-1} d^{-1} and OLR was step-wise increased to 2.3 g COD L^{-1} d^{-1} as illustrated in Figure S1 a. Maximum biodegradation of the cellulose was 75± 5 % (Azman et al., 2015b). At the beginning of this period, there was a peak in hydrolysis efficiencies related to reduced loading rates. That sudden increase reduced with time and hydrolysis efficiencies remained around 45 %. Hydrolysis efficiencies were at the same range with methanogenesis during the period

that indicated relatively stable operation (Figure 5.1). Methane production in replicate reactors showed similar trends over time and average biogas production increased from 1.1± 0.1 L to 1.8± 0.1 L accordingly with increasing OLR (Figure S1 b). Residual VFA was efficiently degraded. Production of C4-C5 VFA stopped when cellulose was used as sole carbon source and the production of these VFAs was not observed until the end of the acclimation period (FigureS1 d-f).

Phase IV (Day 96-190, cellulose and xylan as a feed)

During this phase, all reactors were fed with cellulose and xylan mixture (75:25 w:w) as a carbon source. OLR was kept at 2.3 g COD L^{-1} d^{-1}. Maximum observed biodegradability of the feed was 60±5 % for this period (Chapter 6). During the first 30 days of this phase, hydrolysis efficiencies were around 35 %. Except R4, in all reactors, methanogenesis efficiencies were 10 % lower than the hydrolysis efficiencies (Figure 5.1). This coincided with acetate and propionate accumulation. Except for R4, the average total VFA concentrations reached up to 2 g COD L^{-1}, whereas in R4 total VFA concentrations were 0.8 ±0.4 g COD L^{-1} (Figure S1 d-f). Because of the accumulation of the VFA, low hydrolysis efficiencies and a low measured iron content in the reactors, 100 mg L^{-1} $Fe_2(SO_4)_3$ was included in the media from day 120 onwards. The addition of iron, to improve methane production, was reported by Rao and Seenayya, (1994) and Kim et al., (2002). Indeed, additional $Fe_2(SO_4)_3$ helped to improve process stability and efficiencies. After the addition, both hydrolysis and methanogenesis efficiencies increased to 55 %, which was the observed biodegradation during the experiment of Chapter 6 for the operational conditions (30 °C and HRT of 20 days) of the reactors.

5.3.2 Start-up of lab-scale CSTRs with a transient feeding regime

The presented work revealed that start-up of lab-scale reactors to increase cellulose and xylan degradation capacity of the anaerobic seed sludge is possible when applying a transient feeding strategy. The results and recommendations for the start-up of CSTRs can be summarised as follows:

Crushing of the granules allows for improved contact between substrate and microbial aggregates which is important for initializing particulate compound hydrolysis (Lynd et al., 2002; Azman et al., 2015). However, crushing granular sludge can also cause a disruption in syntrophic associations which can result in lower

conversion efficiencies, compared to granular sludge (Schmidt and Ahring, 1995; Stams and Plugge, 2009). In this study, fluctuations in the conversion efficiencies were also observed at the beginning of the experiment when crushed granular sludge was inoculated to the reactors.

Feeding change from soluble substrate to solid substrate during the start-up period of the CSTRs caused a decrease in methanogenesis efficiencies as a result of the relatively low biodegradability of the solid substrate. During this feeding change, VFA accumulation was observed because of the increased loading rate. Even though, the accumulated VFA was below the inhibitory concentration, the accumulation of acetate and propionate below 50 mM should be considered as a warning for potential imbalances in the conversion processes (Ahring et al., 1995; Mechichi and Sayadi, 2005).

A further important aspect is the presence of sufficient macro and micro nutrients (calcium, magnesium, iron, trace elements etc.) for the efficient anaerobic digestion. Lack of some of these nutrients was shown to cause inconsistent reactor performance (Romero-Güiza et al., 2016; Schattauer et al., 2011). Here, we used five identical reactors to monitor the reactor performance. However, the reactors did not perform in an identical way and had low efficiencies. After completing the substrate medium with an iron source, after 120 days of operation, all reactors recovered and hydrolysis and methanogenesis efficiencies increased considerably to 55 %, approximately. The total start-up period, for efficient hydrolysis and methanogenesis, was certainly increased by this iron deficiency. Iron concentration in the substrate medium up till day 120 was chosen based on literature (Stams et al., 1993; Plugge, 2005). The iron limitation in the present research, show the need to also check the availability of macro and micro nutrients within the reactors during operation since several macro and micro nutrients are crucial cofactors in numerous enzymatic reactions involved in the biochemistry of hydrolysis and methanogenesis (Romero-Güiza et al., 2016).

5.3.3 Bacterial community dynamics

Sequencing of the bacterial 16S rRNA genes gave an average of 115128 sequence reads (Table S1). The reads were assigned to 112 OTUs and these could be assigned to different eleven phyla, seventeen classes, and thirteen orders. Relative abundance $\geqslant$1% of the phylogenetic affiliation of the reads at the order level are considered as significant and

represented individually in Figure 5.2. The bacterial community composition in the five reactors was similar at the first sampling point. The first sampling point showed the inoculum and therefore, analyses of 5 biological replicates were used to indicate the reproducibility of the sampling, DNA extraction, and sequencing methodology. In the inoculum, *Bacteriodales* (12±3%), *Sphingobacteriales* (14±5%), Unassigned *Bacteriodetes* vadin HA17 (29±4%) and *Anaerolineales* (33±7 %) shaped the bacterial community.

After the inoculation, bacterial community composition has changed within the 5 replicate reactors. Relative abundance of *Bacteriodales* increased from 12% to 53-68% while, the relative abundance of unassigned *Bacteriodetes* vadin HA17 decreased from 29 % to 2% (Figure 5.2). In all digesters, *Sphingobacteriales* initially co-dominated the reactors but their relative abundance decreased with time to as low as 1 % (Figure 5.2).

On the other hand, *Anaerolineales* (29 ±11 %) remained as the second most abundant order within the reactors during the start-up period (Figure 5.2). The relative abundance of *Clostridiales* mostly varied during the start-up period and changed between 3-37 % and their relative abundance reduced to 1-4 % at the end of the experiment (Figure 5.2).

Bacteria belonging to phylum *Bacteriodetes* and *Firmicutes* (mostly *Clostridiales*) were most likely forming the main hydrolytic/fermentative population and cellulose and xylan hydrolysis were associated with an increase in relative abundance of these groups. It is known that *Bacteroidales* and *Clostridiales* have a function in the hydrolysis of several particulate compounds and fermentation of sugars to VFA, alcohols, and hydrogen (Azman et al., 2015 a; Campanaro et al., 2016; Goux et al., 2015; Luo et al., 2015; Vanwonterghem et al., 2014). Abundance of *Clostridiales* significantly correlated (p <0.01, r=0.339) with VFA concentrations. After the residual VFA's degraded, the relative abundance of this order decreased and the abundance of *Bacteriodiales* increased. The increase in relative abundance of *Bacteriodiales* coincided with the observations of De Vrieze et al., (2015a). They suggested that members of the phylum *Bacteriodetes* might be dominant in digesters operating at mesophilic conditions and under low levels of VFA concentrations. When VFA concentrations decreased, relative abundance of *Bacteriodiales* increased in all reactors.

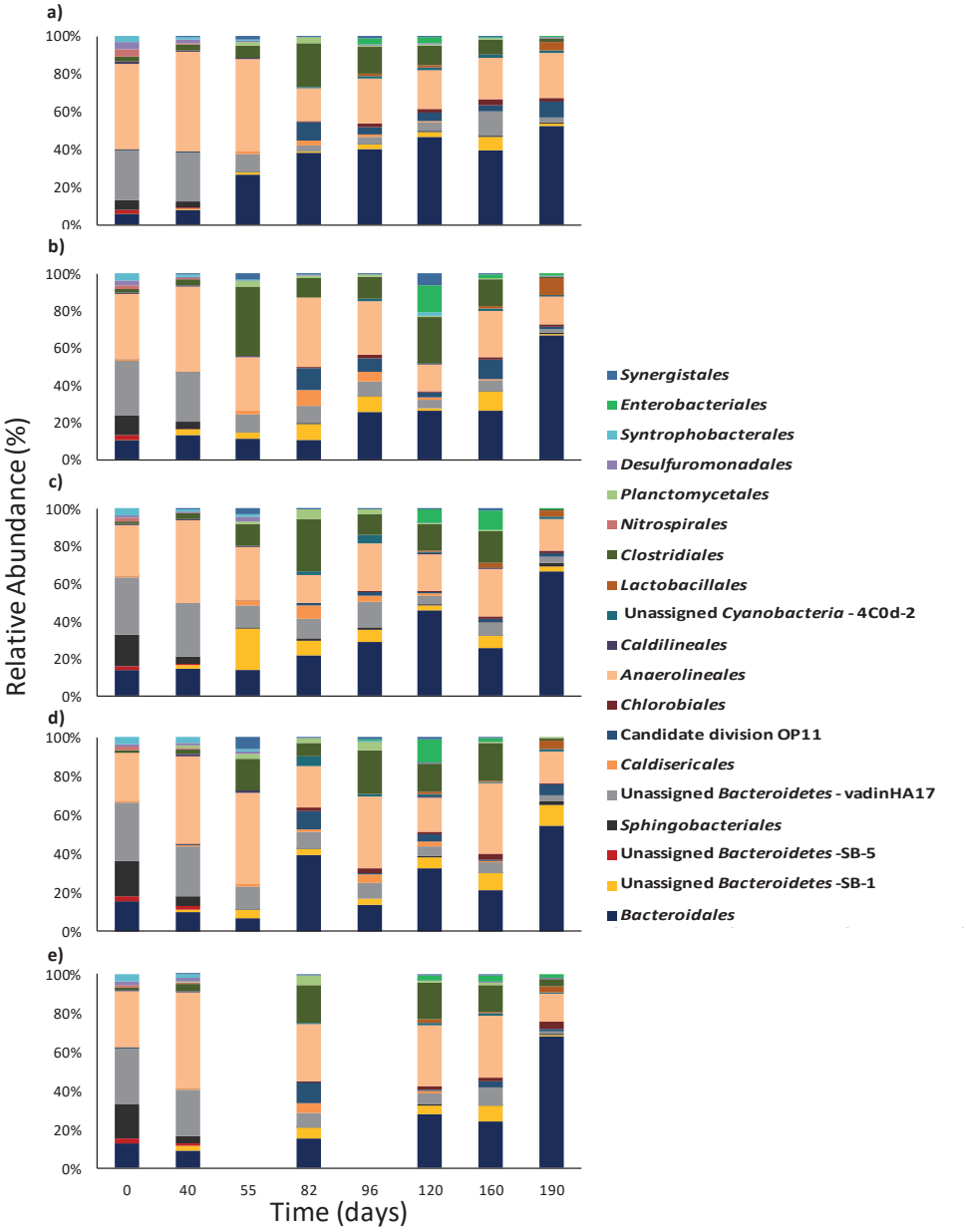


Figure 5.2 Bacterial community dynamics in the reactors a) R1, b) R2, c) R3, d) R4 and e) R5. Phylogenetically annotated at the order level with abundance >1 % in at least one sample. Unassigned was used to indicate bacterial groups that could not be classified at order level.

Increase in the relative abundance of *Bacteroidales*, *Clostridiales* during cellulose and xylan degradation was in parallel with the increase in the relative abundance of the *Methanospirillaceae*. This finding suggests possible interactions between *Bacteroidales*, *Clostridiales* and *Methanosprillum* during the cellulose and xylan degradation. Cellulose and xylan can be converted to hydrogen by these bacterial groups, while hydrogenotrophic methanogens may use the produced hydrogen to produce methane. In addition, high relative abundance of *Anaerolineales* populations can also indicate the possible interaction between fermentative bacteria and hydrogenotrophic methanogens. Even though little information is known about *Anaerolineales*, known *Anaerolineales* species are grown with hydrogenotrophic methanogens (Yamada et al., 2006; Yamada and Sekiguchi, 2009; Sekiguchi et al., 2001).

5.3.4 Archaeal community dynamics

Sequencing of the archaeal 16S rRNA genes gave an average of 27766 sequence reads (Table S1). The reads were assigned to 32 OTUs and these affiliated with *Euryarchaeota* and *Thaumarchaeota,* four classes, five orders and nine families, with at least 1% relative abundance in the samples. Phylogenetic affiliation of the OTUs was represented at family level (Figure 5.3).

Monitoring of the inoculum sludge was performed with four biological replicates. Similar results between four replicates showed the reproducibility of the sequencing results and the methods to prepare the amplicons. *Methanobacteriaceae* (74±5%), unassigned *Halobacteriales* (9±6%) and *Methanosaetaceae* (*Methanotrichaceae*) (5±2%) were the dominant archaeal groups in the inoculum (Figure 5.3).

In contrast to bacterial populations, methanogenic populations remained relatively stable throughout the start-up period. *Methanobacteriaceae* dominated the archaeal community throughout the start-up period with a relative abundance of 60±15%. After eighty days of operation, *Methanospirillaceae* started to co-dominate the reactors and their relative abundance reached approximately 36 % (Figure 5.3). A similar increase in the relative abundance of *Methanospirillaceae* was also observed by Vanwonterghem et al (2014) during α-cellulose degradation. They associated this increase with high VFA concentrations which were also observed in our study.

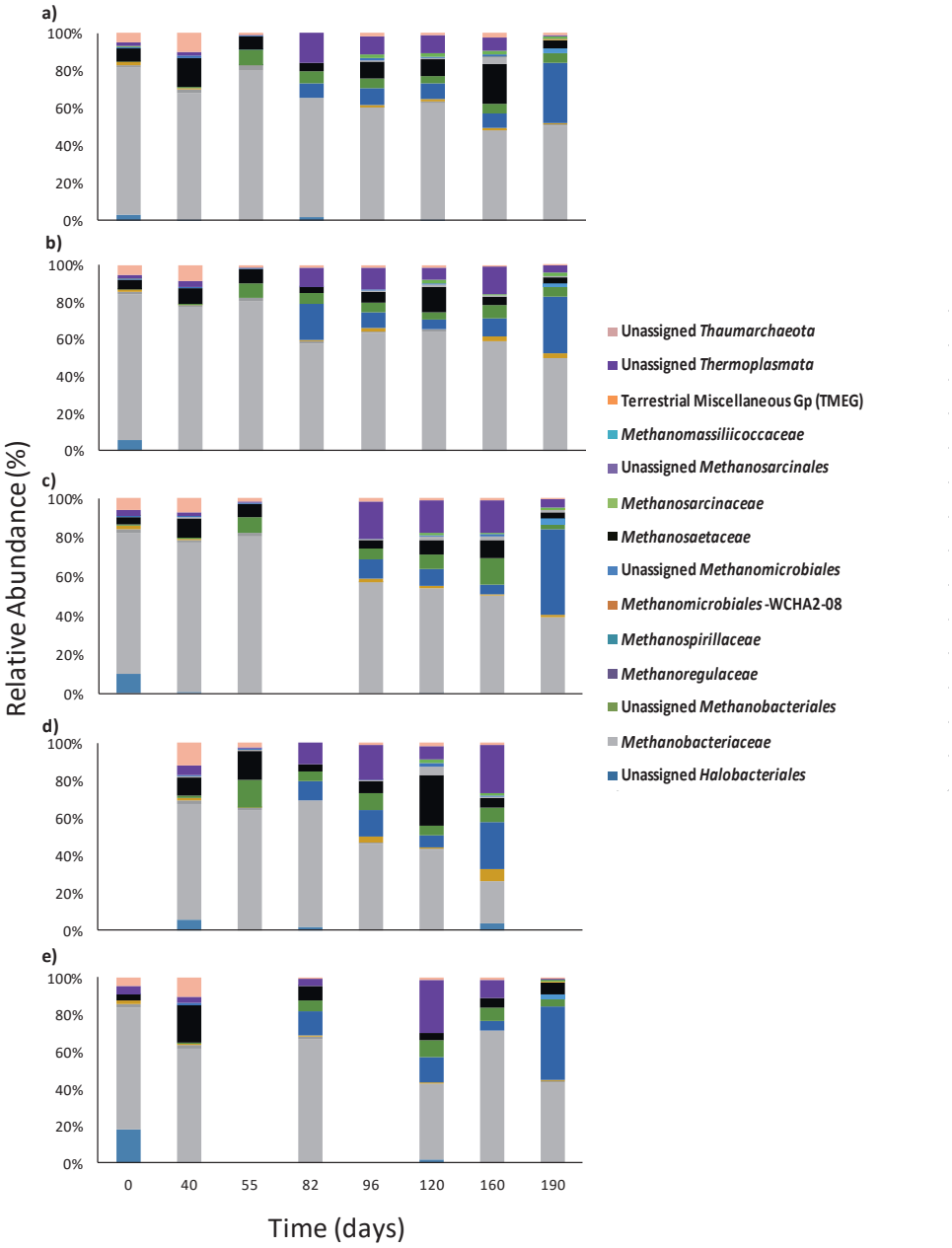


Figure 5.3 Archaeal community dynamics in the reactors a) R1, b) R2, c) R3, d) R4 and e) R5. Phylogenetically annotated at the order level with abundance >1 % in at least one sample. Unassigned was used to indicate archael groups that could not be classified at order level.

Unlike the study of Vanwonterghem et al. (2014), relative abundance of *Methanospirillaceae* remained stable in the reactors even when the VFA concentration decreased. Relative abundance of Unassigned *Halobacteriales* reduced to 1-3 % at the end of the start-up period. On the other hand, the relative abundance of *Methanosaetaceae* (*Methanotrichaceae*) did not show a trend and it varied between 4-20 % during the start-up period.

5.3.5 Richness, diversity and evenness

Diversity, richness and evenness indices were calculated for bacterial and archaeal populations observed in the reactor samples for seven sampling points based on the obtained OTUs. As shown in Table 5.2, richness and evenness of the reactors showed a tendency to increase from day 0 to day 98. Between day 98 and 190, richness decreased compared to the first 98 days of operation, Diversity indices (H and D) also estimated a decrease of the microbial diversity at the end of the start-up period (Table 5.2 a). In the archaeal population, richness and evenness varied during the experiment but showed an increase at the end of the start-up period. H and D also showed similar trends with richness and evenness (Table 5.2 b).

Table 5.2 Ecological parameters for the bacterial (a) and archaeal (b) communities: Simpson's diversity index (D), Shannon – Weaver diversity index (H), evenness (E), and richness (R)

a)

Indices	Reactor: Time(days)	0	40	55	82	96	120	160	190
	R1	0.16	0.14	0.16	0.19	0.08	0.09	0.07	0.09
	R2	0.15	0.22	0.14	0.12	0.08	0.07	0.07	0.07
D	*R3*	0.16	0.20	0.10	0.11	0.08	0.09	0.07	0.09
	R4	0.16	0.14	0.11	0.07	0.11	0.06	0.09	0.06
	R5	0.15	0.24	n.d.	0.10	n.d.	0.08	0.17	0.08
	R1	2.34	2.91	2.46	2.11	2.99	2.91	3.02	2.46
	R2	2.36	3.11	2.47	2.52	2.83	3.11	3.04	2.13
H	*R3*	2.33	2.95	2.71	2.51	2.90	2.95	3.05	2.19
	R4	2.33	3.19	2.71	2.87	2.77	3.19	2.95	2.44
	R5	2.34	2.96	n.d.	2.63	n.d.	2.96	2.44	2.14
	R1	0.20	0.31	0.23	0.17	0.25	0.31	0.41	0.20
	R2	0.19	0.28	0.21	0.21	0.22	0.28	0.25	0.17
	R3	0.20	0.31	0.25	0.23	0.24	0.31	0.26	0.19
	R4	0.24	0.30	0.24	0.25	0.23	0.30	0.27	0.22
E	*R5*	0.20	0.25	n.d.	0.24	n.d.	0.25	0.25	0.18
	R1	35	40	35	24	44	40	44	33
	R2	33	47	30	33	38	47	43	32
	R3	35	47	36	28	35	47	41	29
	R4	35	48	38	31	39	48	49	30
R	*R5*	33	44	n.d.	33	n.d.	44	35	32

b)

0	40	55	82	96	120	160	190
0.54	0.49	0.52	0.27	0.34	0.34	0.28	0.34
0.55	0.59	0.56	0.34	0.43	0.43	0.32	0.33
0.47	0.60	0.55	n.d.	0.31	0.30	0.27	0.30
n.d.	0.41	0.45	0.24	0.24	0.27	0.27	n.d.
0.41	0.42	n.d.	0.40	n.d.	0.28	0.32	0.32
1.12	1.10	1.10	1.64	1.62	1.63	1.73	1.48
1.11	0.94	0.99	1.53	1.34	1.36	1.63	1.52
1.30	0.90	1.02	n.d.	1.63	1.67	1.71	1.68
n.d.	1.39	1.14	1.83	1.79	1.71	1.75	n.d.
1.36	1.23	n.d.	1.41	n.d.	1.57	1.62	1.48
0.12	0.11	0.15	0.18	0.16	0.20	0.22	0.15
0.11	0.09	0.11	0.16	0.13	0.14	0.17	0.16
0.14	0.10	0.12	n.d.	0.16	0.19	0.16	0.23
n.d.	0.18	0.11	0.24	0.18	0.15	0.18	n.d.
0.15	0.12	n.d.	0.16	n.d.	0.17	0.21	0.16
12	12	15	10	15	16	18	17
13	12	9	15	12	12	13	14
14	11	9	n.d.	14	14	14	19
n.d.	14	10	11	14	16	16	n.d.
12	12	n.d.	13	n.d.	10	10	14

The decrease in the diversity and richness reflected the adaptation capacity of the microbial populations to cellulose and xylan as a carbon source. Rich and diverse microbial populations are usually associated with good reactor performances (Carballa et al., 2015). However, there are also studies, indicating that a diverse microbial population is not required to have a better function when the environmental conditions are stable (Shin et al., 2016; Goux et al., 2016). Even though there was a decrease in bacterial diversity and richness in our study, reactor performances increased at the end of the start-up period. Our results were similar to the study of Goux et al., (2016) in which they also observed decreased microbial diversity and richness during the start-up period of a full-scale farm reactor treating plant biomass.

5.3.6 Microbial community dynamics and reactor performance

One of the aims of our study was to get insight into the microbial community changes during a start-up period of five highly controlled replicate reactors. The composition and diversity of the replicate reactors showed similar patterns throughout the experiment (Figure 5.2, 5.3 and 5.4). Although the microbial communities in the replicates showed some differences at individual time points, all reactors showed similar reactor performances at the end of the start-up period. Changes in the microbial communities were observed during the change in the feeding regime from mostly soluble carbon sources to insoluble carbon sources (Figure 5.4). There were no strong correlations between microbial community dynamics and reactor performance in terms of VFA and biogas production and methane yields (Table S1 and S2). Deterministic factors on microbial community dynamics throughout the acclimation period were mainly the feeding regime and OLR. This finding showed consistency with the literature that showed the influence of different feedstocks on microbial communities (De Francisci et al., 2015; Shin et al., 2016; Treu et al., 2016).

Bacterial and archaeal communities showed a shift from the seed sludge after 40 days of reactor operation. There were no significant differences in the microbial community compositions between Day 0 and Day 40. The reason for the stable microbial communities could be related to the feeding regime. Feeding regime duringthis period included a similar influent composition as the wastewater influent of the anaerobic reactor from which the seed sludge was taken (Oude Elferink et al., 1998).

When cellulose was added to the reactors, microbial communities shifted from the seed sludge community profile.

Microbial communities present at very low abundance in the seed sludge were partially washed out due to the drastic changes in the feeding regime. While the feeding regime changed to cellulose and xylan, the change favoured specific populations in all reactors and new clustered microbial community profiles (Figure 5.4).

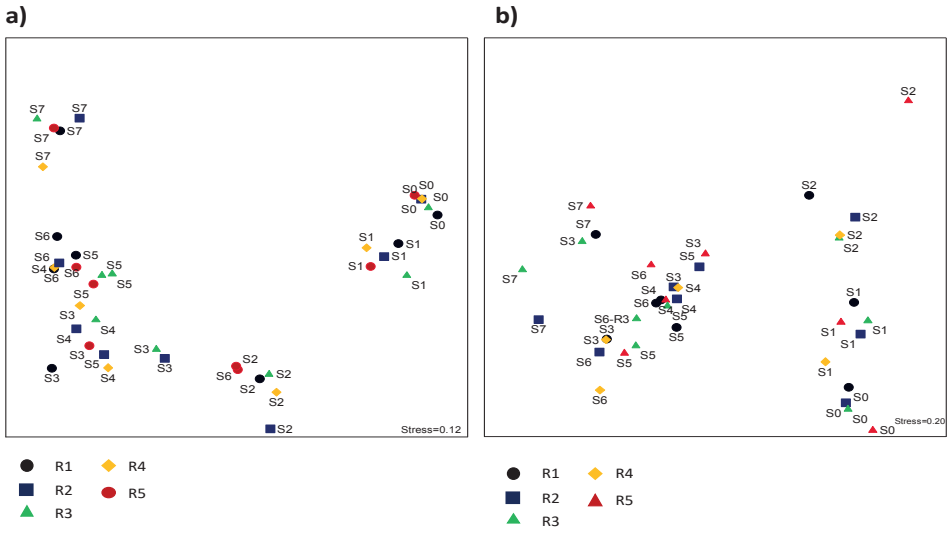


Figure 5.4 Non-metric multidimensional (NMDS) scaling of the bacterial (a) and archaeal (b) communities in five replicate anaerobic digesters derived from Bray–Curtis distance matrix. S1- S7 indicates the sampling days of 0, 40, 55, 82, 96, 120, 160 and 190, respectively.

These results indicate that the microbial composition changed, at the end of the start-up period, compared to the microbial population of the seed sludge and their similarity decreased with time.

5.4 Conclusions

This study shows that start-up of mesophilic CSTRs for cellulose and xylan degradation is possible by adopting a transient feeding strategy despite the origin of seed sludge. However, macro and micro nutrient content within the reactors should be controlled (especially iron) since these nutrients have distinct roles in regulating biochemical reactions. In this way, more rapid establishment of microbial community for efficient cellulose and xylan degradation can be obtained. Furthermore, NGS is an indispensable

tool to determine the complex microbial community. Frequent utilization of this tool during start-up helps to monitor establishment of microbial communities within the bioreactors and understand possible degradation pathways. Therefore, NGS is recommended to be used together with biochemical data for reactor performance analysis during start-up of anaerobic reactors.

5.5 Supplementary Material

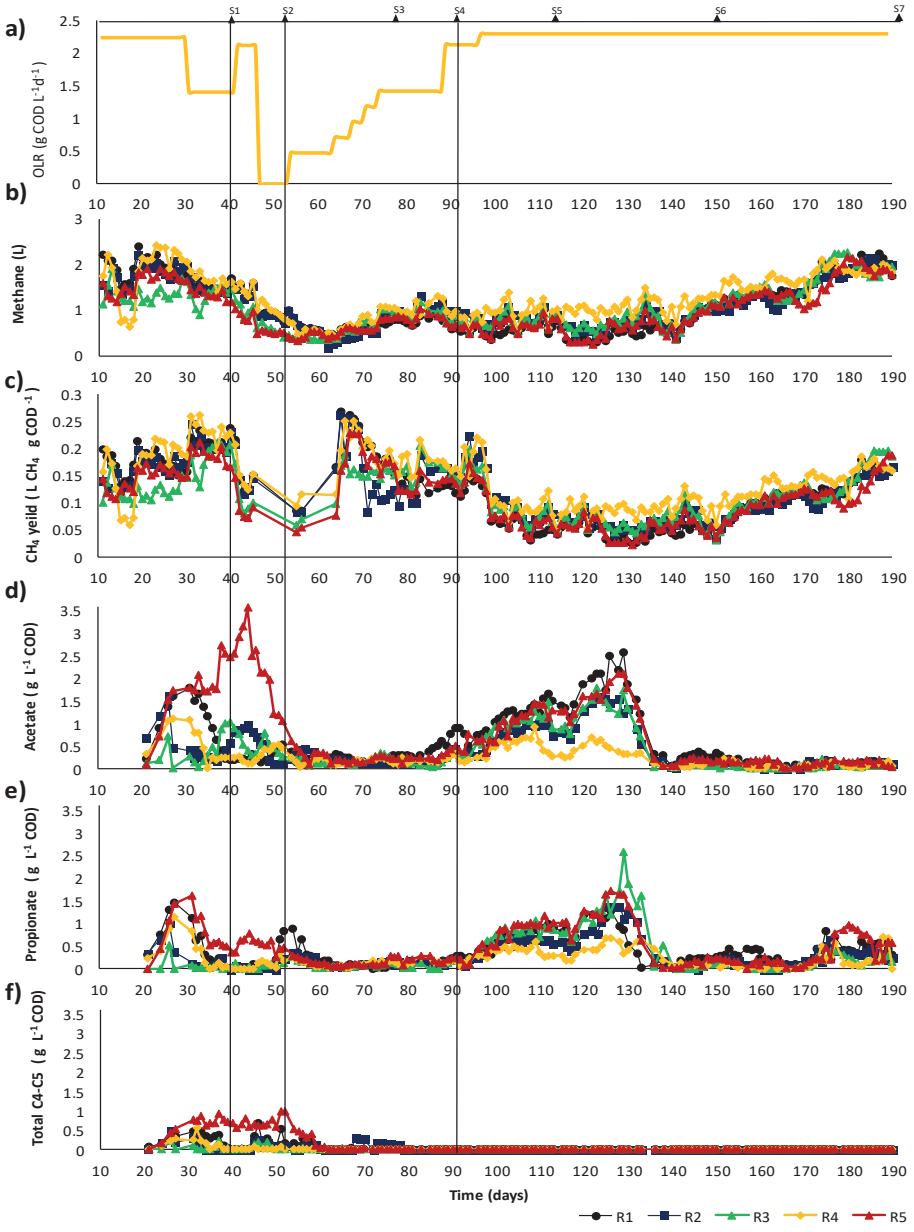


Figure S1: Reactor performances and metabolic parameters of the replicate reactors. a) OLR b) biogas production, c) methane yields d) acetate, e) propionate concentrations and f) sum of the produced butyrate, iso-butyrate and valerate concentrations."▲" marks show the sampling points for the microbiological analyses.

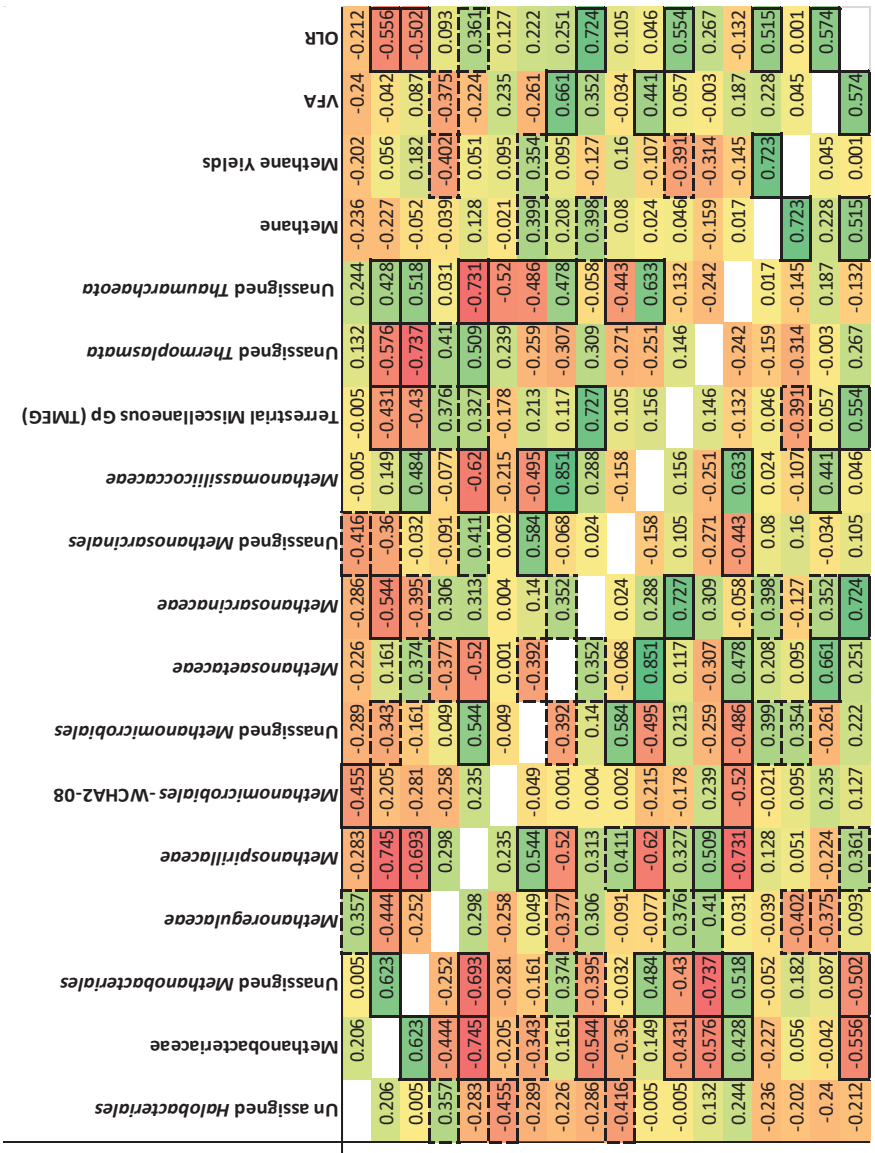
Table S1: The number of reads per sample generated after a 2 step PCR and sequencing using HiSeq Illumina sequencing platform

Sampling days	Reactor	Bacterial reads	Archaeal reads
Day 0	R1	127927	11639
	R2	232584	16816
	R3	88987	11713
	R4	19511	_
	R5	158773	12233
Day 40	R1	68239	30916
	R2	301802	24019
	R3	25922	5475
	R4	26910	2631
	R5	64207	40403
Day 55	R1	41060	1878
	R2	136353	9238
	R3	44602	5651
	R4	82778	29561
	R5	_	_
Day 82	R1	200779	49213
	R2	151470	17579
	R3	47535	_
	R4	104508	11529
	R5	71712	9468
Day 96	R1	192431	77409
	R2	509897	48780
	R3	233988	60908
	R4	188904	27412
	R5	_	_
Day 120	R1	13842	12136
	R2	63961	50857
	R3	16133	11486
	R4	41694	79770
	R5	167898	34440
Day 160	R1	1655	3137
	R2	189406	64890
	R3	110048	68904
	R4	55843	34611
	R5	17865	_
Day 190	R1	208892	18532
	R2	277196	23782
	R3	110525	1905
	R4	85254	30868
	R5	163465	14527

Table S2 Correlation matrix between operational data and the a) bacterial orders, b) archaeal families. Green colours indicates positive correlations while red colour indicates negative correlations Correlation is significant at the p= 0.05 level (2-tailed) for the groups in the solid brackets, while the dashed brackets indicates the significant correlations at p=0.01 (2-tailed)

	Bacteroidales	Unassigned *Bacteriodetes* -SB-1	Unassigned *Bacteriodetes* - SB-5	*Sphingobacteriales*	Unassigned vadinHA17	*Caldisericales*	Candidate division OP11	*Chlorobiales*	*Anaerolineales*	*Caldilineales*	Unassigned *Cyanobacteria* -4C0d-2	*Lactobacillales*	*Clostridiales*	*Nitrospirales*	*Planctomycetales*	*Desulfuromonadales*	*Syntrophobacterales*	*Enterobacteriales*	*Synergistales*	*Methane*	Methane Yields	VFA	OLR
Bacteroidales		0.062	-0.52	-0.213	-0.751	-0.184	0.584	0.647	-0.814	-0.674	0.701	0.693	-0.056	-0.54	-0.09	-0.635	-0.764	0.592	-0.169	0.225	0.026	-0.049	0.503
Unassigned ***Bacteriodetes*** **-SB-1**	0.062		-0.628	-0.296	-0.22	0.426	0.271	0.402	-0.035	-0.285	0.424	0.163	0.497	-0.632	0.537	-0.504	-0.452	0.079	0.492	0.122	0.089	0.237	0.315
Unassigned ***Bacteriodetes*** **- SB-5**	-0.52	-0.628		0.767	0.76	-0.398	-0.439	-0.646	0.371	0.518	-0.658	-0.44	-0.591	0.992	-0.574	0.672	0.762	-0.44	-0.563	-0.191	-0.185	-0.259	-0.464
Sphingobacteriales	-0.213	-0.296	0.767		0.617	-0.489	-0.244	-0.45	0.125	0.291	-0.274	-0.159	-0.727	0.76	-0.55	0.422	0.479	-0.336	-0.67	-0.031	-0.142	-0.28	-0.185
Unassigned vadinHA17	-0.751	-0.22	0.76	0.617		-0.037	-0.618	-0.702	0.614	0.611	-0.563	-0.734	-0.286	0.754	-0.104	0.61	0.78	-0.674	-0.152	-0.301	-0.161	-0.136	-0.588
Caldisericales	-0.184	0.426	-0.398	-0.489	-0.037		0.195	0.024	0.062	-0.227	0.187	-0.449	0.732	-0.396	0.817	-0.28	-0.083	-0.116	0.554	-0.33	-0.031	0.012	-0.339
Candidate division OP11	0.584	0.271	-0.439	-0.244	-0.618	0.195		0.603	-0.58	-0.735	0.614	0.378	0.152	-0.435	0.174	-0.775	-0.723	0.384	-0.179	0.084	0.023	-0.103	0.244
Chlorobiales	0.647	0.402	-0.646	-0.45	-0.702	0.024	0.603		-0.437	-0.644	0.712	0.655	0.246	-0.647	0.134	-0.594	-0.764	0.703	0.154	0.131	-0.136	0.212	0.618
Anaerolineales	-0.814	-0.035	0.371	0.125	0.614	0.062	-0.58	-0.437		0.579	-0.528	-0.503	-0.036	0.393	0.075	0.606	0.53	-0.507	0.236	-0.001	0.148	0.259	-0.279
Caldilineales	-0.674	-0.285	0.518	0.291	0.61	-0.227	-0.735	-0.644	0.579		-0.72	-0.363	-0.18	0.539	-0.256	0.714	0.727	-0.333	0.168	-0.039	-0.011	0.299	-0.206
Unassigned ***Cyanobacteria*** **-4C0d-2**	0.701	0.424	-0.658	-0.274	-0.563	0.187	0.614	0.712	-0.528	-0.72		0.468	0.266	-0.658	0.355	-0.756	-0.758	0.46	0.066	0.134	-0.02	0.025	0.424
Lactobacillales	0.693	0.163	-0.44	-0.159	-0.734	-0.449	0.378	0.655	-0.503	-0.363	0.468		-0.11	-0.44	-0.349	-0.358	-0.614	0.715	-0.079	0.339	-0.036	0.159	0.717
Clostridiales	-0.056	0.497	-0.591	-0.727	-0.286	0.732	0.152	0.246	-0.036	-0.18	0.266	-0.11		-0.581	0.708	-0.43	-0.269	0.258	0.754	-0.182	-0.043	0.339	0.057
Nitrospirales	-0.54	-0.632	0.992	0.76	0.754	-0.396	-0.435	-0.647	0.393	0.539	-0.658	-0.44	-0.581		-0.578	0.683	0.76	-0.44	-0.562	-0.188	-0.179	-0.251	-0.464
Planctomycetales	-0.09	0.537	-0.574	-0.55	-0.104	0.817	0.174	0.134	0.075	-0.256	0.355	-0.349	0.708	-0.578		-0.388	-0.25	-0.155	0.559	-0.157	0.152	0.136	-0.152
Desulfuromonadales	-0.635	-0.504	0.672	0.422	0.61	-0.28	-0.775	-0.594	0.606	0.714	-0.756	-0.358	-0.43	0.683	-0.388		0.786	-0.408	-0.148	-0.151	-0.064	-0.061	-0.389
Syntrophobacterales	-0.764	-0.452	0.762	0.479	0.78	-0.083	-0.723	-0.764	0.53	0.727	-0.758	-0.614	-0.269	0.76	-0.25	0.786		-0.455	-0.063	-0.285	-0.206	-0.058	-0.47
Enterobacteriales	0.592	0.079	-0.44	-0.336	-0.674	-0.116	0.384	0.703	-0.507	-0.333	0.46	0.715	0.258	-0.44	-0.155	-0.408	-0.455		0.246	0.118	-0.305	0.343	0.714
Synergistales	-0.169	0.492	-0.563	-0.67	-0.152	0.554	-0.179	0.154	0.236	0.168	0.066	-0.079	0.754	-0.562	0.559	-0.148	-0.063	0.246		-0.107	-0.081	0.563	0.173
Methane	0.225	0.122	-0.191	-0.031	-0.301	-0.33	0.084	0.131	-0.001	-0.039	0.134	0.339	-0.182	-0.188	-0.157	-0.151	-0.285	0.118	-0.107		0.752	0.227	0.566
Methane Yields	0.026	0.089	-0.185	-0.142	-0.161	-0.031	0.023	-0.136	0.148	-0.011	-0.02	-0.036	-0.043	-0.179	0.152	-0.064	-0.206	-0.305	-0.081	0.752		0.094	0.098
VFA	-0.049	0.237	-0.259	-0.28	-0.136	0.012	-0.103	0.212	0.259	0.299	0.025	0.159	0.339	-0.251	0.136	-0.061	-0.058	0.343	0.563	0.227	0.094		0.552
OLR	0.503	0.315	-0.464	-0.185	-0.588	-0.339	0.244	0.618	-0.279	-0.206	0.424	0.717	0.057	-0.464	-0.152	-0.389	-0.47	0.714	0.173	0.566	0.098	0.552	

a)


Un assigned Halobacteriales
Methanobacteriaceae
Unassigned Methanobacteriales
Methanoregulaceae
Methanospirillaceae
Methanomicrobiales -WCHA2-08
Unassigned Methanomicrobiales
Methanosaetaceae
Methanosarcinaceae
Unassigned Methanosarcinales
Methanomassiliicoccaceae
Terrestrial Miscellaneous Gp (TMEG)
Unassigned Thermoplasmata
Unassigned Thaumarchaeota
Methane
Methane Yields
VFA
OLR
Un assigned Halobacteriales 0.206 0.005 0.357 -0.283 -0.455 -0.289 -0.226 -0.286 -0.416 -0.005 -0.005 0.132 0.244 -0.236 -0.202 -0.24 -0.212
Methanobacteriaceae 0.206 0.623 -0.444 -0.745 -0.205 -0.343 0.161 -0.544 -0.36 0.149 -0.431 -0.576 0.428 -0.227 0.056 -0.042 -0.556
Unassigned Methanobacteriales 0.005 0.623 -0.252 -0.693 -0.281 -0.161 0.374 -0.395 -0.032 0.484 -0.43 -0.737 0.518 -0.052 0.182 0.087 -0.502
Methanoregulaceae 0.357 -0.444 -0.252 0.298 -0.258 0.049 -0.377 0.306 -0.091 -0.077 0.376 0.41 0.031 -0.039 -0.402 -0.375 0.093
Methanospirillaceae -0.283 -0.745 -0.693 0.298 0.235 0.544 -0.52 0.313 -0.062 -0.62 0.327 0.509 -0.731 0.128 0.051 -0.224 0.361
Methanomicrobiales -WCHA2-08 -0.455 -0.205 -0.258 0.235 -0.049 0.001 0.004 0.002 -0.215 -0.178 0.239 -0.52 -0.021 0.095 0.235 0.127
Unassigned Methanomicrobiales -0.289 -0.343 -0.161 0.049 0.544 -0.392 0.14 0.584 -0.495 0.213 -0.259 -0.486 0.399 0.354 -0.261 0.222
Methanosaetaceae -0.226 0.161 0.374 -0.377 -0.52 0.352 -0.068 0.851 0.117 -0.307 0.478 0.208 0.095 0.661 0.251
Methanosarcinaceae -0.286 -0.544 -0.395 0.306 0.313 0.352 0.024 0.288 0.727 0.309 -0.058 0.398 -0.127 0.352 0.724
Unassigned Methanosarcinales -0.416 -0.36 -0.032 -0.091 0.411 0.584 -0.068 0.024 -0.158 0.105 -0.271 -0.443 0.08 0.16 -0.034 0.105
Methanomassiliicoccaceae -0.005 0.149 0.484 -0.077 -0.62 -0.495 0.851 0.288 -0.158 0.156 -0.251 0.633 0.024 -0.107 0.441 0.046
Terrestrial Miscellaneous Gp (TMEG) -0.005 -0.431 -0.43 0.376 0.327 0.213 0.117 0.727 0.105 0.156 0.146 -0.132 0.046 -0.391 0.057 0.554
Unassigned Thermoplasmata 0.132 -0.576 -0.737 0.41 0.509 0.239 -0.259 -0.307 0.309 -0.271 -0.251 0.146 -0.242 -0.159 -0.314 -0.003 0.267
Unassigned Thaumarchaeota 0.244 0.428 0.518 0.031 -0.731 -0.52 -0.486 0.478 -0.058 -0.443 0.633 -0.132 -0.242 0.017 -0.145 0.187 -0.132
Methane -0.236 -0.227 -0.052 -0.039 0.128 -0.021 0.399 0.208 0.398 0.08 0.024 0.046 -0.159 0.017 0.723 0.228 0.515
Methane Yield -0.202 0.056 0.182 -0.402 0.051 0.095 0.354 0.095 -0.127 0.16 -0.107 -0.391 -0.314 -0.145 0.723 0.045 0.001
VFA -0.24 -0.042 0.087 -0.375 -0.224 0.235 -0.261 0.661 0.352 -0.034 0.441 0.057 -0.003 0.187 0.228 0.045 0.574
OLR -0.212 -0.556 -0.502 0.093 0.361 0.127 0.222 0.251 0.724 0.105 0.046 0.554 0.267 -0.132 0.515 0.001 0.574

b)

Chapter 6

Microbial community dynamics in CSTRs degrading cellulose and xylan in the presence or absence of humic acid under anaerobic conditions

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Alfons J. M. Stams, Sabina Bec, Grietje Zeeman

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Abstract

Inhibition effect of humic acid (HA) on anaerobic digestion of cellulose and xylan and the mitigation potential of the inhibition were evaluated in controlled fed batch reactors at 30°C and a HRT of 20 days. Reactor performances were evaluated by biogas production and metabolite measurements for 220 days. Microbial population dynamics of the reactors were monitored with next generation 16S rRNA gene sequencing at nine different sampling times. Our results showed that increasing levels of HA inhibited the hydrolysis efficiency of the digestion by 40 % and concomitantly reduced the methane yield. Addition of hydrolytic enzymes helped to reverse the negative effects of HA, whereas calcium addition did not reverse HA inhibition. Microbiological analyses showed that abundance of hydrolytic/fermentative bacterial groups such as *Clostridiales, Bacteroidales* and *Anaerolineales* was significantly lowered by the presence of HA. HA also affected the archaeal populations. Mostly hydrogenotrophic methanogens were negatively affected by HA. Abundance of *Methanobacteriaceae, Methanomicrobiales*-WCHA208 and *Unassigned Thermoplasmata*-WCHA1-57 were negatively affected by the presence of HA, whereas *Methanosaetacea (Methanotrichaceae)* was not affected.

6.1 Introduction

Recently, sustainable energy production has drawn great interest. Although there are many sources of sustainable energy (e.g. wind, solar, thermal etc.), specifically biomass is an attractive energy source due to its high energy potential. Traditional biomass processing is the most common way to produce energy (Kopetz, 2013; Lauri et al., 2014; Toka et al., 2014). Approximately 47% of the sustainable energy production is derived from biomass (Sawin et al., 2015). Anaerobic digestion is one of the prominent technologies to conserve energy in biomass as biogas (Appels et al., 2011; Tiwary et al., 2015; van Merbeek et al., 2015). However, available technologies for anaerobic biomass digestion can only recover around 50% of the potential energy (Liu et al., 2015a; Raposo et al., 2012). The reason for the lower energy recovery is mainly related to biodegradability of the biomass and the presence of several inhibitors (Azman et al., 2015a; Chen et al., 2008).

Pre-treatment technologies have been extensively studied to improve the biodegradability of the biomass and increase the biogas yield during anaerobic biomass digestion (Hendriks and Zeeman, 2009; Zheng et al., 2014). Physical, chemical and biological pre-treatments and combinations of these pre-treatment methods are generally applied. In many cases pre-treatment has a positive effect on biogas yield. However, inhibitory compounds usually remain within bioreactors and even additional recalcitrant molecules can be produced after the pre–treatment (Klinke et al., 2004; Negro et al., 2004). Because of that reason, more insight in the effect of inhibitory compounds on anaerobic digestion is required to achieve a more efficient methane production.

HA are inhibitors of anaerobic biomass digestion. HA have a very complex chemical structure that their presence can alter the chemistry of the environment (Davies et al., 2001). HA can be found in several environments as they are formed as a result of biological decay. HA are abundant in soil as well as in natural waters, sewage, leaching sites, anaerobic digesters treating manure and agricultural biomass (Fernandes 2010; Kang et al., 2002; Li et al., 2011). Although, the ecological role of the HA in nature is well documented, there are only few papers that describe the (negative) effects of HA on anaerobic cellulosic biomass hydrolysis (Azman et al., 2015b; Brons et al., 1985; Fernandes et al., 2015). The exact mechanism of HA inhibition on hydrolysis is not

known, but binding properties of HA to hydrolytic enzymes are proposed to explain the inhibition (Fernandes et al., 2015). HA may play an important role in the low biogas production within biogas plants in which cellulose and xylan are highly abundant (Vassilev et al., 2010). Thus, negative effects of HA on anaerobic digestion should be taken away to improve biogas production.

Removal of HA can be an option to overcome the negative effects. Indeed, removal of HA from drinking water treatment plants by membrane filtration systems has been successfully achieved (Ren et al., 2015). On the other hand, extraction, absorption, ion exchange, coagulation and flocculation processes have been proposed to remove HA from several matrices (Li et al., 2014a; Song et al., 2013; Tan, 2014). Aforementioned methods are not suitable for anaerobic digesters due to the high solid content of the biomass. Thus, different approaches are needed to reverse the inhibitory effects of HA. Two different approaches have been described to overcome HA inhibition in anaerobic digesters. These are hydrolytic enzyme addition and polyvalent cation addition (Azman et al., 2015b; Brons et al., 1985; Fernandes et al., 2015). Addition of hydrolytic enzymes and polyvalent cations can reduce the active binding sites of the HA. In this way, scavenging of the hydrolytic enzymes by HA might be minimised and microbial conversion can proceed. Although these methods reversed the HA inhibition on anaerobic digestion, all the reported experiments were conducted in batch incubations. Therefore, their application possibilities to large scale digester operations are still unclear.

This study investigated HA inhibition on anaerobic cellulose and xylan digestion and the mitigation of the HA inhibition with three objectives. Firstly, to confirm the HA inhibition in fed batch reactors and secondly, to show the feasibility of calcium and hydrolytic enzyme addition to reverse the inhibitory effects of HA. Thirdly, to investigate the effect of HA on the microbial community. In this scope, we operated five fed batch anaerobic reactors in parallel. One reactor was used as a control reactor (R1), whereas the other reactors were used as test reactors (R2-R5). In the test reactors, increasing levels of HA were applied (R2-R5), while additional treatments of calcium addition (R3), hydrolytic enzyme addition (R4) and combination of hydrolytic enzyme and calcium addition (R5) were applied. Reactor performance and microbial community composition were evaluated for 210 days. Correlations between microbial

population dynamics and operational parameters were made to couple reactor performances to microbial population dynamics.

6.2 Material and methods

6.2.1 Operation of CSTRs

In total, 5 lab-scale anaerobic double wall CSTRs (Completely Stirred Tank Reactor) (total volume 6L; working volume 5L) were operated in parallel. All reactors were inoculated with crushed anaerobic granular sludge which was taken from a full scale UASB reactor, treating pulp and paper industry effluents (Industriewater Eerbeek, Eerbeek, The Netherlands). Each reactor was equipped with water jackets that were connected to a water bath, circulating water to the water jackets. Constant temperature was assured for each individual reactor at 30±0.5 °C and operational pH was kept between 6.8 and 7.2 by adding 5 M NaOH when necessary. Continuous stirring of the reactors was obtained by anchor type propellers at 100 rpm.

Following the inoculation of the reactors, seed sludge was acclimatised to the reactor environment at 30 °C with 1.8 g VS $L^{-1}day^{-1}$ organic loading rate (OLR) and a hydraulic retention time (HRT) of 20 days for 210 days. A starch, glucose and VFA mixture (acetate, propionate and butyrate) was fed for 55 days; after that the feeding continued with a cellulose and xylan mixture till day 210. After the acclimation period, five reactors were fed every day for another 220 days with synthetic medium, using cellulose (avicel; PH-101, Fluka, Darmstadt) and beech wood xylan (Roth, Karlsruhe, Germany) at a ratio of 75:25 (w/w). OLR was kept at 1.8 g VS $L^{-1}day^{-1}$. The feed was mixed with mineral based medium which was previously described (Plugge, 2005; Stams et al., 1993), with additional 100 mg L^{-1} $Fe_2(SO_4)_3$ and omitting reducing agents to maintain a HRT of 20 days. After methane production stabilised (P0), HA addition and inhibition mitigation experiments were started. Humic acid (Sigma-Aldrich; CAS Number: 68131-04-4) addition was initiated, starting from day 30, for four reactors (R2, R3, R4 and R5) while R1 was used as a control reactor. HA was added every day in increasing concentrations 2, 20, 40, 100 and 400 mg L^{-1} for different periods (P1 (day 31-50), P2-P3 (day 50-91), P4 (day 92-125), P5 (day 126-146) and P6 (147-173), respectively) until reaching inhibition. Starting from P5, a few drops of silicon oil (Sigma-Aldrich, Darmstadt, Germany) were added to the reactors to prevent foaming, when necessary.

After an observed inhibition, HA addition was stabilised (P7- P8; day 173-220) to test whether HA inhibition remained stable. Operation conditions are summarised in Table 6.1.

Table 6.1 Operational conditions of the reactors and the time periods that were used in the experiments.

Operation days	R1			R2			R3			R4			R5		
	HA ad. (mg L d^{-1})	Ca ad.	Enzyme ad.	HA ad. (mg L d^{-1})	Ca ad.	Enzyme ad.	HA ad. (mg L d^{-1})	Ca ad.	Enzyme ad.	HA ad. (mg L d^{-1})	Ca ad.	Enzyme ad.	HA ad. (mg L d^{-1})	Ca ad.	Enzyme ad.
0-30 (P0)	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_
31-50 (P1)	_	_	_	2	_	_	2	+	_	2	_	+	2	+	+
51-70 (P2)	_	_	_	20	_	_	20	+	_	20	_	+	20	+	+
71-91 (P3)	_	_	_	20	_	_	20	+	_	20	_	+	20	+	+
92-125 (P4)	_	_	_	40	_	_	40	+	_	40	_	+	40	+	+
126-146 (P5)	_	_	_	100	_	_	100	+	_	100	_	+	100	+	+
147-173 (P6)	_	_	_	400	_	_	400	_	_	400	_	+	400	_	+
174-194 (P7)	_	_	_	400	_	_	400	_	_	400	_	_	400	_	_
195-220 (P8)	_	_	_	400	_	_	400	_	_	400	_	_	400	_	_

R3, R4 and R5 were used to test three different mitigation strategies for HA inhibition. $CaCl_2$ (Sigma-Aldrich) was added to R3 and R5; 0.11g $CaCl_2$ g HA $_{added}^{-1}$ was supplied within different periods (P1, P2, P3, P4 and P5). Three enzyme cocktails were obtained from DSM (Delft, The Netherlands) and were used for the enzyme addition experiments. All enzymes were multicomponent mixtures that had side enzyme activities. The first enzyme was a cellulase, which had cellulase, β-glucanase and xylanase activity. Cellulase was produced by a commercial *Trichoderma* strain and contained 100 mg protein ml^{-1}. The second enzyme was an endoglucanase, which had β-glucanase, cellulase and xylanase activity. Endoglucanase was produced by a commercial *Talaromyces* strain and contained 90 mg protein ml^{-1} (suspension was prepared with demi-water at 10 mg protein ml^{-1}). The third enzyme was a xylanase which had xylanase and β-glucanase activity. Xylanase was produced by a commercial *Aspergillus* strain and contained 150 mg protein g^{-1}. Both enzymes were dosed to the reactors based on protein content. The amount of added enzymes was expressed as mg protein per humic acids (HA) added to the reactors: 0.6 mg cellulase mg HA $_{added}^{-1}$, 0.075 mg xylanase mg HA $_{added}^{-1}$ and 0.55 mg endoglucanase mg HA $_{added}^{-1}$ for P1 to P5

and the enzyme amount was reduced by half for P6. Enzyme addition was stopped in P7 and P8.

Biogas production was monitored by a gas flow measurement device (μflow, Bioprocess Control, Lund, Sweden) and recorded daily. Cumulative biogas production was recorded daily and expressed in mL at STP (Standard Temperature & Pressure: 0° C, 1 atm). Biogas composition was quantified biweekly via gas chromatograph (Interscience GC 8000 series) equipped with a thermal conductivity detector and two columns (Molsieve 5A 50 m × 0.53 mm for hydrogen, nitrogen and methane and Porabond Q 50 m × 0.53 mm for CO_2. Temperatures of injector, detector and oven were 110, 99 and 50 °C, respectively. Organic acids were quantified using a Thermo Scientific Spectrasystem HPLC system, equipped with a Varian Metacarb 67H 300 × 6.5 mm column kept at 45°C, running with 0.005 M sulphuric acid as eluent. The eluent had a flow rate of 0.8 $ml.min^{-1}$. The detector was a refractive index detector. Data analyses were performed using ChromQuest (Thermo Scientific, Waltham, MA). The total organic acid concentrations were expressed as their COD equivalents (mg L^{-1} COD) of measured acetate and propionate concentrations. Hydrolysis, acidogenesis and methanogenesis efficiencies were calculated with the formulas that were described previously (Azman et al., 2015b). The biological methane potential (BMP: ml CH_4 ml enzyme $mixture^{-1}$) of the enzyme mixture was measured as described previously (Azman et al., 2015b). Since the methane production from the enzyme mixtures contributed to the total methane yields significantly, methane production in R4 and R5 were corrected for the amount of methane that was derived from the enzyme mixtures.

6.2.2 Microbial community monitoring by next generation 16S rRNA amplicon sequencing

50 mL sludge samples were collected in the beginning and at the end of each period. Samples were kept at -20 °C prior to genomic DNA extraction. Genomic DNA extraction from the nine sampling points (P0 toP8) was performed using Fast DNA® SPIN kit for soil (MP Biomedicals, OH, USA) following the manufacturers protocol with additional washing steps before starting to the extraction. 1X PBS solution with 0.5 mM EDTA was used to wash pellets two times to remove the HA from the solids which could be inhibitory for the PCR reactions. The DNA yields were measured with a Nanodrop®

(ND-1000) spectrophotometer (Nanodrop Technologies, Wilmington, DE). DNA qualities were checked using the OD 260/280 ratio. Samples that had 1.80± 0.15 260/280 values considered as good quality DNA and amplicon sequencing was performed with those samples.

Extracted DNA from selected samples was used for bacterial and archaeal community analysis. The amplification of bacterial and archaeal gene fragments was done using a 2-step PCR. First amplification of bacterial 16 S rRNA gene fragments was done using the 27 F- DegS (5′-GTT[TC]GAT[TC][AC]TGGCTCAG-3′) (van den Bogert et al., 2011 and 2013) and equimolar mix of two reverse primers; 338R-I and 338-R-II (5′-GC[AT]GCC[AT]CCCGTAGG[TA]GT-3′) (Daims et al., 1991) and the first amplification of archaeal 16S rRNA gene fragments was done using primers 518F (5′-CAGC[AC]GCCGCGGTAA-3′) (Wang and Qian, 2009) and 905R (5′-CCCGCCAATTCCTTTAAGTTTC-3′) (Kvist et al., 2007). PCR amplifications were carried out in technical duplicates in a total volume of 50 µl containing 500 nM of each forward and reverse primer (Biolegio BV, Nijmegen, The Netherlands), 1 unit of Phusion DNA polymerase (Thermo Scientific, USA), 10 µl of HF-buffer, 200 µM dNTP mix, 1 µl DNA template, made to a total volume of 50 µl with nuclease free sterile water. The PCR program was as follows: denaturing at 98°C for 30 s, followed by 25 cycles of denaturing at 98°C for 10 s, annealing at 56°C for bacterial and 60 °C for archaeal for 20 s, extension at 72°C for 20 s, followed by a final extension step at 72°C for 10 min. After positive amplifications, technical duplicates were pooled and prepared for the second step PCR amplification. A second amplification was performed to extend 8 nt barcodes to the amplicons, as described previously (Hamady et al., 2008). Barcoded amplification was performed in a total volume of 100 µl containing 5 µl of the first PCR product, 500 nM of each forward and reverse primer (Biolegio BV, Nijmegen, The Netherlands), 2 units of Phusion DNA polymerase (Thermo Scientific, Waltham, MA), 20 µl of HF-buffer, 200 µM dNTP mix, made to a total volume of 100 µl with nuclease free water. The PCR program was as follows: denaturing at 98°C for 30 s, followed by five cycles of denaturing at 98°C for 10 s, annealing at 52°C for 20 s, extension at 72°C for 20 s, followed by a final extension at 72°C for 10 min. Barcoded PCR products were cleaned using the HighPrep PCR clean-up system (MagBio Genomics Inc., Gaithersburg, MD). DNA was quantified using Qubit (Invitrogen, Bleiswijk, The Netherlands). After the

second PCR, barcoded samples were pooled in equimolar quantities to create a library. The libraries were purified again by using the same purification protocol Prepared libraries were sent to GATC company (Konstanz, Germany) for Hiseq sequencing on the Illumina platform.

6.2.3 Sequencing data analyses

16S rRNA gene sequencing data was analysed using NG-Tax, an in-house pipeline (Ramiro-Garcia et al., 2016). Paired-end libraries were filtered to contain only read pairs with perfectly matching barcodes, and those barcodes were used to demultiplex reads by sample. Resulting reads were separated by sample using the affiliated barcodes. Taxonomy affiliation was done with the SILVA 16S rRNA reference database by using an open reference approach as described by Quast et al. (2013). Quantitative Insights into Microbial Ecology (QIIME) v1.2 (Caporaso et al., 2010) was used to define microbial compositions based on the described pipeline. The project was deposited to NCBI's Sequence Read Archive (SRA) under project number PRJNA320994.

6.2.4 Statistical analyses

Significant differences between reactor operational parameters were checked with one-way ANOVA test. When the ANOVA rules were matched, Posthoc tests (Tukey's honest significant difference test) were applied to further compare the operational data. Differences were considered statistically significant at a p value <0.05, or otherwise stated.

The influence of process parameters on the microbial community composition was analysed using Redundancy analyses (RDA) with the CANOCO software (version 5) (Šmilauer and Jan, 2014). The significance test for RDA was carried out by Monte Carlo permutation (499 times) and correlations were considered significant at a p value <0.05. Ranked Spearman correlation was also applied to determine the correlation between microbial groups and operational conditions. All statistical and correlation analyses were performed by IBM SPSS Statistics 23.

6.3 Results

6.3.1 Anaerobic digester performance

'Steady-state' conditions, in which stable methane production and effluent VFA concentrations were reached (El-Mashad et al., 2004), were achieved before initiation of the HA inhibition experiments with an HRT of 20 days. The complete operation time of the reactors was divided into 8 different periods as given in Table 6. 1. Different time periods also reflect the sampling points for the microbiological analyses.

Figure 6.1, Figure 6.2 and Table S1 show the process parameters of each reactor. Until the end of P3, all the reactors followed similar trends in terms of hydrolysis, acidogenesis and methanogenesis efficiencies. During these periods, Hydrolysis, acidogenesis and methanogenesis efficiencies of the reactors were calculated to be 51±4%; a stable reactor performance was shown. In all reactors, some acetate and propionate were present and acetate was the dominant VFA.

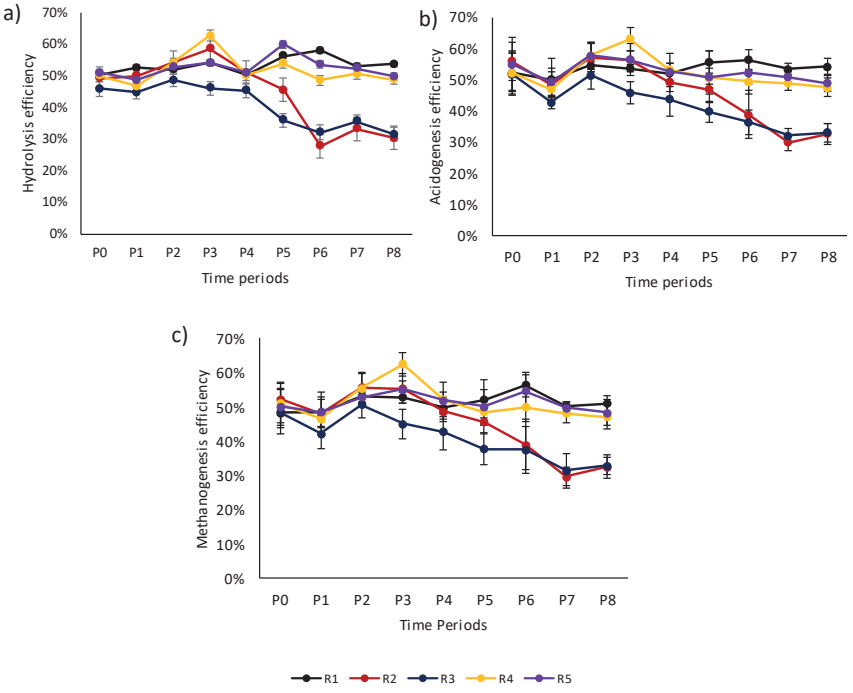


Figure 6.1 Hydrolysis, acidogenesis and methanogenesis efficiencies (%) of the reactors throughout the experiment. Each data point represents the average efficiencies within the mentioned time period. The reactors were represented with R1 (control), R2 (inhibition), R3 (Ca addition), R4 (enzyme addition) and R5 (Ca and enzyme addition).

The average total VFA concentration in the reactors was 188±140 mg L^{-1} COD. Measured average daily biogas production in the reactors was 3995±362 mL and the average methane content of the produced biogas was 51±1% (Figure 6.2 and Table S1). After P3, hydrolysis efficiencies in R2 and R3 decreased gradually from 51±4 % to 30 ± 5 % throughout the experiment due to the increasing concentration of HA (Figure 6.1). Acidogenesis and methanogenesis efficiencies concomitantly decreased due to the restrained hydrolysis. The average total VFA concentration in those reactors remained similar as compared that in the former periods and below 100 mg L^{-1} COD (Figure 6.2 and Table S1). Average daily biogas production and the methane content of the reactors also reduced after P4. During the P7 and P8, measured average daily biogas production in R2 and R3 was 2680±10 mL which was significantly lower than that in the other reactors (Figure 6.2 and Table S1). In contrast to R2 and R3, the performance of the other reactors stayed stable throughout the experiment. Hydrolysis efficiencies were calculated to be 53±3 % for R1, R4 and R5 after P4. Acidogenesis and methanogenesis efficiencies coincided with hydrolysis efficiencies which showed the process stability of the reactors. VFA concentration in R1 was significantly higher than the other reactors, around 350 mg L^{-1} COD from P5 to P8, whereas, VFA concentration in R4 and R5 remained low and was not significantly different. In R1, R4 and R5 daily biogas production showed similar trends; 4019 ±111 mL with a methane content of 50±1% (Table S1).

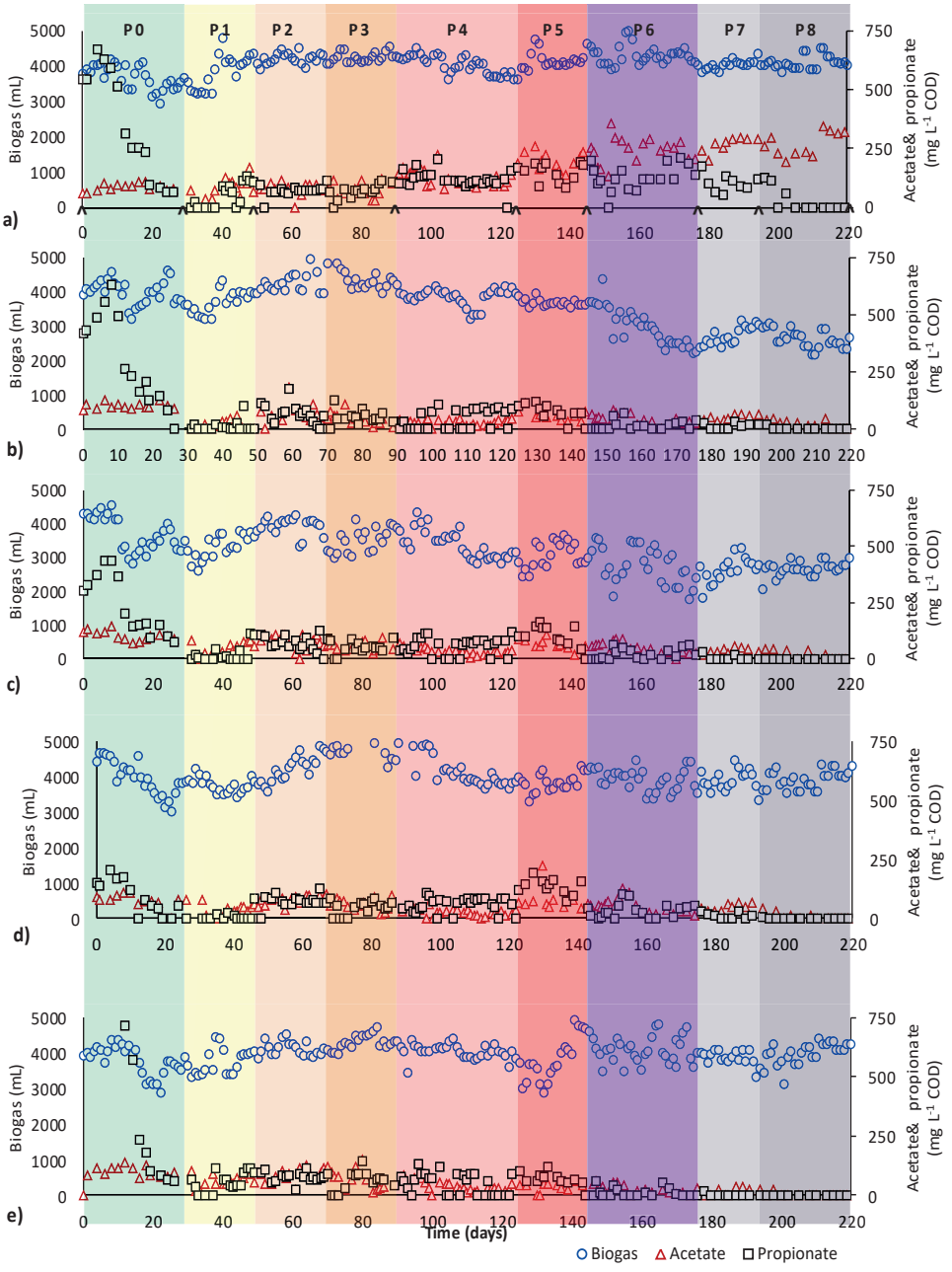


Figure 6.2 Daily biogas and VFA production of the a) R1 (control), b) R2 (inhibition), c) R3 (Ca addition), d) R4 (enzyme addition) and e) R5 (Ca and enzyme addition). "^" marks the sampling time points for the microbiological analyses.

6.3.2 Bacterial community composition

The number of reads per sample that was obtained by next generation sequencing varied from 1015 to 418163 (Table S2). The reads were assigned to eleven different phyla, seventeen classes and twenty orders that were abundant at least 1% of the reads in the samples. The dominant bacterial populations for all the reactors affiliated with the phyla *Bacteriodetes*, *Firmicutes* and *Chloroflexi*. In total 78±12 % of the total reads were assigned to those 3 phyla (Figure 6.3).

Variations in diversity in lower taxa levels were also observed. In average 28 ±11 % of the reads could not be assigned at family level, indicating that some of the bacterial populations within the anaerobic sludge remained uncharacterised. At the level of order, *Lactobacillales* (20±12%), *Anaerolineales* (19±9%), *Bacteroidales* (15 ±9%) and *Clostridiales* (13±7%) were the most abundant within all the reactors throughout the whole experiment (Figure 6.3). Members of these 4 orders were present in all samples, suggesting that they shaped the core bacterial population involved in anaerobic cellulose and xylan degradation.

6.3.3 Archaeal community composition

The number of reads per sample that was obtained by next generation sequencing varied from 1029 to 170459 (Table S2). The samples (R1-P6, R5-P3, P6 and P8) that had lower than 1000 reads were not included in the statistical analyses but they were represented in Figure6.4. All reads were assigned to phylum *Euryarchaeota*, within four different classes, six orders and nine families abundant in at least 1% of the reads in the samples. 4±3 % of the reads could not be assigned at family level. The dominant archaeal population in all reactors at the family level were members of *Methanospirillaceae* (37±21%), *Methanobacteriaceae* (27±19%), *Methanoregulaceae* (10±14%) and *Methanosaetaceae* (*Methanotrichaceae*) (10 ±8%) (Figure 6.4). On average, 81±11 % of the reads affiliated with those 4 families in all the reactors. Except *Methanosaetaceae* (*Methanotrichaceae*) and *Methanosarcinaceae*, which have members that perform acetoclastic methanogenesis, the other family groups included hydrogenotrophic methanogens. Beside the aforementioned families, members of the order *Methanosarcinales, Methanomicrobiales, Thermoplasmatales* were also detected at low levels (<5%) within the reactors in various abundance (Figure 6.4).

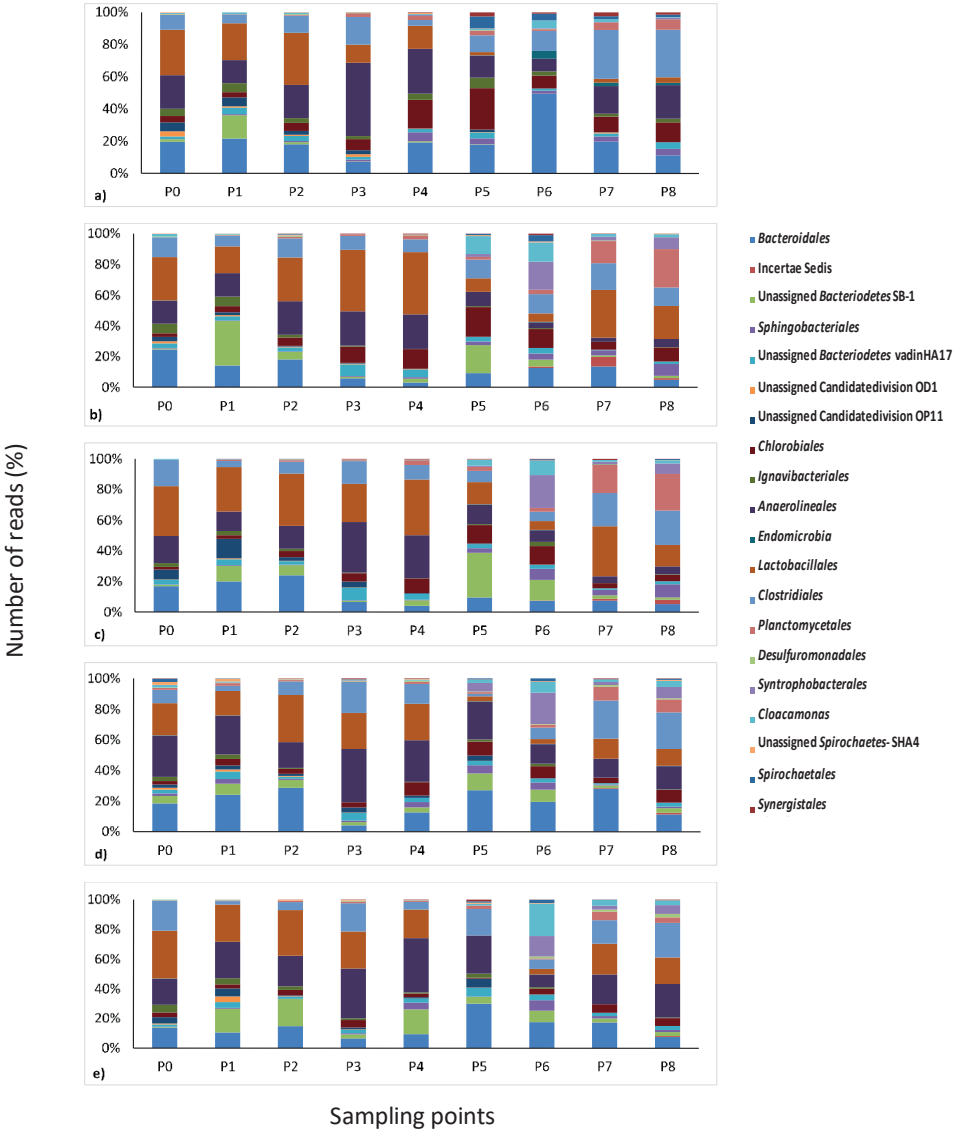


Figure 6.3 Relative abundance of the bacterial community in the reactors a) R1 (control), b) R2 (inhibition), c) R3 (Ca addition), d) R4 (enzyme addition) and e) R5 (Ca and enzyme addition). Only the orders were presented that were abundant at an abundance >1 % in at least one sample. The term unassigned was used to indicate the bacterial groups that were not classified at order level.

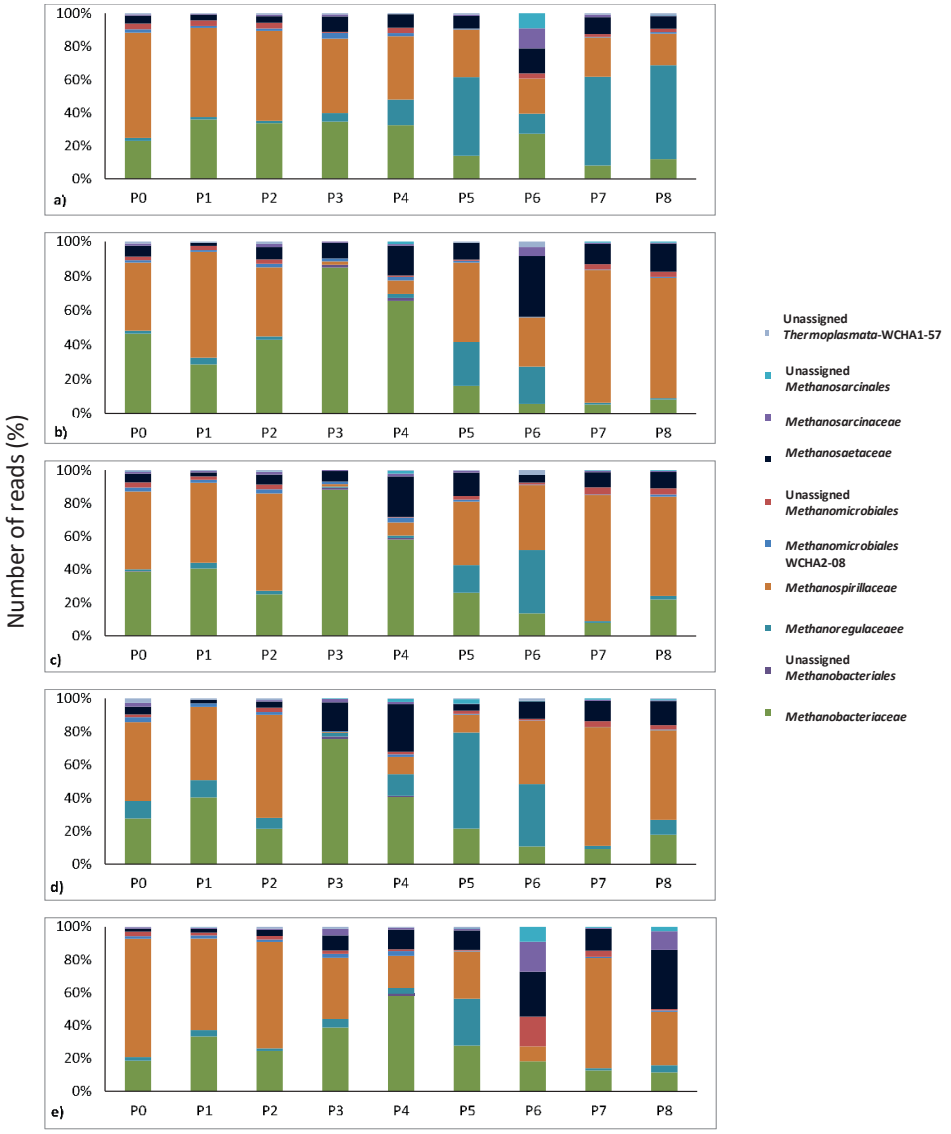


Figure 6.4 Relative abundances of the archaeal community in the reactors a) R1 (control), b) R2 (inhibition), c) R3 (Ca addition), d) R4 (enzyme addition) and e) R5 (Ca and enzyme addition). Only the orders were presented that were abundant at an abundance>1 % in at least one sample. The term unassigned was used to indicate the bacterial groups that were not classified at order level.

6.4 Discussion

6.4.1 Effect of humic acid addition on digester performance

The effect of HA on the anaerobic digestion of xylan and cellulose was evaluated by calculating hydrolysis, acidogenesis and methanogenesis efficiencies. Daily addition of HA at concentration as high as 40 mg L^{-1} to the reactors did not show any significant effect till the end of P3. After that period, performance of R2 reduced compared to R1 (blank control). Slow reduction in hydrolysis efficiency was related to increased HA addition. Hydrolysis efficiency of R2 decreased from 50±5 % to 30±1% during the 400 mg $L^{-1}d^{-1}$ HA addition (Figure 6.1, Table S1). The reduction of the acidogenesis and methanogenesis efficiencies was connected to the hydrolysis inhibition as no increase in intermediate products was observed. Hydrolysis efficiency of R2 was reduced by 40%, compared to the control reactor when the HA concentrations reached up to around 8 g L^{-1}. This confirms the previous observations of HA inhibition in batch incubations (Azman et al., 2015b; Fernandes et al., 2015). Restrained hydrolysis in R2 influenced the subsequent steps of the anaerobic digestion, causing reduced biogas production after P3. However, the degree of inhibition was not similar between the reported inhibition levels. Fernandes et al. (2015) extracted HA from maize and manure and reported inhibitory concentrations of HA on batch-wise enzymatic cellulose degradation as low as 0.5 g L^{-1}, whereas Azman et al. (2015b) reported 50 % inhibition on anaerobic degradation of cellulose by using commercially available humic acid salts at 5 g L^{-1} concentrations in batch incubations. As can be understood from previous studies, when comparing the reported inhibition values, it is not possible to define a specific HA inhibition value for anaerobic digestion. This is mainly related to the composition and dosing strategies of the HA used in the studies. HA are complex molecules, their compositions vary drastically with the source of HA, extraction and preparation methods (Tan, 2014). The effects of HA seem to be case specific and should be evaluated separately for each bioreactor and used feedstock. In this study, we observed hydrolysis inhibition around 8 g L^{-1} by using the same stock of HA that was used previously (Azman et al., 2015). The observed inhibition concentration in our study are much higher than the reported HA concentrations from plant material and manure (Fernandes, 2010). The main reason for the differences in the observed inhibition levels can be related to

adaptation capacity of the microbial community to elevated HA concentrations. Step wise increase of the HA might enable the microbial communities to adapt to the higher concentrations. Potential acclimation of microorganisms can be possible either via production of more hydrolytic enzymes as Fernandes et al. (2015) suggested or production of EPS like molecules by different microbial communities to prevent HA to penetrate the active cells and disrupt the cell integrity (Prokhotskaya and Steinberg, 2007). Additionally, aforementioned studies mainly reported acute effects of HA. Because of that, HA inhibition may be different in continuous reactor systems when compared to batch systems and show more chronic effects.

6.4.2 Mitigation of humic acid inhibition by calcium and hydrolytic enzyme addition

$CaCl_2$ was added daily to R3 and R5 to evaluate the potential of calcium to mitigate HA inhibition. Positive effects of calcium addition to overcome HA inhibition were reported previously in batch-wise incubations. Brons et al. (1985) reported the mitigation of HA inhibition on potato starch hydrolysis via $CaCl_2$ addition and Azman et al. (2015b) observed similar effects on cellulose hydrolysis. In general, calcium is considered an essential macro nutrient to support microbial growth and aggregate formation. Addition of $CaCl_2$ is known to have a stimulatory effect on anaerobic digestion in the range of 100 to 3000 mg L^{-1} (Chen et al., 2008; Romero-Guiza et al., 2016). However, our study showed different results compared to previous studies. In R3, in which 0.11g $CaCl_2$ g HA $_{added}^{-1}$ was added as mitigation agent for HA inhibition, hydrolysis efficiencies remained similar to the reactor in which HA were added daily without $CaCl_2$ addition (R2). Our results might be explained by restrained surface availability of cellulose and xylan particles due to the formation of HA-calcium precipitates (Alverez et al., 2004). Precipitates that accumulated in the CSTRs could have prevented adhesion of the microorganisms to the cellulose and xylan particles which were crucial for hydrolytic activity. Another reason could be related with the calcium addition strategy. Azman et al. (2015b) used pulse addition of 5 mM $CaCl_2$ whereas, in this study semi-continuous addition of $CaCl_2$ was applied. Pulse addition of 2.5 mM $CaCl_2$ at short HRTs (2-4 hours) was shown to even enhance anaerobic digestion of sucrose by a mixed culture (Yuan et al., 2010). Therefore, the observations of Azman et al. (2015b) might

be more related to enhancement of microbial activity, leading to more enzyme production rather than mitigation of the HA inhibition.

On the other hand, enzyme addition to R4 and R5 showed a positive effect to overcome HA addition. Since the enzyme mixtures were partially a source for methane production in R4 and R5, the amount of methane that could be derived from enzymes was subtracted from overall methane production. The methane production from 1 ml enzyme mixture was determined as 70, 80 and 60 ml methane for cellulase, xylanase and endoglucanase, respectively. After subtraction, hydrolysis, acidogenesis and methanogenesis efficiencies were calculated. As a result, the calculated net efficiencies in these reactors were found to be similar to the efficiency of the control reactor (Figure 6.1, Table S1). These results indicate that the effects of HA were reversed by enzyme addition. Two hypotheses can be postulated to explain the positive effect of the enzyme addition: i) additional hydrolytic enzymes can attach to humic acids, preventing their scavenging behaviour against intrinsic hydrolytic enzyme production by abundant hydrolytic bacteria within anaerobic sludge (Fernandes et al., 2015). ii) competition between HA and enzymes to bind the cellulose particles. Lignin has similar functional groups as HA and Vermaas et al. (2015) found that lignin preferentially binds to the hydrophobic side of the cellulose and also to the specific residues on the cellulose-binding modules of the enzymes that are critical for cellulose binding to cellulases. Our results can support both hypotheses by showing that the intrinsic enzyme production from hydrolytic bacteria was capable of maintaining the hydrolytic activity when binding sites of HA were inactivated by enzyme addition or preventing HA to bind cellulose particles.

Surprisingly, when enzyme and calcium were added together, the same effect was observed as with the sole enzyme addition. For R3, we hypothesised that precipitates of the HA-Ca complex might cover cellulose and xylan particles, preventing enzyme adhesion and consequently lower the biodegradation. In contrast to R3, in R5 enzyme-humic acid binding might be stronger than enzyme-calcium bindings and affinity of enzymes to HA might be higher than to calcium. It is known that hydrolytic enzymes (especially β-glucosidases) form very strong bonds in soil environments (Ceccanti et al., 2008). Once active binding sites of the HA are occupied by hydrolytic enzymes, it is more likely that calcium-HA complexes are not formed and consequently

calcium cations could enhance anaerobic digestion as discussed previously (Yuan et al., 2010; Romero-Guiza et al., 2016). However, this hypothesis needs further studies to be proven.

6.4.3 Effect of humic acid, enzyme and calcium addition on bacterial and archaeal community composition.

The composition of bacterial and archaeal communities plays an important role in anaerobic cellulose and xylan degradation. Addition of HA showed a selective effect on bacterial and archaeal communities. As the HA concentration increased, hydrolysis became restrained and therefore bacterial and archaeal compositions shifted in the reactors R2 to R5. Shifts in the microbial communities occurred after P4 when HA inhibition was observed. Variations in bacterial and archaeal community composition for all reactors in different operational periods were given in Figure S1.

Members of *Bacteriodetes* and *Firmicutes* are well known fermentative hydrolytic bacteria that are responsible for anaerobic cellulose degradation in many biogas plants (Azman et al., 2015a; Campanaro et al., 2016; De Vrieze et al., 2015a; Stolze et al., 2015; Westerholm et al., 2016). In all reactors, abundance of *Bacteroidales* and *Clostridiales* indicated that these were the key players in the hydrolysis of cellulose and xylan. *Bacteroidales* were more dominant in the reactors in which hydrolysis was not inhibited (R1, R4 and R5) than R2- R3 (hydrolysis inhibition). Their abundance was associated with biogas production and correlated with VFA concentrations ($r = 0.372$, $p<0.01$) (Figure 6.5). Therefore, they are important for xylan and cellulose degradation forming intermediate products such as short chain fatty acids and H_2. In the presence of HA, abundance of *Bacteroidales* was reduced by up to 30 % ($r=-0.326$, $p<0.05$) at increasing concentrations of HA in R2 and R3 while their abundance within the other reactors stayed relatively stable (Figure 6.3). In the presence of HA, abundance of *Clostridiales* was correlated with the presence of HA than *Bacteroidales,* suggesting that ongoing hydrolysis in R4 and R5 might be maintained by members of this order (Figure 6.5). *Anaerolineales* was the other abundant bacterial order within all reactors. *Anaerolineales* significantly correlated with biogas production ($r = 0.477$, $p<0.001$) (Table S3). Members of *Anaerolineales* are known to ferment sugars in anaerobic

digesters (Ambuchi et al., 2016; de Vrieze et al., 2015b) and they have a role in degradation of a variety of carbohydrates, including xylan (Yamada et al., 2007).

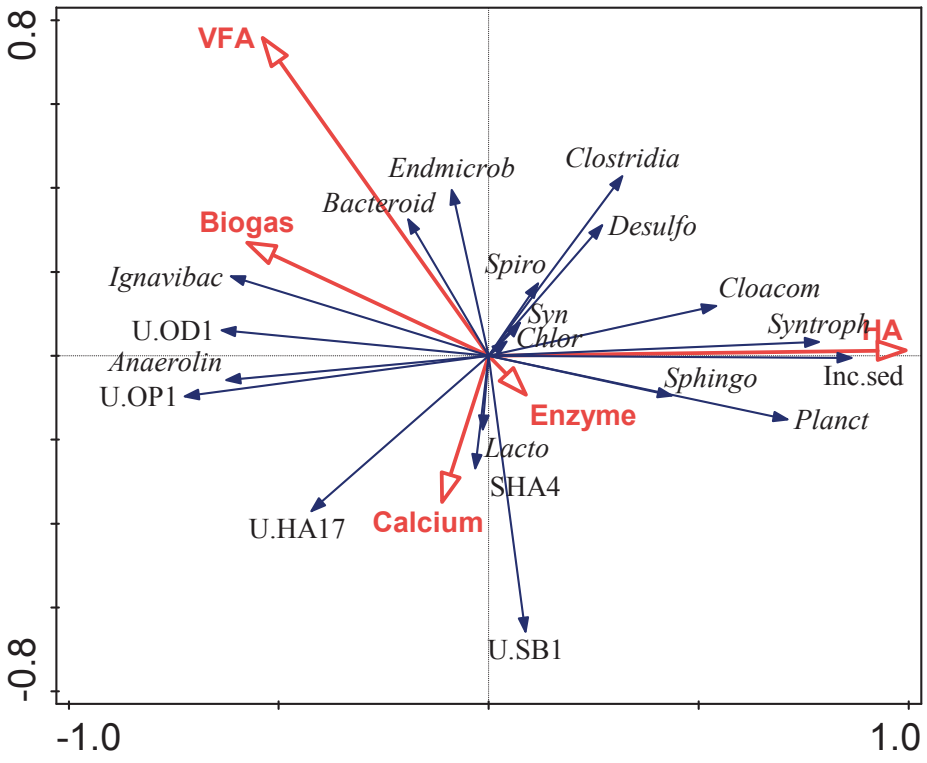


Figure 6.5 Redundancy analysis (RDA) ordination diplot for the bacterial community. *Red vectors* represent the influence of operational parameters biogas production (Biogas), total volatile fatty acids (Total VFA), humic acids (HA), calcium addition (calcium), enzyme addition (enzyme). Blue vectors represent bacterial orders : *Bacteroidales* (*Bacteroid*), Incertae Sedis (Inc.sed), Unassigned *Bacteriodetes* SB-1 (U.SB1), *Sphingobacteriales* (*Sphingo*), Unassigned *Bacteriodetes* vadin HA17 (U.HA17),Unassigned Candidate division OD1 (U.OD1), Unassigned Candidate division OP11 (U.OP1), *Chlorobiales* (*Chlor*), *Ignavibacteriales* (*Ignavibac*), *Anaerolineales* (*Anaerolin*), Lineage I (*Endmicrobe*), *Lactobacillales* (*Lacto*), *Clostridiales* (*Clostridia*), *Planctomycetales* (*Planct*), *Desulfuromonadales* (*Desulfo*), *Syntrophobacterales* (*Syntroph*), *Cloacamonas* (*Cloacom*), Unassigned *Spirochaetes*-SHA4 (SHA4), *Spirochaetales* (*Spiro*), *Synergistales* (*Syn*). A detailed correlation matrix is provided in Table S3.

The high frequency and dominant co-occurrence of *Anaerolineales* with cellulolytic species indicated the possible interaction between them during cellulose and xylan hydrolysis. There are not many cultured *Anaerolineales* species, but cultured *Anaerolineales* species grow together with a hydrogenotrophic partner (Yamada et al., 2006; Sekiguchi et al., 2001). Because of that reason growth of *Anaerolineales* could be dependent on hydrogenotrophic methanogens. *Anaerolineales* was negatively affected ($r = -0.355$, $p<0.05$) by the increasing concentrations of HA (Table S3). In R2 and R3 (hydrolysis inhibition) abundance of *Anaerolineales* was reduced 3 folds as compared to the other reactors (Figure 6.3). Decrease in the abundance of *Anaerolineales* suggests the disruption in their possible microbial interactions with methanogens, leading to decreased methane production. When the abundance of *Anaerolineales* reduced due to the presence of HA, a well-known syntrophic group *Syntrophobacterales* increased in abundance in the R2 to R5. Their abundance was correlated with the HA ($r = 0.569$, $p<0.001$) (Table S3). Surprisingly, we did not detect *Syntrophobacterales* in R1. Most probably, they could not compete with the *Anaerolineales* species. *Planctomycetales* was the other bacterial order that was positively correlated with HA ($r = 0.584$, $p<0.001$) (Figure 6.5, Table S3). Members of *Planctomycetales* are highly diverse and their role in nature is mostly unclear. Some members are thought to be involved in humus degradation in termite gut (Kudo, 2009; Ward et al., 2006). Therefore, their abundance in R2 to R5 might be related to HA degradation. However, more research is required to test this occurrence.

Not many bacterial groups correlated with the presence of calcium and enzyme addition. *Lactobacillales, Spirochaetes*-SHA-4 and Unclassified *Bacteriodetes* SB-1 were mainly clustered with enzyme and calcium addition (Figure 6.5). In all reactors, *Lactobacillales* was represented by only *Trichococcus* genus. *Trichococcus* species can be frequently found in wastewater treatment plants. Especially, *Trichococcus flocculiformis* was reported to cause foaming and bulking of the sludge which is not desirable for reactor operations (Nielsen et al., 2009; Schef et al., 1984). We did observe foaming in R3-R5 whereas no foaming in R1 and moderate foaming in R2 was observed. Abundance of *Trichococcus* in these reactors most probably caused the foaming problem. Though foaming problems were prevented with the addition of equal amounts of silicone oil to all reactors, the abundance of *Trichococcus* was not reduced in R2-R5.

In general, archaeal communities in all reactors were represented by hydrogenotrophic methanogens. Although hydrogenotrophic methanogens were more abundant in all reactors, acetoclastic and hydrogenotrophic methanogenesis should have taken place at the same time. Most probably, lower abundance of the *Methanosaetaceae* (*Methanotrichaceae*) was related to the biomass yield. When the *Methanosaetaceae* (*Methanotrichaceae*) grows in filamentous form, their biomass yields decrease with an altered carbon metabolic flux that favours the conversion of acetate to methane (Zhang et al., 2012). Obtained low reads of *Methanosaetaceae (Methanotrichaceae)* can be explained by this phenomenon since Eerbeek sludge contains mainly *Methanosaetaceae (Methanotrichaceae)* (Roest et al., 2005).

The methanogenic communities were also affected by the operational conditions. However, the community was more stable than the bacterial community (Figure S2). Members of *Methanobacteriaceae* and *Methanoregulaceae* were found related with biogas production (Figure 6.6). Especially *Methanobacteriaceae* were significantly ($r = 0.300$, $p < 0.05$) correlated with biogas production (Table S4). Abundance of *Methanobacteriaceae* with positive correlation with biogas production was reported previously in high rate AD systems (de Vrieze et.al., 2015b; Hao et al., 2013 Steinberg and Regan, 2011). *Methanoregulaceae* became relatively dominant at the end of the experiment in R1. The members of *Methanoregulaceae* use H_2/CO_2 and some also formate (Oren, 2014). They have been reported in relatively low amounts (relative abundance 1-15 %) in anaerobic digesters (Vanwonterghem et al., 2015; Wilkins et al., 2015). It is not clear why *Methanoregulaceae* is highly abundant in R1.

The presence of HA affected the archaeal composition. Abundance of *Methanobacteriaceae, Methanomicrobiales* WCHA208 and Unassigned *Thermoplasmata* WCHA1-57 were negatively affected by the presence of HA. Their abundance showed significant negative correlation ($r = -0.400$, $p<0.01$) with the presence of HA (Table S4). On the other hand, members of the acetoclastic methanogenic family *Methanosaetaceae (Methanotrichaceae)* showed significant positive correlation ($r = 0.589$, $p <0.001$) with the presence of HA (Table S4). This result suggested that abundance of *Methanosaetaceae (Methanotrichaceae)* increased while the abundance of hydrogenotrophic methanogens decreased in R2-R5.

Calcium and enzyme addition were not deterministic for archaeal composition. *Methanobacteriaceae* were positively correlated with elevated calcium concentrations whereas, *Methanospirillaceae* (r=-0.340, $p<0.05$) and Unclassified *Methanomicrobiales* (r= -0.350, $p<0.01$) were found negatively correlated (Table A4). There was only one family that showed a negative correlation with enzyme additions, which was *Methanospirillaceae* (r= -0.302, $p<0.05$) (Table S4).

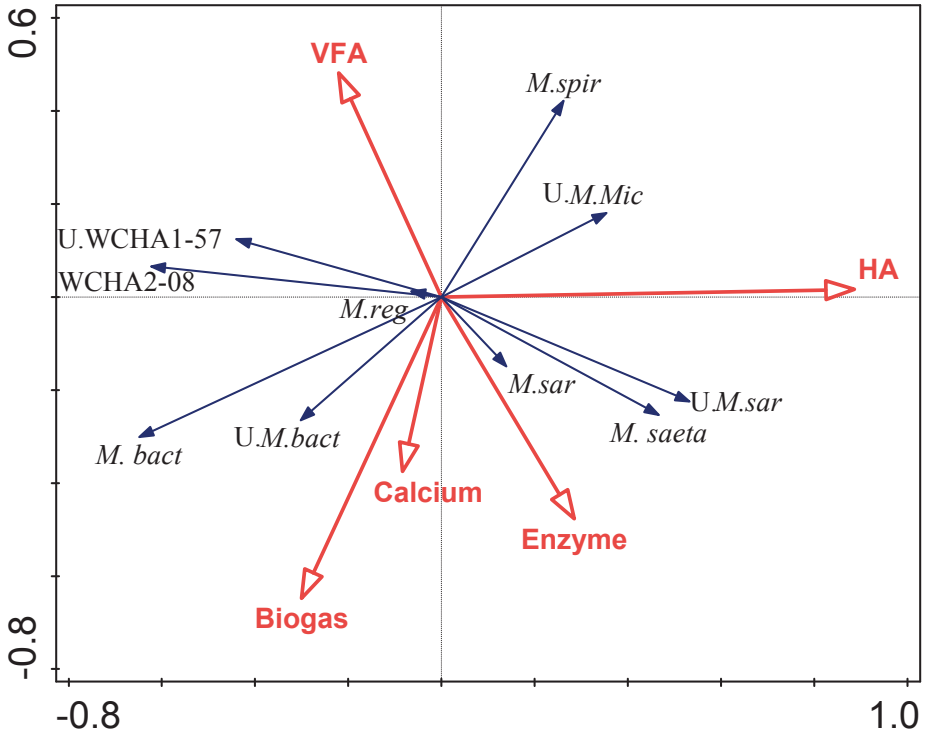


Figure 6.6 Redundancy analysis (RDA) ordination diplot for the archaeal community. Red vectors represent the influence of operational parameters biogas production (Biogas), total volatile fatty acids (Total VFA), humic acids (HA), calcium addition (calcium), enzyme addition (enzyme). Blue vectors represent archaeal families: *Methanobacteriaceae* (*M. bact*), Unassigned *Methanobacteriales* (U.*M. bact*), *Methanoregulaceae* (*M.reg*), *Methanospirillaceae* (*M. spir*), *Methanomicrobiales* WCHA2-08 (WCHA2-08), Unassigned *Methanomicrobiales* (U.*M. Mic*), *Methanosaetaceae (Methanotrichaceae)* (*M. saeta*), *Methanosarcinaceae* (*M.sar*), Unassigned *Methanosarcinales* (U.*M.sar*), Unassigned *Thermoplasmata* WCHA- 15-7 (U. WCHA1-57). A detailed correlation matrix provided as S4.

6.5 Conclusions

In conclusion, HA inhibited especially the hydrolysis step of the digestion up to 40 %. Addition of hydrolytic enzymes helped to reverse the negative effects of HA whereas calcium addition did not show any effects to reverse HA inhibition. Microbiological analyses showed that fermentative hydrolytic bacteria and hydrogenotrophic methanogens were affected by the presence of HA, whereas acetoclastic methanogens were not affected by HA addition. Our results showed that intrinsic enzyme production was sufficient to maintain hydrolytic activity when there were no active enzyme scavengers in the environment. For that reason, we propose to control enzyme additions based on the influent HA rather than volatile solid concentration, to limit costs.

6.6 Supplementary materials

Table S1 Summary of reactor operation data within different time periods

Reactor	Parameter	Unit	P0		P1		P2		P3		P4		P5		P6		P7		P8	
			x̄	σ	x̄	σ	x̄	σ	x̄	σ	x̄	σ	x̄	σ	x̄	σ	x̄	σ	x̄	σ
R1	Acetate concentration	mg/L COD	82	16	75	42	76	24	75	34	122	36	203	38	244	56	251	113	270	48
	Propionate concentration	mg/L COD	346	219	52	49	70	21	70	29	119	35	149	40	116	51	36	42	34	51
	Biogas production	mL	3677	322	3826	469	4197	153	4243	139	4001	260	4187	246	4309	265	4029	117	4098	189
	Methane content	%	52.0	1.0	51.0	0.0	51.0	0.0	50.5	0.5	50.0	0.0	50.0	0.0	50.0	0.5	50.0	0.0	50.0	0.0
	Hydrolysis efficiency	%	50.5	5.5	52.7	1.0	52.0	1.0	54.2	0.5	50.5	2.5	56.0	0.0	58.0	0.0	53.0	0.0	54.0	0.0
	Acidogenesis efficiency	%	52.3	6.0	50.0	6.6	54.5	2.0	53.7	1.3	51.9	3.4	55.6	3.5	56.3	3.4	53.4	1.7	54.1	2.6
	Methanogenesis efficiency	%	48.3	4.3	48.5	5.9	53.2	1.9	52.8	1.7	49.7	3.2	52.0	3.1	56.4	3.6	50.1	1.5	50.9	2.3
R2	Acetate concentration	mg/L COD	100	12	15	19	62	40	46	25	28	16	61	21	43	23	45	13	26	0
	Propionate concentration	mg/L COD	303	189	12	25	64	43	48	31	45	44	64	41	18	21	13	15	0	0
	Biogas production	mL	3974	349	3706	318	4307	324	4362	324	3838	246	3672	89	3016	488	2615	259	2669	272
	Methane content	%	52.5	0.5	52.0	0.0	52.0	0.0	51.5	0.5	50.5	0.5	50.0	0.0	49.0	2.0	45.5	0.5	49.0	0.0
	Hydrolysis efficiency	%	49.3	0.4	50.0	2.2	54.2	0.4	58.6	3.2	51.1	0.6	45.7	0.0	27.8	0.0	33.2	0.0	30.4	0.0
	Acidogenesis efficiency	%	55.9	6.0	48.3	4.1	57.1	4.5	56.4	5.2	49.2	3.1	46.9	1.6	38.9	7.5	29.9	2.6	32.6	3.4
	Methanogenesis efficiency	%	52.3	4.7	47.9	4.1	55.6	4.2	55.4	4.4	48.6	3.1	45.6	1.1	38.9	7.3	29.6	2.7	32.5	3.3
R3	Acetate concentration	mg/L COD	103	20	46	25	76	31	57	18	33	18	61	20	50	25	35	12	17	18
	Propionate concentration	mg/L COD	239	131	23	37	71	31	50	27	61	38	93	55	21	21	13	20	0	0
	Biogas production	mL	3710	500	3330	346	3912	288	3554	347	3366	405	3094	371	2877	518	2560	421	2691	205
	Methane content	%	52	1	52	0.5	52	0	51.5	0.5	50.0	1.0	49.0	0.0	50.0	1.0	49.0	0.0	50.0	0.0
	Hydrolysis efficiency	%	46.0	1.0	44.8	4.3	48.8	3.2	46.1	5.8	45.5	7.2	36.0	0.0	32.1	0.0	35.6	0.0	31.6	0.0
	Acidogenesis efficiency	%	51.9	8.1	42.8	4.7	51.4	4.0	45.8	4.4	43.6	5.4	39.8	3.9	36.4	6.9	32.0	6.1	33.0	1.7
	Methanogenesis efficiency	%	48.5	6.6	42.2	4.4	50.5	3.7	45.1	4.3	42.6	5.1	37.5	4.5	37.5	6.9	31.4	5.1	32.8	2.4
R4	Acetate concentration	mg/L COD	72	32	58	24	80	16	58	22	28	18	75	46	54	32	44	17	8	14
	Propionate concentration	mg/L COD	86	72	93	29	74	25	46	26	66	36	107	60	35	32	17	18	2	4
	Biogas production	mL	3967	452	3736	179	4383	364	4933	276	4110	397	3901	277	3810	298	3913	257	3910	274
	Methane content	%	51	1	51	0.5	51	0	51	0	50.5	0.5	50	0	50	0.5	49.5	0.5	48	0
	Hydrolysis efficiency	%	50.0	0.6	46.8	3.9	54.2	3.7	62.9	1.7	50.3	0.2	54.0	0.0	48.7	0.0	50.6	0.0	48.9	0.0
	Acidogenesis efficiency	%	52.3	6.9	46.9	2.3	57.8	4.3	63.0	3.6	53.3	5.4	50.6	3.2	49.4	3.9	48.9	2.2	47.5	3.0
	Methanogenesis efficiency	%	51.0	5.8	46.5	2.2	55.5	4.6	62.5	3.5	52.1	5.0	48.5	3.4	49.9	4.1	48.2	2.9	47.1	3.5
R5	Acetate concentration	mg/L COD	95	33	64	22	87	27	69	37	38	22	35	22	31	18	16	17	5	11
	Propionate concentration	mg/L COD	510	345	61	40	80	20	63	40	52	46	61	33	17	21	2	6	0	0
	Biogas production	mL	3805	392	3762	345	4161	189	4351	196	4095	204	4035	623	4140	370	3919	141	3971	319
	Methane content	%	52.5	0.5	52	0.5	51	0	51	0	50.5	0.5	50	0	50	0.8	51.5	0.5	49	0
	Hydrolysis efficiency	%	50.9	0.5	48.7	3.9	52.8	3.7	54.2	1.7	51.1	0.2	60	0	53.6	0	52.4	0	49.9	0
	Acidogenesis efficiency	%	54.9	8.4	49.3	4.5	57.8	4.3	56.4	3.0	52.5	2.7	50.9	8.3	52.2	3.6	50.9	1.5	49.0	2.9
	Methanogenesis efficiency	%	50.0	5.2	48.5	4.4	52.7	2.4	55.1	2.5	51.9	2.6	50.1	7.7	54.7	4.8	49.8	1.7	48.3	3.8

Table S2 The number of reads per sample that was obtained by next generation sequencing

Sampling points	Reactor	Bacterial reads	Archaeal reads
	R1	206686	51312
	R2	183824	71481
	R3	190662	38170
	R4	73253	32974
P0	R5	67189	12665
	R1	141518	21686
	R2	241952	30432
	R3	122191	17946
	R4	46220	8974
P1	R5	161299	13941
	R1	4748	2569
	R2	30187	17569
	R3	71988	30052
	R4	228725	47558
P2	R5	106020	27049
	R1	87630	7281
	R2	133360	22089
	R3	75941	16452
	R4	98322	31967
P3	R5	1015	1000<
	R1	4672	1029
	R2	168718	23809
	R3	138606	23555
	R4	122813	14585
P4	R5	74325	3739
	R1	191092	71665
	R2	220401	40089
	R3	62691	6807
	R4	65146	3279
P5	R5	40541	67287
	R1	418163	1000<
	R2	413256	170459
	R3	322267	57700
	R4	165088	17538
P6	R5	77314	1000<
	R1	21270	3459
	R2	50424	11688
	R3	185568	29807
	R4	270967	31139
P7	R5	229511	25456
	R1	220733	17580
	R2	159008	25149
	R3	376954	29153
	R4	121302	8229
P8	R5	133203	1000<

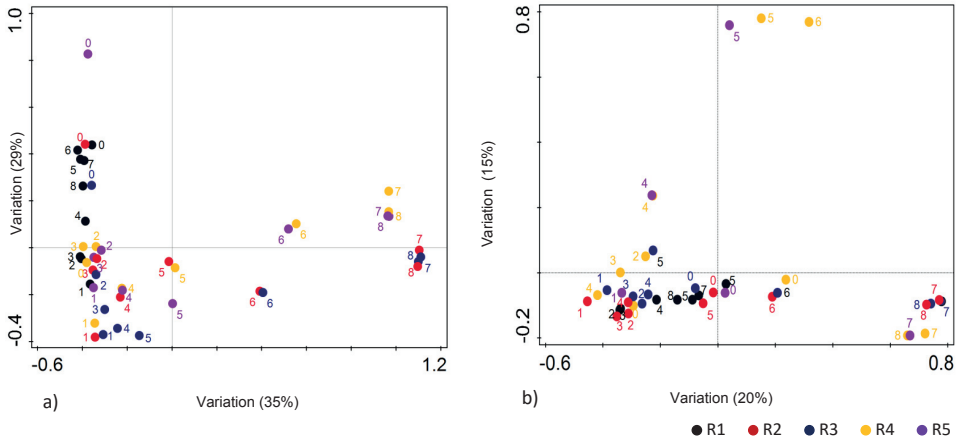


Figure S1 Redundancy analysis (RDA) scatter plots of each individual samples. Numbers represents the sampling point of each individual samples. These graphs show the variations between samples according to a) bacterial community composition and b) archaeal community composition.

Table S3 Correlation matrix between operational data and the bacterial orders. Negative correlations (red) and positive correlations (green) are shown. Correlations were determined by the two- tailed Spearman's Rank Order Correlations statistics.

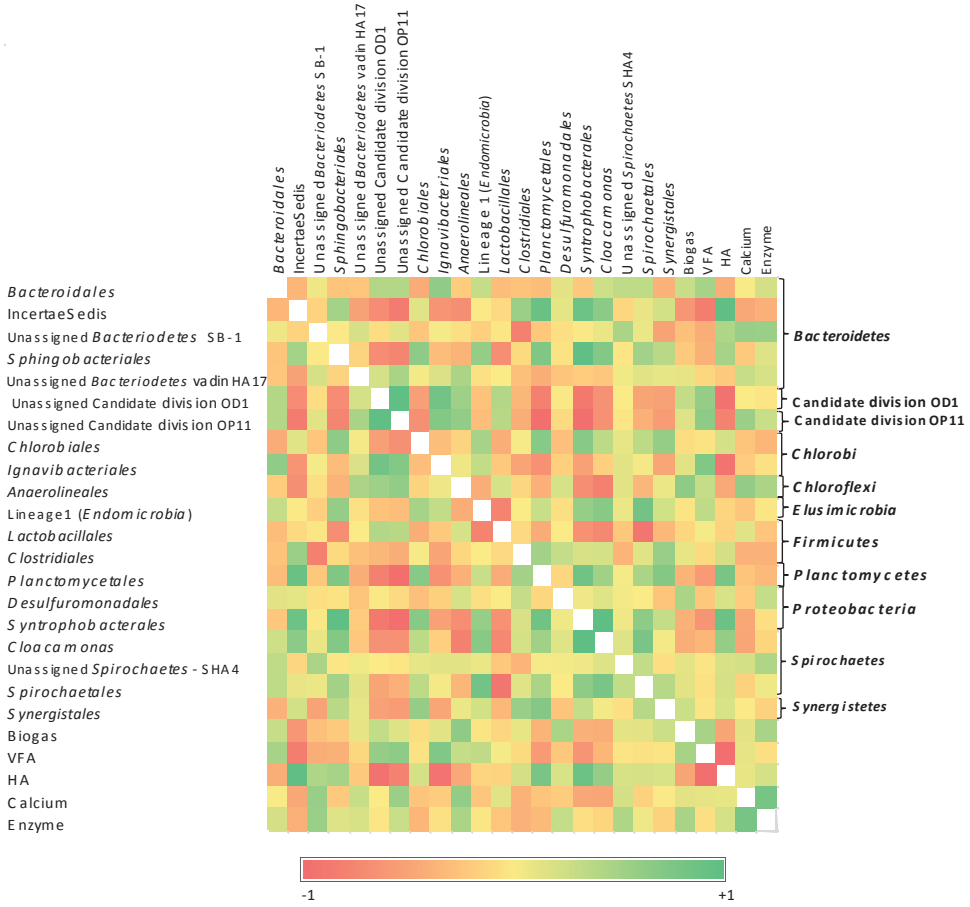
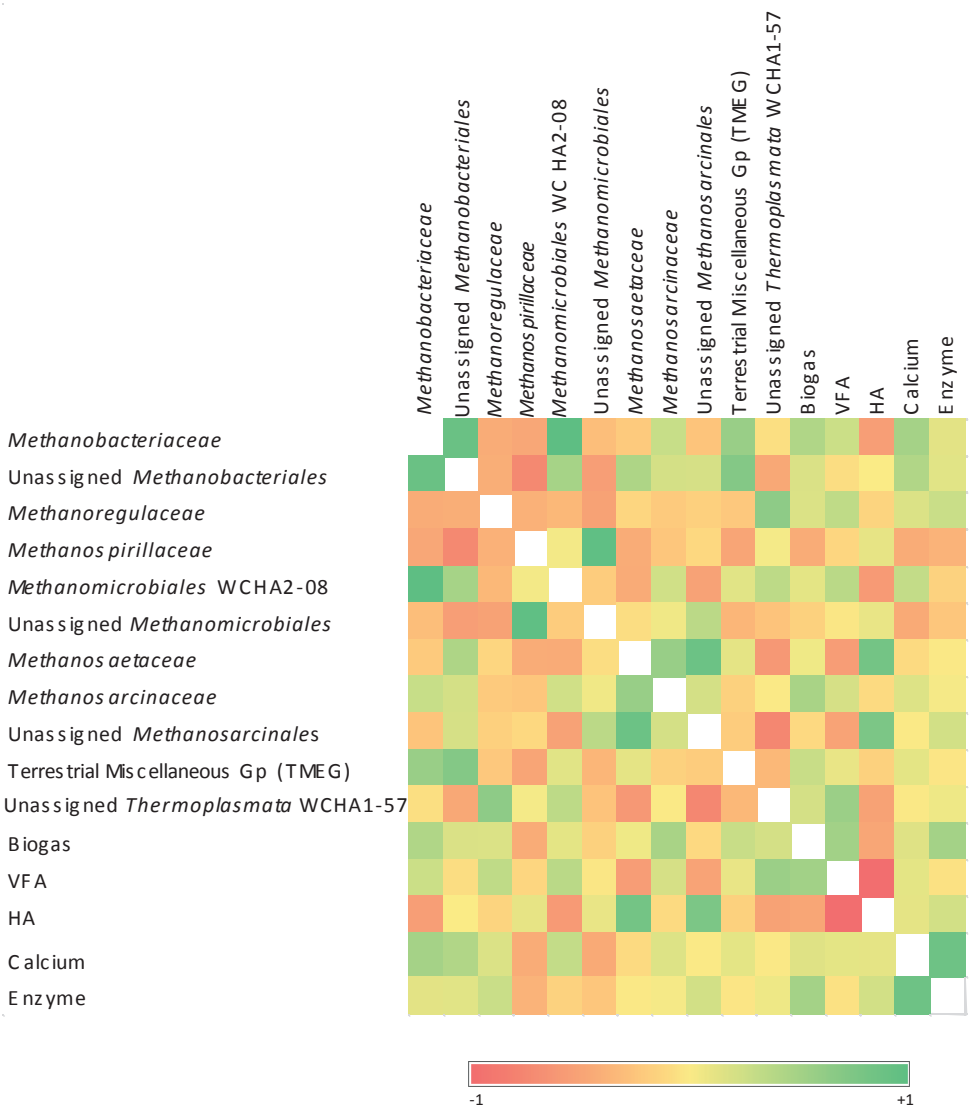


Table S4 Correlation matrix between operational data and the archaeal families. Negative correlations (red) and positive correlations (green) are shown. Correlations were determined by the two- tailed Spearman's Rank Order Correlations statistics.



Chapter 7

General Discussion

Samet Azman

7.1 Introduction

Hydrolysis is the rate limiting step of the anaerobic digestion (AD) of biomass (Ma et al., 2013; Vavilin et al., 2008). The presence of inhibitors lowers the hydrolysis efficiency and therefore the methane yield in biogas plants. Humic acids (HA) are complex molecules which are formed during the decay of the organic fraction of the biomass and can also be present in raw biomaterials. HA usually contain aromatic rings, alkyl, carboxyl and phenol groups which give HA their polyelectrolyte behaviour (Saiz-Jimenez, 1993; Tan, 2014). Moreover, HA are known to inhibit hydrolysis of biopolymers (Azman et al., 2015b; Brons et al., 1985; Fernandes et al., 2015). Although inhibition mechanisms are not fully understood, binding of HA to hydrolytic enzymes is proposed to cause the inhibition. Additionally, their effect on overall AD of (hemi)cellulosic materials and microbial community dynamics during the AD process is not known.

The main purpose of this thesis research was i) to determine the inhibitory effect of HA on AD of cellulose and hemicellulose, ii) to analyse the microbial population dynamics in the presence/absence of HA, iii) to develop mitigation strategies to overcome HA inhibition.

7.2 Hydrolytic/ fermentative microorganisms

Hydrolytic microorganisms are abundant in diverse natural environments and engineered systems (Lynd et al., 2002; Azman et al., 2015a). Hydrolytic microorganisms initiate organic material degradation via the production of hydrolytic enzymes. Hydrolytic microorganisms are important for efficient hydrolysis and acidification and therefore high methane yields in biogas plants. As explained in **Chapter 2**, that reviews the role of anaerobic hydrolytic microbes in the conversion of lignocellulosic biomass, abundant hydrolytic/fermentative microbes in biogas plants are identified with the aid of molecular tools. Also, some of these microbes are available in pure cultures for controlled experiments, using different lignocellulosic fractions to understand the role of hydrolytic/fermentative microorganisms in the biogas plants. Anaerobic hydrolytic microorganisms are commonly found in the phylum *Firmicutes* and *Bacteroidetes* (**Chapter 2**). The knowledge about the relative abundance of microbial groups alone does not give information about their activity. Therefore, knowledge on the physiology and biochemistry of hydrolytic bacteria and their interactions with other microbes

involved in the anaerobic digestion processes is needed to implement new technologies for enhanced methane formation.

7.3 Optimal conditions for hydrolytic microorganisms

Hydrolytic activity is affected by many factors, as summarised in Figure 2.2 of **Chapter 2**. When a single bioreactor is used for biogas production, conditions in the bioreactor need to facilitate activity of all microbes involved in the processes. If hydrolysis is physically separated from methanogenesis, in a two-step process, optimal conditions for the hydrolytic/acidifying microbes can be optimized separately from the acetogenic and methanogenic bioreactors. Reported pH for hydrolytic microorganisms varies between pH 5-7. Even though hydrolytic enzymes have higher activity at moderately acidic conditions, neutral pH is usually selected to maintain both hydrolytic and methanogenic activities in a one-reactor process. Different types of hydrolytic bacteria can grow at different temperatures ranging from psychrophilic to thermophilic. The activity of the hydrolytic enzymes in relation to temperature is usually proportional. However, hydrolytic activity increases until an optimum temperature, after which it rapidly decreases. Consequently, different types of hydrolytic enzymes can have a different optimum temperature. (Veeken and Hamelers, 1999; Viikari et al., 2007). Besides temperature and pH, also the structure of lignin, cellulose and hemicellulose determines the bioavailability of the raw materials for the hydrolytic enzymes and their biodegradability. Especially particle size and crystallinity of the substrates determine the hydrolysis efficiency. Decreasing these two parameters substantially increases the hydrolysis rates and methane yields. This can be done via physical and thermochemical pre-treatment methods which are frequently applied for biogas production (**Chapter 1**). Another important obstruction for efficient hydrolysis and methanogenesis is the presence of inhibitory compounds and mitigate their inhibition.

7.4 Anaerobic digestion of cellulose and hemicellulose in the presence of humic acids

7.4.1 Humic acid inhibition on hydrolysis

Inhibition of anaerobic hydrolysis by HA was studied and described in **Chapter 3** and **Chapter 6**. The results confirm the known information on HA inhibition (Brons et al. 1985; Fernandes et al. 2015) and add information about the inhibitory effect of HA on long term CSTR reactor operation and the abundance of microbial community. In this

thesis, inhibition of anaerobic cellulose and hemicellulose digestion was studied in batch incubations (**Chapter 3**) and in long-term CSTR reactor operations for cellulose and xylan degradation (**Chapter 6**). In these experiments, the same batch of humic acid salts (Sigma-Aldrich, Darmstadt, Germany) was used. The results of these experiments clearly show that 5 g L^{-1} HA inhibits anaerobic hydrolysis of cellulose by 50%. Moreover, anaerobic cellulose and xylan digestion in CSTRs that are operated at 20 days of HRT and 30°C are inhibited by 40% at HA concentrations around 8 g L^{-1}. The observed inhibitory effect of HA confirms the observation of Brons et al. (1985) and Fernandes et al. (2015). Therefore, it is concluded that HA negatively affects hydrolysis of cellulose and hemicellulose.

7.4.2 Humic acid inhibition on methanogenesis

Different concentrations of HA in the range of 1 g.L^{-1} to 5 g L^{-1} were tested to determine HA inhibition in batch incubations of pure methanogenic cultures and a mixed culture (Chapter 4). The results from this study clearly show that methanogenesis is also affected by HA. Nearly all hydrogenotrophic methanogens are inhibited by 1 g L^{-1} HA while *Methanospirillum hungatei* is resistant to inhibition up to 7 g L^{-1}. Also, acetoclastic methanogens are negatively affected by HA. *Methanosarcina barkeri* is completely inhibited at concentrations of 1 g L^{-1} HA and *Methanothrix concilii* is not severely affected by the presence of HA up to a concentration of 5 g L^{-1}. The difference in cell wall structure between different type of methanogens is proposed to explain the inhibitory effect of HA on pure methanogenic cultures. *Methanospirillum hungatei* and *Methanothrix concilii* have the most complex cell envelope of any archaea that have been described (Albers and Meyer, 2010). Apparently, the complex, thick, proteinaceous impermeable envelope layer (sheats) of these methanogens prevents the attachment/penetration of the HA to the cells. However, this hypothesis should be experimentally proven.

A mixed culture (community in anaerobic sludge) is more resistant to inhibition both of acetoclastic and hydrogenotrophic methanogenesis. The mixed methanogenic community, present in the crushed granular sludge is sufficiently diverse to sustain methanogenesis, regardless of HA presence. These results show similarity with the study of Ghasimi et al. (2016). They also did not observe a negative effect of HA on mesophilic and thermophilic batch specific methanogenic activity tests, using anaerobic

sludge treating fine sieve fraction of municipal waste in the range of 0.4 -2 g L^{-1}. The results presented in **Chapter 4** indicate that pure cultures of methanogens are more vulnerable to HA inhibition, while a mixed community can resist the inhibition and therefore can maintain the methanogenic activity. In this respect, hydrolysis is still the rate limiting step of the overall AD of lignocellulosic material and it is more susceptible to HA inhibition.

7.4.3 Effect of humic acid on microbial community dynamics

Microbial population dynamics for bacteria and archaea were investigated in CSTR systems, operated at 20 days of HRT, 30 °C, with cellulose and xylan as a substrate, in the presence and absence of HA for a long-term incubation experiment (with a steady state period of 220 days) in **Chapter 6**. The relative abundance of *Clostridiales, Bacteroidales* and *Anaerolineales* is significantly lowered by the presence of HA. Especially, *Clostridiales* and *Bacteroidales* are well known hydrolytic/fermentative bacteria in biogas plants as discussed in **Chapter 2**. The decreased relative abundance of these bacterial orders coincides with decreased hydrolysis efficiency. Therefore, HA negatively affects not only the relative abundance of the hydrolytic/fermentative microorganisms but also anaerobic conversion of cellulose and xylan.

The presence of HA also affects the archaeal populations. Hydrogenotrophic methanogens, especially the relative abundance of *Methanobacteriaceae* and *Methanomicrobiales*-WCHA*208*, are negatively affected by the increasing HA concentrations. The relative abundance of *Methanosaetaceae* (*Methanotrichaceae*) shows a positive correlation with increasing HA concentrations. These results confirmed the results of **Chapter 4** in which the effect of HA was investigated in batch wise incubations. Apparently, Methanosaetaceae (*Methanotrichaceae*) becomes relatively abundant in the presence of HA.

Binding properties of HA to hydrolytic enzymes is proposed to explain the HA inhibition. With this study, it is shown that HA also decreases the relative abundance of key hydrolytic/fermentative bacteria and methanogens which can affect the overall reactor performance.

7.5 The humic acid dilemma. Is the inhibition severe for biogas production?

The inhibitory effect of HA was suggested by Zeeman (1991), based on the studies of Zeeman (1991) and van Velsen (1981). Zeeman (1991) suggested that observed inhibition during anaerobic manure digestion might be related to some other compounds present in manure rather than ammonia. Ammonia inhibition is one of the most common causes for reduced methane yields in biogas plants treating agricultural biomass. The inhibitory effect of ammonia on methanogenesis is well studied (Yenigun and Demirel, 2013), while the effect of ammonia on hydrolysis is seems to be less (Fernandes et al., 2012). Fernandes et al. (2012) showed that ammonia nitrogen in the range of 2.4 to 7.8 g NH_4^+ N L^{-1} did not inhibit hydrolysis of cellulose or tributyrin. These results support the hypothesis of Zeeman (1991) that ammonia might not be inhibitory for the hydrolysis step of lignocellulosic material. Later, Fernandes et al. (2015) also showed that humic and fulvic acid-like molecules, extracted from cow manure and maize, strongly inhibit the hydrolysis of cellulose. The combined results of these recent studies showed the inhibitory effect of humic compounds on hydrolysis, which was already suggested several decades ago.

Results presented in this thesis support the hypotheses about observed HA inhibition on hydrolysis and also show the inhibition on methanogenesis. However, there is inconsistency on reported inhibitory HA concentrations. Brons et al. (1985) reported 0.25 and 1 g L^{-1} sodium humate as inhibitory concentrations for potato protein hydrolysis. Fernandes et al. (2015) found that humic acid-like compounds inhibit cellulose hydrolysis completely at concentrations as low as 0.5 g L^{-1}, whereas in this thesis 5 g L^{-1} HA causes 50 % inhibition of anaerobic degradation of cellulose in batch wise incubations. Differences between found inhibitory concentrations might be due to differences in the HA structural characteristics, and therefore, the inhibition might depend on the HA source. It is known that HA are complex molecules and that their chemical structure varies with the source of HA, extraction and preparation methods (Tan, 2014). A recent study from Liu et al. (2015b) supported this hypothesis when they added two different HA to the anaerobic sludge digestion process. The added HA differed in chemical structure, hydrophobicity, surfactant properties, and degree of aromaticity. Their results showed that HA, containing lesser alkyl groups improved the hydrolysis rates but inhibited the methanogenesis while HA that had more aromatic

rings did not affect anaerobic digestion. Because of these reasons, it is advisable to extract HA from the raw materials prior to anaerobic digestion to determine the inhibition potential of the specific HA. When the HA is extracted from the raw materials, the inhibitory concentration of extracted HA can be determined in the batch tests for the selected inoculum. If the inoculum is susceptible to the HA inhibition, mitigation strategies should be considered to overcome the negative effect of HA.

Another important point for HA inhibition is adaptation capacity of the microorganisms to the inhibitory compounds. Step-wise addition of increased concentrations HA to the CSTRs, rather than a direct full exposure, helps the microbial community in the inoculum to adapt to the inhibitory conditions as demonstrated in **Chapter 6**. In this way, a higher degree of operational stability, instead of instant collapse, can be established in the bioreactors. A stepwise increase of HA concentrations within the reactors increases the endurance of the microbial activity. Difference in observed inhibitory concentrations between **Chapter 3** and **Chapter 6** supports this idea. Pulse addition of HA (5 g L^{-1}) has more severe inhibitory effect on hydrolysis, compared to the stepwise increased HA concentrations that reach 8 g L^{-1}. This adaptation of microorganisms to higher HA concentrations implies that HA inhibition in anaerobic digesters can be prevented by applying suitable acclimation periods and avoiding shock loadings.

7.6 Mitigation of HA inhibition and improvement of anaerobic digestion

It is clear from the results of this thesis that mitigation strategies for HA inhibition should be applied for improving anaerobic digestion of lignocellulosic biomass. The mitigation strategies for HA inhibition should be selected carefully to not interfere with the microbial activity.

HA should either be removed from the environment or their active binding sites should be inactivated to overcome the inhibitory effect of the HA. Removal of humics is required to improve drinking water quality therefore, removal of these compounds from feed water is very well documented. Several methods are used to remove humics from water environments such as coagulation, flocculation, electrocoagulation, removal via active organic compounds, precipitation with chemicals such as iron and aluminium sulphides and oxidation processes (Brum and Oliveira, 2007; Matilainen et al., 2010;

Trellu et al., 2016). However, these methods are not suitable for anaerobic digestion process due to the high solids content of lignocellulosic substrate. Extraction and removal of humic compounds from raw materials with solid-liquid fractionation and alkali pre-treatment are also suggested to recover humic acids from anaerobic digesters (Biswas et al., 2012; Li et al., 2014a; Vu et al., 2016). Although extraction and separation techniques are expensive methods for small scale biogas plants, they can be economically feasible for large-scale biogas plants if the humic rich sludge is sold as soil conditioner (Piccolo et al., 1997).

The removal of HA from the environment and feed biomass may lead to improvement of the anaerobic digestion. Yet, inactivation of the binding sites of the HA in one bioreactor still can be more feasible for anaerobic digesters (Azman et al., 2015b; Brons et al., 1985; Fernandes et al., 2015). When one digester is used for the biogas production including the mitigation of HA inhibition, economic costs for extra storage and buffering tanks can be minimized. Calcium (Brons et al., 1985) and hydrolytic enzyme addition (Fernandes et al., 2015) were proposed to overcome the inhibitory effect of HA on hydrolysis. Therefore, these strategies were tested in **Chapter 3** and **Chapter 6** of this thesis.

7.6.1 Cation addition to mitigate humic acid inhibition

Results from **Chapter 3** showed that addition of divalent or trivalent cations such as Fe^{+3} reversed the inhibitory effect of HA. The addition of 5 mM of magnesium, calcium and iron cations clearly mitigated the inhibitory effects of HA and hydrolysis rates reached to the similar levels of the control groups that were incubated without HA. In the batch tests, calcium showed stronger affinity to HA, compared to the other cations. Therefore, calcium addition was selected as a mitigation strategy, as described in **Chapter 6**.

Despite the positive effect of calcium addition in batch wise incubations, a similar positive effect could not be reached in long-term CSTR operations. The reason for that is unclear but it is likely related to the structure of calcium-HA precipitates that formed within the reactors. Calcium binds to active sites of HA and calcium-HA complexes are formed. Most probably, this complex covers the cellulose and xylan particles. The rigid cover acts like a shield and prevents hydrolytic microorganisms and

their exoenzymes to reach available substrates. In this case, calcium addition may even enhance the inhibitory effect of HA. Because of that reason, addition of calcium directly to the anaerobic digestion to overcome HA inhibition is not promising for continuous bioreactor systems. However, alkaline calcium salts ($CaCO_3$ or $Ca(OH)_2$) can still be used as pre-treatment. As discussed in **Chapter 1**, alkaline pre-treatment causes the partial removal of lignin and an increase in methane yields. Fernandes (2010) discussed the feasibility of the alkaline treatment of biomass for inactivation and precipitation of humic material and utilization of the formed precipitates as a soil conditioner. Modelling of calcium addition at increased pH showed the possibility of formation of inorganic (calcium) rich precipitates which can be used as soil conditioner. However, this modelling study needs to be experimentally proven for full-scale applications. Although in current practice alkaline pretreatment alone is not preferred due to the economic costs, its feasibility should be reconsidered by further evaluating its potential of mitigating HA inhibition.

7.6.2 Enzyme addition to mitigate humic acid inhibition

Results from **Chapter 6** showed that enzyme addition can be a solution to overcome the negative effect of HA. Daily addition of hydrolytic enzyme mixture (1.2 mg enzyme.mg HA added $^{-1}$) to the CSTRs reversed the HA inhibition and the reactors remained as stable and active as the control reactor. These results indicate that a microbial community well-adapted to the degradation of cellulose and xylan (**Chapter 5**) can maintain hydrolytic activity. When HA is added to the reactors, hydrolytic enzymes that are produced by the indigenous microbial community may bind to HA and this leads to lower hydrolysis rates. The lower hydrolysis rates can be increased by addition of commercially available enzymes. In this way, the active binding site of HA will be occupied with the added enzymes and indigenous hydrolytic activity can proceed as if there is no inhibitory compound in the environment. A microbiome, that is well-adapted to cellulose and xylan degradation has sufficient activity of hydrolytic enzymes and enzyme addition can be considered when hydrolytic/fermentative microorganisms are inhibited or bioavailability of the substrates is limited. Therefore, HA concentrations present in the feed should be considered when enzyme addition is

applied. In this way, economic concerns about excess enzyme addition can be minimized.

7.7 Future research

Results from this thesis helped to increase the knowledge about HA inhibition. However, there are still unanswered questions about the inhibition. Current knowledge about HA inhibition is derived from experimental set-ups in which only model substrates are used. Hence, HA experiments should be performed with actual biowaste to mimic more realistic conditions of biogas plants. Using real biowaste will give the opportunity to investigate the effect of HA in the presence of other inhibitors such as high VFA, ammonia, and high/low pH. Moreover, the complex structure of HA needs to be elucidated and each functional group should be tested to determine which functional groups are responsible for the inhibition.

The application of DNA-based next generation sequencing allows to determine the effect of HA on the microbial population dynamics. However, RNA, protein, and metabolite based –omics methods, such as metaproteomics and metabolomics can be applied to reveal the effect of HA on activity of specific microbial communities in the microbiomes.

The technical and economic feasibility of the hydrolytic enzyme addition to mitigate HA inhibition should also be investigated further. Optimum conditions for the hydrolytic enzymes for mitigating HA inhibition should be determined. In addition, cost-benefit analyses should be carried to find the feasibility of hydrolytic enzyme addition to overcome HA inhibition for large-scale applications.

Appendices

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Summary

Research on the hydrolysis step of AD became more important with the increased use of recalcitrant waste products such as manure, sewage sludge and agricultural biomass for biogas production. Hydrolysis is often the rate limiting step of the overall AD. Hydrolysis enhancement is one of the required steps to optimise biogas production. Despite the progress to overcome the limitations of hydrolysis, inhibition of hydrolysis is still poorly researched. Humic acid-like molecules (HA) are one of the inhibitors of the anaerobic hydrolysis and their effect on the overall AD process is generally overlooked.

In this thesis, the HA inhibition on anaerobic digestion of (hemi)cellulosic material and inhibition mitigation strategies, using cation and enzyme addition were investigated. In addition, the microbial community dynamics during AD in the presence and absence of HA were examined. In this scope, in **Chapter 2**, we reviewed the literature and pinpointed the urgent need for comprehensive studies on the role of hydrolytic microorganisms and environmental factors that affect their abundance and activity within biogas plants. Consequently, the hydrolysis mechanism and involved hydrolytic enzymes were discussed. The review advocates that a holistic approach, including microbiological and engineering studies should be chosen to disclose the role of hydrolytic microbes within biogas reactors. In **Chapter 3 and Chapter 4** the effect of HA on anaerobic cellulose hydrolysis and methanogenesis, in batch wise incubations is reported, respectively. Our results showed that pulse addition of 5 g L^{-1} HA caused a 50 % decrease in hydrolysis rate of anaerobic cellulose degradation (**Chapter 3**). Moreover, VFA accumulation was observed in the presence of HA during anaerobic cellulose degradation, which indicated the possible inhibition of HA on methanogenesis. Based on the results of Chapter 3, pure cultures of methanogens including *Methanothrix concilii*, *Methanosarcina barkeri*, *Methanobacterium formicicum*, *Methanospirillum hungatei and Methanobrevibacter arboriphilicus* and a mixed culture were tested to study the vulnerability of methanogenesis to HA inhibition. Hydrogenotrophic methanogenesis in pure cultures was inhibited by more than 75% in the presence of 1 g L^{-1} HA (except *M. hungatei*) whereas acetoclastic methanogenesis by *M. concilii* was only slightly affected by HA up to 3 g L^{-1}. When methanogenic granular sludge was incubated with HA, the specific methanogenic activity tests showed less inhibition, when compared to the pure cultures of

methanogens. HA inhibition was also observed during long-term CSTR operation at an HRT of 20 days, 30°C and a mixture of cellulose and xylan as substrate (**Chapter 6**). Hydrolysis efficiency of the cellulose and xylan digestion was inhibited by 40 % in the presence of 8 g L^{-1} HA and concomitantly reduced the methane yields.

Mitigation of the HA inhibition is required to increase the hydrolysis efficiency and methane yields of cellulosic biomass digestion. Therefore, two different strategies were tested for their potential use as mitigation agents, viz. addition of cations such as calcium, magnesium and iron (**Chapter 3 and Chapter 6**), and addition of hydrolytic enzymes (**Chapter 6**). Addition of magnesium, calcium and iron salts mitigated the HA inhibition, and hydrolysis efficiencies reached up to 75, 65 and 72%, respectively, compared to the control groups in the batch wise incubations (**Chapter 3**). However, in long term CSTR operations, calcium addition did not show a positive effect on hydrolysis inhibition. On the other hand, enzyme addition helped to reverse the negative effect of HA.

The microbial communities involved in AD of cellulose and hemicellulose were also studied. **Chapter 5** and **Chapter 6** describe microbial community analyses using 16S rRNA next generation sequencing. In **Chapter 5**, five replicate reactors were monitored during the start-up period. Transient feeding strategy was used to acclimatise anaerobic sludge for efficient cellulose and xylan degradation. During the experiment, *Bacteriodales*, *Clostridiales* and *Anaerolineales* became dominant bacterial populations, while *Methanobacteriaceae* and *Methanospirillaceae* were the dominant archaeal populations within the reactors. In **Chapter 6**, the microbial population dynamics in the presence and absence of HA were monitored. Microbiological analyses showed that the relative abundance of hydrolytic/fermentative bacterial groups such as *Clostridiales, Bacteroidales* and *Anaerolineales* was significantly lowered by the presence of HA. HA also affected the archaeal populations. Mostly hydrogenotrophic methanogens were negatively affected by HA.

In conclusion, this thesis confirms that HA inhibit the hydrolysis and methanogenesis in both batch incubations and CSTR systems. Microbial populations were also affected by HA. Therefore, hydrolytic enzyme and cation addition can be an option to mitigate HA inhibition and enhance hydrolysis and methanogenesis during conversion of biomass to biogas.

Samenvatting

Sinds de toegenomen productie van moeilijk afbreekbaar afval, zoals: mest, slib vanuit afvalwaterzuivering installaties en land en –tuinbouw afval, is onderzoek naar de hydrolyse stap in anaerobe digestie (AD) van groter belang geworden. Deze hydrolyse stap is vaak limiterend voor de snelheid van het gehele AD proces en verbetering van deze stap is vereist voor het verder verbeteren van biogas productie. Ondanks de ontwikkelingen in het bevorderen van de hydrolyse, is inhibitie van hydrolyse weinig onderzocht. Humus zuren (HZ) zijn een van de inhibitoren van het hydrolyse proces en het effect van deze stoffen op het gehele AD proces wordt vaak over het hoofd gezien.

In deze thesis is het remmende effect van HZ op de anaerobe vergisting van (hemi)cellulose onderzocht. Ook is er gekeken naar het effect van HZ op hydrolyse bevorderende strategieën als toevoeging van kationen of enzymen. Daarnaast is het effect van HZ op de microbiologische populatie dynamica onderzocht. In **hoofdstuk 2** is relevante literatuur samengevat waaruit geconcludeerd kan worden dat er noodzaak is voor uitgebreide studies naar de rol van hydrolytische micro-organismen in anaerobe vergisting processen, en naar de rol van omgevingsfactoren die hun aanwezigheid en activiteit beïnvloeden. Vervolgens worden de hydrolyse mechanismen en de betrokken enzymen besproken. De samenvatting pleit voor een aanpak die zowel microbiologische als technische aspecten bekijkt om de rol van hydrolytische micro-organismen in biogas reactoren te bestuderen.

In **hoofdstuk 3 en 4** wordt het effect van HZ op anaerobe afbraak van cellulose en productie van methaan in batch processen besproken. De resultaten lieten zien dat een toevoeging van 5 g L^{-1} HZ resulteerde in vermindering van hydrolyse efficiëntie tot 50% (**hoofdstuk 3**). Bovendien was er ophoping van vluchtige vetzuren in deze conditie, wat erop kan duiden dat HZ methaan productie remmen. Gebaseerd op deze resultaten zijn pure cultures van methanogenen getest op hun gevoeligheid voor HZ, waaronder: *Methanothrix concilii*, *Methanosarcina barkeri*, *Methanobacterium formicicum*, *Methanospirillum hungatei, Methanobrevibacter arboriphilicus.* Daarnaast is ook een gemixte cultuur getest op de gevoeligheid voor HZ. In hydrogenotrofe pure cultures werd methaanproductie tot meer dan 75% gereduceerd in de aanwezigheid van 1 g L^{-1} HZ (met uitzondering van *M. hungatei*). Acetoclastische methaan productie door *M. concilii* werd maar matig beïnvloed door HZ met concentraties tot 3 g L^{-1}. Methaan

productie in granulair slib in aanwezigheid van HZ, werd minder beïnvloed in vergelijking met pure cultures. HZ inhibitie was ook waargenomen tijdens CSTR intubaties met hydraulische retentie tijden van 20 dagen, bij een temperatuur van 30 °C en gebruik makend van een mix van cellulose en xylaan als substraat (**hoofdstuk 6**). Hydrolyse van cellulose en xylaan waren 40% minder efficiënt in de aanwezigheid van 8 g L^{-1} HZ, met als gevolg een verlaagde methaan opbrengst.

De remmende effecten van HZ moeten verminderd worden om de hydrolyse efficiëntie en de methaan opbrengst vanuit cellulose materiaal hoog te houden. Daarom zijn twee verschillende strategieën getest om de negatieve effecten van HZ te verminderen. Er is gekeken naar toevoeging van kationen as calcium, magnesium en ijzer (**hoofdstuk 3 en hoofdstuk 6**), en de toevoeging van hydrolytische enzymen (**hoofdstuk 6**). In aanwezigheid van magnesium-, calcium- en ijzerzouten, werd een verhoogde efficiëntie van respectievelijk 75, 65 en 72% geobserveerd in vergelijking met de controle groep (**hoofdstuk 3**). Echter, tijdens langere termijn operatie van een CSTR, was er geen positief effect van calcium op hydrolyse activiteit waargenomen. Toevoeging van enzymen droeg wel bij aan het tegengaan van de negatieve effecten van HZ.

De microbiële groepen betrokken bij de vertering van cellulose en hemicellulose zijn ook bestudeerd. **Hoofdstuk 5 en Hoofdstuk 6** beschrijven de analyse van deze microbiële groepen via 16S rRNA next generation sequencing. In **hoofdstuk 5** zijn 5 reactoren gevolgd gedurende een opstart periode. Voeding strategieën zijn toegepast om het anaerobe slib aan te passen aan het efficiënt afbreken van cellulose en xylaan. Gedurende het experiment werden *Bacteriodales*, *Clostridiales* en *Anaerolineales* dominante groepen, terwijl *Methanobacteriaceae* en *Methanospirillaceae* de dominantie archaea waren. In **hoofdstuk 6** werd de microbiële populatie dynamica in aan- en afwezigheid van HZ gevolgd. Microbiële analyse liet zien dat de relatieve aanwezigheid van hydrolytische/fermentatieve bacteriële groepen, als *Clostridiales, Bacteroidales* en Anaerolineales significant minder aanwezig waren in aanwezigheid van HZ. HZ had ook effect op de archaea populatie. Met name hydrogenotrofe methanogenen werden negatief beïnvloed door HZ.

Translation by Martijn Diender

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Samet Azman was born on July 20th 1986, in Bursa Turkey. He studied Biology, with specialization in Microbiology at Istanbul University between 2004 and 2008. After his graduation from Istanbul University, he joined the Istanbul Technical University, Environmental Biotechnology program. Between 2009-2010, he took environmental engineering courses to fulfill the merits of the master program. During his time at Istanbul Technical University, he participated in several projects, including microbial profiling of hydrocarbon degradation in marine sediments and anaerobic degradation of antibiotics. He received his MSc. Degree in 2011. After a short time his graduation, he moved to Wageningen to pursue a Ph.D. degree at Wageningen University about humic acid inhibition on anaerobic digestion.

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List of Publications

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- Azman S, Khadem AF, Zeeman G, van Lier JB, Plugge CM (2015). Mitigation of humic acid inhibition in anaerobic cellulose digestion by addition of various salts. Bioengineering, 2:54-65.
- Cetecioglu Z, Ince B, Azman S, Ince O (2014). Biodegradation of tetracycline under various conditions and effects on microbial community. Appl Biochem Biotechnol 172:631-640.
- Cetecioglu Z, Ince B, Azman S, Gokcek N, Coskun N, Ince O (2013). Determination of anaerobic and anoxic biodegradation capacity of sulfamethoxazole and the effects on mixed microbial culture. In: Biodegradation-engineering and technology. Chamy R (ed), InTech, Rijeka pp 223-250.

Manuscripts - under review

- Khadem AF*, Azman S*, Plugge CM, Zeeman G, van Lier JB, Stams AJM. Effect of humic acids on the activity of pure and mixed methanogenic cultures.
- Azman S, Khadem AF, Plugge CM, Stams AJM, Bec S, Zeeman G. Microbial community dynamics in CSTRs degrading cellulose and xylan in the presence or absence of humic acid under anaerobic conditions.

Manuscripts-in preparation

- Azman S, Khadem AF, Plugge CM, Stams AJM, Zeeman G. Microbial community dynamics in five replicate CSTRs during start-up period with transient feeding strategy to achieve anaerobic digestion of cellulose and xylan.

* Equal Contribution

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Wageningen-Bennekom-Gemlik

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The SENSE Research School declares that **Mr Samet Azman** has successfully fulfilled all requirements of the Educational PhD Programme of SENSE with a work load of 37.6 EC, including the following activities:

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- *Humic and fulvic acid inhibition on anaerobic degradation of cellulosic material*. 2nd International Conference on Biogas Microbiology (ICBM), 10-12 June 2014, Uppsala, Sweden

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