Hydrolysis inhibition of complex biowaste

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Thesis

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To my mother,
saudade!

Abstract

The increasing demand of renewable energy sources and reuse of wastes, challenges our society for better technological solutions for energy production. Co-digestion of agricultural biowaste, such as animal manure and plant residues, offers an interesting contribution to the renewable energy strategies. The biogas plants, where the complex substrates, such as agricultural biowaste, get converted into biogas, are then able to produce electricity and heat, which can be used in the farm and delivered to the main electricity grid. Moreover, due to its decentralised nature, the implementation of small-scale biogas plants can supply renewable energy to people without the need for large-scale infrastructural networks such as electricity grids, thereby solving part of the populations' energy demands.

The production of biogas from complex biowaste is rate-limited by the hydrolysis step of the anaerobic digestion process. However the hydrolysis step has been poorly described and not very well understood, resulting in non-optimized anaerobic digester volumes. Therefore, in this thesis, a review on the anaerobic hydrolysis step is presented, together with ways to accelerate hydrolysis. Solutions are proposed by either mitigating the revealed inhibiting compounds, by pre-treating difficultly hydrolysable substrates, or as is nowadays also applied, by adding hydrolytic enzymes to full scale biogas co-digestion plants.

In this thesis two compounds were studied in terms of its inhibiting effect on hydrolysis: ammonia nitrogen and humic matter (HM). In contrast to previous suggestions in literature, ammonia nitrogen did not show an inhibiting effect on anaerobic hydrolysis. On the other hand Humic acids-like (HAL) and Fulvic acids-like (FAL) extracted from fresh cow manure and silage maize, which are extensively described in terms of its chemical characteristics in this thesis, showed a strong inhibiting effect on the hydrolysis step.

Plant matter is high in lignocellulosic biomass. Lignocellulosic biomass consists of lignin, which is resistant to anaerobic degradation, cellulose and hemicelluloses. The degree of lignin encrustation generally determines the bio-methane potential (BMP) of organic matter. Pre-treatment of plant material, can increase the BMP and therefore the biogas production during co-digestion of manure. Calcium hydroxide pre-treatment was shown, in this thesis, to improve the BMP of lignocellulosic biomass, especially for high lignin content substrates. Maleic acid generated the highest percentage of dissolved COD during pre-treatment, however, compared to calcium hydroxide, its high market price limits its applicability.

Enzyme addition has recently gained the attention of biogas plants' operators in order to accelerate hydrolysis, however further research is needed to elucidate its potential in biowaste digesters.

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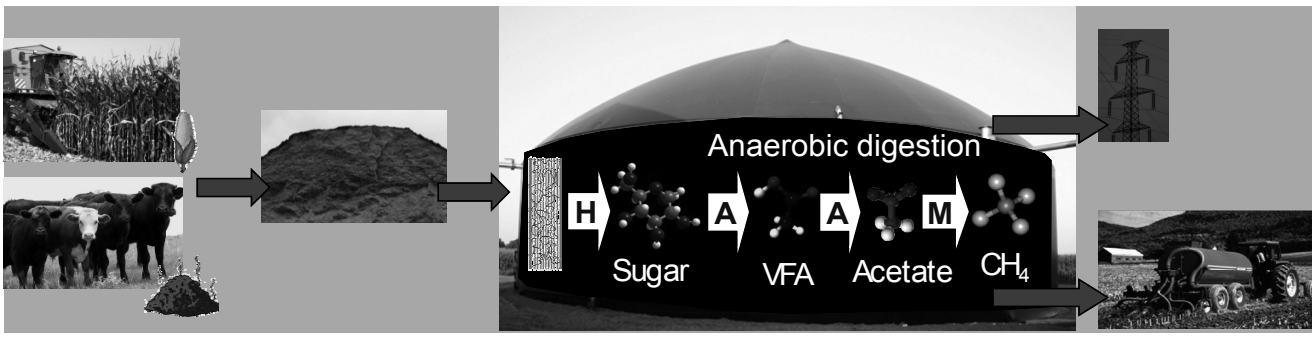
Chapter 1

General Introduction



Renewable energies are the solution for future energy supply as they increase energy independency by reducing fossil fuels use and mitigate greenhouse gas emissions. It is predicted that renewable energies will become the fastest growing energy source in the world in the next 20 years (EIA, 2009).

1.1 World's energy consumption

Currently, more than 85% of the world total primary energy consumption originates from fossil fuels, such as oil (36%), coal (27%) and gas (23%) (EIA, 2009). This fossil fuel dependency is found all over the world, independently of the area of the world and the level of economical development, as can be seen in Figure 1.1.

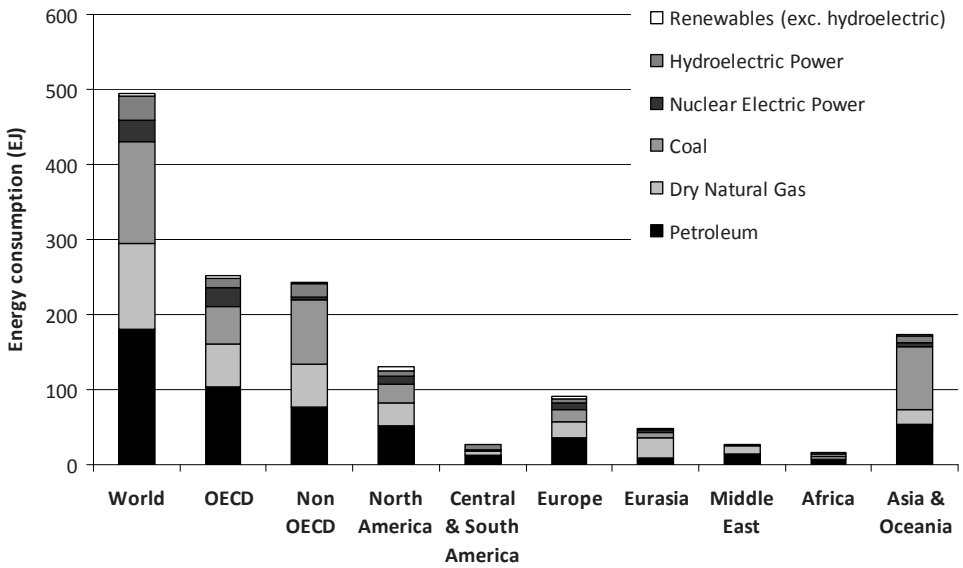


Figure 1.1. Energy consumption in the different areas of the world according to primary energy source. Data from 2007, adapted from IEA report (EIA, 2009).

Even though differences can be seen in the type of fossil fuel used, since in the Middle East including the Gulf Area, the major source of primary energy is oil (53%), followed by natural gas (45%) and coal (1.5%), but in Asia and Oceania the opposite is verified with coal (49%) as the major source, followed by oil (32%) and natural gas (11%). Other clear differences are that from the total primary energy consumption sources: Central and South America consumes less than 4% of coal,

while for Africa this value raises to almost 30%, and that Eurasia consumes 53% of natural gas while in Asia and Oceania this value is only about 10%. North America and Europe show similar tendencies, about 40% from oil, 24% from natural gas and about 20% from coal. These two regions also show the highest consumption of energy originating from nuclear power, 11% for Europe and 8% for North America. Worldwide, the percentage of energy originating from renewable sources, excluding the hydroelectric power, is less than 4% (EIA, 2009).

It is clear that the world is too dependent on fossil fuel energy sources, however this is not the only issue that needs to be addressed in the near future. The energy inequality between people of the different areas of the world is also striking. The 1,2 billion people belonging to the OECD (18% of world population) consume 257 EJ of the total 510 EJ of the world's energy consumption, leaving the other half (253 EJ) to the remaining 5,5 billion people belonging to the Non OECD (82% of world population). North America is the biggest consumer of energy in the world (293 GJ·person^{-1}·year^{-1}), followed by Eurasia, Europe and the Middle East, as shown in Figure 1.2.

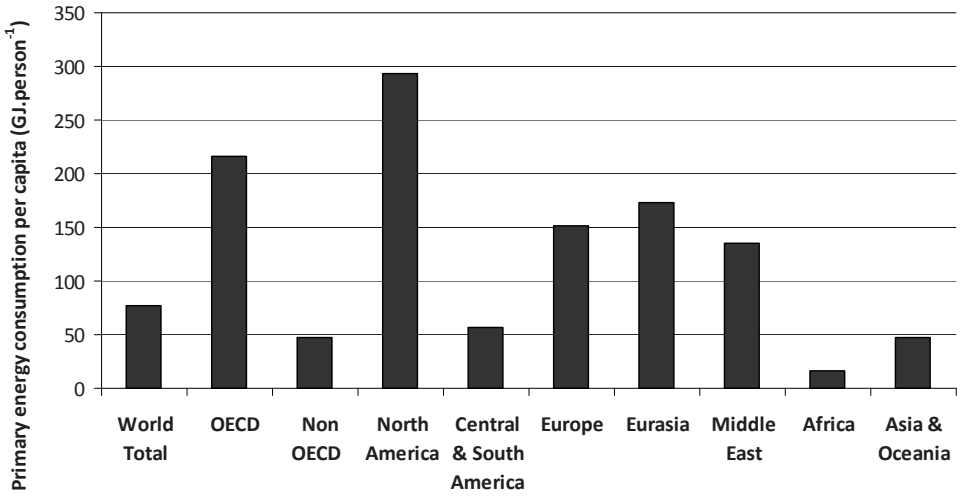


Figure 1.2. Primary energy consumption in the different areas of the world per capita. Data from 2007, adapted from IEA report (EIA, 2009).

On the other hand certain African countries, such as Ethiopia, Somalia and Rwanda consume about 1 GJ·person^{-1}·year^{-1}. Due to its decentralised nature, the implementation of specific renewable energy technologies can supply energy to people in a fairer and more sustainable way.

1.2 Europe's energy consumption

Europe imports more than half of its primary energy consumption. Its dependency on imported fossil fuels, which equals ≈ 90% from oil, > 60% from natural gas, and > 40% from coal, is steadily increasing. As a result, Europe is one of the most import-depending areas with regard to energy supply (AEBIOM, 2009; EEA, 2008). Russia is the main energy supplier of Europe, supplying about 25% of the natural gas consumption, approximately 30% of the oil consumption and about 10% of the coal consumption (EEA, 2008).

When evaluating only the 27 countries belonging to the European Union (EU-27), thus excluding countries such as Norway, Turkey and countries in the Balkans, nuclear power as primary energy source equals 13.4% (Figure 1.3). This is higher than the previously mentioned 11% of Europe's consumption, since none of these countries have nuclear power plants. The renewable energies in the EU-27 only amount to 7.8% in 2007 (Figure 1.3). However it has increased by 28% since 2002, and the tendency is that it will increase further owing to the fact that new energy sources are increasingly more attractive (Eurostat, 2009b). Within the renewables, biomass, and specifically wood and wood waste, is the main primary energy source, as illustrated by Figure 1.3. The wood and wood wastes are mostly combusted in Cogeneration Plants, therefore converting this solid biomass into heat and electricity (EurObserv'ER, 2009). Biogas only represents 4.0% of the total renewables and 5.8% of the biomass.

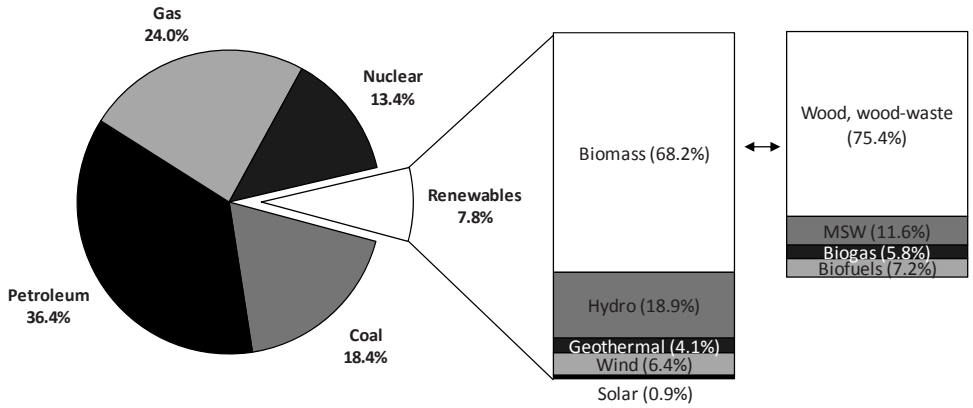


Figure 1.3. Energy consumption in the Europe-27 countries zone according to the primary energy source. Data from 2007, adapted from Eurostat reports (Eurostat, 2009a; Eurostat, 2009b).

To be able to increase the consumption of primary energy originating from renewable sources, and specifically biomass, which is abundant and dispersedly available, it is necessary to access the available resources, develop and implement suitable and sustainable strategies supported by governmental policies for energy harvesting and improve the energy conversion rates of present and future technologies.

1.3 Biowaste as a primary energy source

According to the Renewable Energy Directive (2009/28/EC), biomass is the biodegradable part of products, waste and residues from biological origin from agriculture, forestry, food-processing industries including fisheries and aquaculture, and municipal and industrial waste. Currently in Europe, the total amount of biomass accounts for two thirds of the renewable energy sources, however it has been given less attention than solar and wind (EEA, 2006). The Renewable Energy Directive (2009/28/EC) approved the targets energy derived from renewable sources by 2020, which is 20% for Europe and 14% for the Netherlands. In order to achieve these targets more attention needs to be given to biomass, and especially to the biomass originating from agriculture, which is so far highly unexploited (EEA, 2006), taking into account that half of the European Union's land is farmed (EEA, 2007). However, increasing the use of biomass from agriculture should not result in competing land use claims between food production, forestry biodiversity, and biomass production. Preferably, biomass conversion into bioenergy should be restricted to biowaste, thereby sustainably solving agro-waste disposal problems.

Almost 3 billion tonnes of waste are produced each year in Europe, from which 3% are hazardous. Almost 60% of the total waste is composed of mineral materials from mining, construction and demolition, and from manufacture of construction materials (Eurostat, 2009a). Agriculture biowaste, such as animal manure and agricultural waste & residues, account for about 10%. Household waste, such as mixed municipal waste and kitchen waste, accounts for 8%, whereas sludges from domestic and industrial waste water treatment plants (WWTP) (including digestate from anaerobic digesters) account for about 2% of the total waste production (Kloek and Blumenthal, 2009). Even though the percentage of biowaste is low compared to the total waste produced in Europe, it represents a large potential for renewable energy generation. The energy potential from biomass for the coming 40 years, has been presented by many authors in scientific papers and technical reports (e.g. AEBIOM, 2009; CEC, 2005;

EEA, 2006; Faaij, 2006; Smeets et al., 2007). All scenarios, presented by these references, include the increase in usage of biowaste as an important source for the biomass energy production, but also the increase in dedicated energy crops and the usage of wood from forest growth. According to Smeets et al. (2007), the potential amount of energy produced in Europe from agricultural residues is between 1 and 4 EJ·year^{-1} in 2050. However, they do not consider animal manure, which represents 5% of Europe's total waste production, and about half of the agriculture biowaste (Kloek and Blumenthal, 2009). On the other hand, in the scenarios evaluation of the European Biomass Action Plan (CEC, 2005) and European Environmental Agency (EEA) report (EEA, 2006), a greater part of the agricultural residues are assumed to be used for food products and animal feeding instead of energy production. Therefore, making it difficult to predict and compare scenarios in terms of biomass potential. Moreover, the energy potential of the different scenarios is differently calculated, resulting in distinctive scenario outcomes.

According to the EEA, the five main sources of biowaste that accounted in 2010 for almost 90%, or 4.2 EJ of the biowaste, are: agricultural solids residues (most of it as straws), wet manures, wood processing residues, municipal solid waste (MSW) and black liquor from the paper production process. Furthermore, it is assumed that due to the waste minimisation practises, the total amount of biowaste will remain stable in the coming 20 years (EEA, 2006). In percentage this means that about 24% of the biomass will be derived from agricultural solids residues, about 15% from wet manures, about 15% from wood processing residues, between 16 and 19% from municipal solid waste (MSW) and between 15 and 17% from black liquor (EEA, 2006).

1.4 Technologies available for biowaste conversion into energy

There are many technologies available to convert biomass, and particularly biowaste, into energy. They can be divided into thermo-chemical, biochemical and physical-chemical, as shown in Figure 1.4.

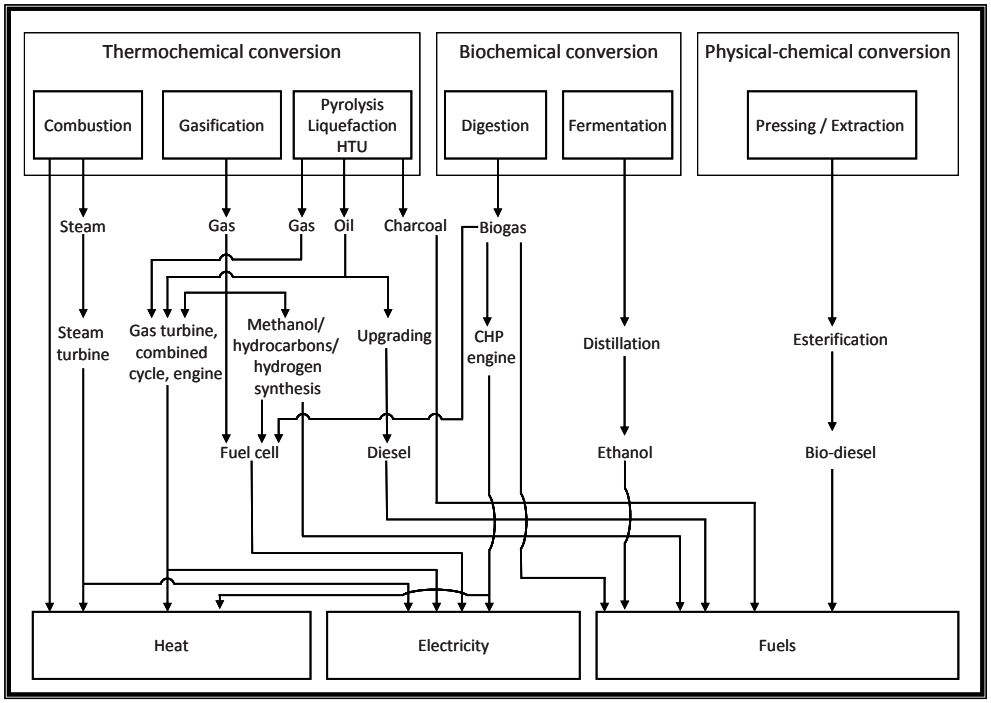


Figure 1.4. Technologies available for biomass conversion into secondary energy source. Adapted from Turkenburg and Faaij (2000) and Deublein and Steinhauser (2008).

The main problem with most technologies is their low electrical conversion efficiencies, reaching as low as 30% for a gas turbine (Deublein and Steinhauser, 2008). For technologies, such as anaerobic digestion, that produce biogas, Combined Heat and Power (CHP) generators are a good option, since the overall efficiency can increase to 80 – 90%. This is achieved by using the remaining thermal energy to produce heat that can be used for industrial or domestic purpose (Loesoenen, 2003). Increasing energy conversion efficiencies is essential for improving the energy supply and for using less primary energy sources. In the Renewable Energy Directive (2009/28/EC), conversion technologies achieving at least 85% conversion efficiencies for residential and commercial applications and at least 70% for industrial applications are suggested.

1.5 Biogas plants for biowaste conversion into energy

According to the Renewable Energy Directive (2009/28/EC), biogas installations can play a major role in the conversion of agriculture biowaste into secondary energy sources, since it offers sustainable and economical development to rural areas, due to its decentralised nature and the regional investment structure. Most of biogas installations applied in Europe consist of a vertical CSTR followed by a storage tank for post digestion and full CH_4 capturing. In countries, such as China, where the number of household biogas plants is the highest in the world (> 26 million) and keeps rapidly rising, most of the reactors are a concrete pit of a about 8 m^3 of volume supplying enough energy for a household or farm. This type of reactor was implemented already in the 1970's. In the last decade though, new glass fiber reinforced plastic (GRP) reactors are being used, since they do not need maintenance and have a longer life span (Chen et al., 2010).

At co-digestion biogas plants, where more than one substrate is added to the anaerobic digester, there are usually separate storage tanks for the individual substrates, as shown in Figure 1.5, with a liquid manure storage and a co-substrate storage. The total solids content in these digesters is usually between 8 and 10% TS (wet fermentation) and reactors are operated at an HRT of 60 to 90 days (Weiland, 2006) under either mesophilic or thermophilic conditions (Deublein and Steinhauser, 2008).

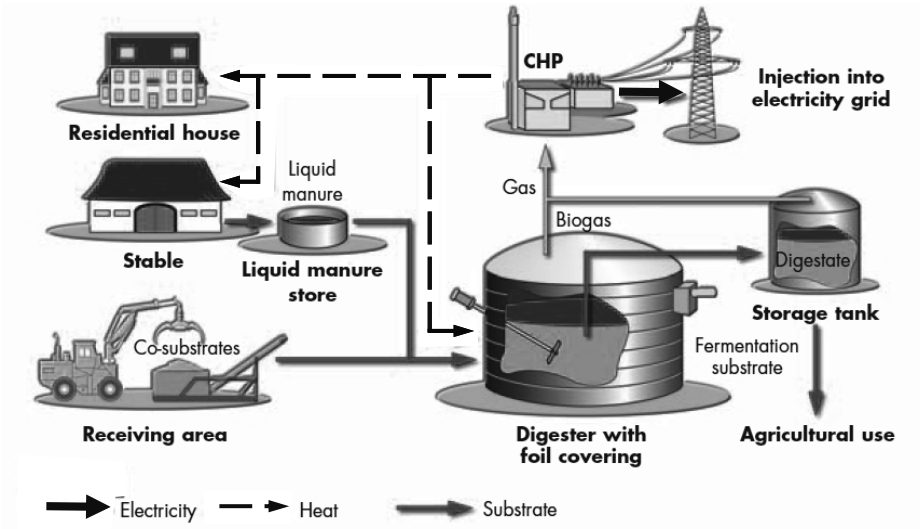


Figure 1.5. Example of agriculture co-digestion biogas plant. Adapted from FNR (2009).

The major advantages of co-digestion are the increase in volumetric biogas production, increased process stability by manure-coupled alkalinity addition, and the improvement of the nutrient ratio C:N:P, resulting in a higher fertilizing value of the digestate (Álvarez et al., 2010).

1.6 Substrates for co-digestion

Many substrates can be used for co-digestion, however for stable reactor operation and producing a valuable fertilizer an appropriate combination of substrates needs to be applied. Most co-digestion plants in Europe add animal manure with a plant material. The high ammonia concentrations of the manure will provide a good buffer capacity in the reactor, therefore improving the anaerobic digestion, comparing to the digestion of plant material alone. On the other hand, the plant material will enhance the biogas yield, since its yield can be 2 to 8 times higher than the manure's yield (AEBIOM, 2009).

Regarding biowaste co-digestion many sources of substrates can be used, originating from agriculture, as shown in Figure 1.6, but also from food processing industry, such as fish and olive oil industries, from pulp and paper industry, from yeast producing industry, such as breweries, from markets and canteens, from municipal solid waste and sewage sludge (AEBIOM, 2009). However, as some of these substrates contain compounds that are harmful to the environmental, such as heavy metals, strict legislation of some countries forbids its co-digestion in biogas plants.

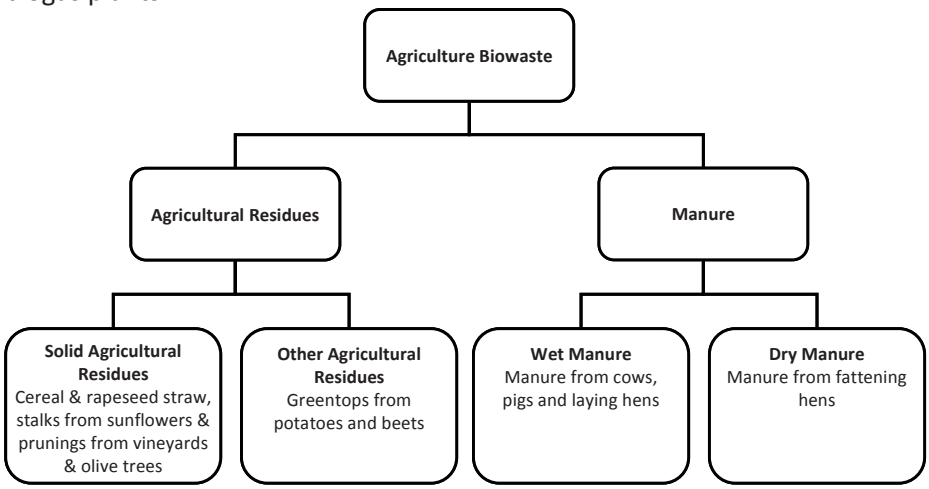


Figure 1.6. Agriculture biowaste sources according to EEA report (EEA, 2006).

Maize silage is one of the agricultural substrates with the highest biogas yield, 230 m^3 biogas·ton^{-1} of wet biomass (Weiland, 2006). On the other hand, cow and pig slurry have the lowest biogas yield, 25 to 30 m^3biogas·ton^{-1} of wet biomass (Weiland, 2006). Other biowaste, such as slaughterhouse waste which has biogas yields that go up to 1000 m^3 biogas·ton^{-1} of wet biomass could greatly increase the energy output, however, in most European countries it cannot be added to manure co-digestion biogas plants due to strict legislation. On the other hand, some industrial waste, such as baking waste and used greases, which also contain high biogas yields, 700 to 1000 m^3 biogas.ton^{-1} of wet biomass, can be considered for co-digestion (Weiland, 2006). Biowaste from households, that has a biogas yield of about 120 m^3 biogas·ton^{-1} of wet biomass, can also be used for co-digestion in countries were source separation is effective, such as Germany and The Netherlands (Weiland, 2000). This is also the case for canteen waste, which contains high lipids, therefore resulting in high biogas yields (Weiland, 2000).

The biogas production yield and rate will vary depending on the characteristics of the substrates, such as dry matter content, lignin content, inhibiting compounds content, carbohydrates, lipids and proteins content, and the accessibility of the substrate molecules to the anaerobic bacteria and archea, but also how these substrates balance each other's characteristics inside the anaerobic digester.

1.7 Anaerobic digestion process

The anaerobic digestion process can be divided in four distinguishable steps: hydrolysis, acidogenesis, acetogenesis and methanogenesis, as shown in Figure 1.7. During hydrolysis, biopolymers, such as carbohydrates, proteins and lipids, are converted into monomeric compounds, such as sugars, amino acids and fatty acids and glycerol (Sanders, 2001). This conversion of biopolymers is catalysed by exoenzymes (hydrolases) secreted from facultative and obligatory anaerobic acidogenic bacteria (Gallert and Winter, 2005). The monomeric compounds released during hydrolysis are then small enough to enter the acidogenic cell membrane and subsequently converted to VFA, alcohols, NH_3, lactic acid, H_2, CO_2 and H_2S. These products are then excreted from the acidogenic bacteria and anaerobically oxidised to acetate, formate, H_2 and CO_2 by acetogenic bacteria. Finally, the strictly anaerobic methanogenic archaea reduces the CO_2 + H_2 and cleaves the acetate molecule to produce methane and CO_2 (Insam et al., 2010; van Lier et al., 2008).

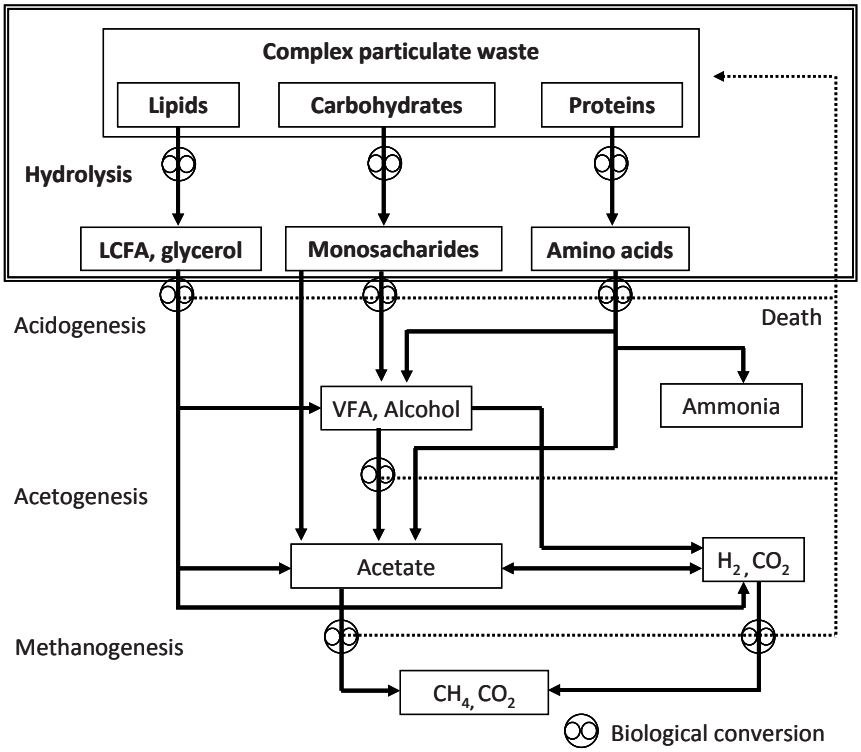


Figure 1.7. Anaerobic digestion process, adapted from Sanders (Sanders, 2001).

Hydrolysis – the first step of anaerobic digestion - is considered as the rate limiting step in the anaerobic digestion of complex waste, such as animal manure and agricultural residues (Pavlostathis and Giraldo-Gomez, 1991). However, the hydrolytic process is still poorly described (Vavilin et al., 2008), which results in non-optimized digester volumes. Therefore, for properly designing anaerobic digesters, not only the biodegradability, or bio-methane potential (BMP), of complex waste needs to be assessed, but also the hydrolysis rate, which determines the maximum biogas production rate (El-Mashad, 2003). Because of that, ways to accelerate the hydrolysis step, either by mitigating inhibiting compounds, pre-treating difficultly hydrolysable substrates, or adding hydrolysis boosters, such as hydrolytic enzymes, needs to be studied and adopted in full scale biogas co-digestion plants when successfully proven.

1.8 Scope of the thesis

The major goal of this thesis is to increase the knowledge on the hydrolysis step during anaerobic digestion of complex wastes. Therefore, this thesis starts with defining what is the present world situation in terms of energy consumption and how anaerobic digestion, through biogas plants, can help solving the renewable energy demand of the future (**chapter 1**). Special emphasis is then given to the anaerobic digestion of biowaste, and particularly from the agriculture sector, where hydrolysis is clearly rate limiting the process. In order to better understand the hydrolysis process, focussing on the limiting factors, an overview on hydrolysis is given in **chapter 2**. In this review it is clear that an important point for improving hydrolysis is to determine its inhibiting compounds. **Chapter 3** describes the impact of ammonia, which is present at high concentrations in animal manures and has been reported to inhibit other steps of anaerobic digestion, such as methanogenesis (El-Mashad, 2003; Van Velsen, 1981; Zeeman, 1991). The results of chapter 3 indicate that ammonia does not inhibit the hydrolysis of complex substrates, however, the results also suggest that there is another compound, present in the liquid fraction of manure that does inhibit the hydrolysis, as has been suggested by Zeeman (1991). Humic matter is considered as potential inhibitor, whereas humic acids and fulvic acids have been reported to inhibit the hydrolysis of protein by Brons et al. (1985). Therefore, in **chapter 4**, extraction of humic acid-like (HAL) and fulvic acid-like (FAL) from cow manure and maize silage is performed and a full chemical characterization of these compounds is presented, in order to later be able to identify, by the inhibiting patterns, which mechanism might be responsible for inhibition. In **chapter 5**, the inhibiting effect of the HAL and FAL is tested, and indeed, inhibition on hydrolysis is found. Although even at the lowest tested concentration of HAL and FAL severe inhibition is found, no clear relation could be assessed, hampering mechanistic or mathematical interpretation of the results. In **chapter 7**, a possible mechanism is hypothised and discussed. In this thesis several ways of accelerating the hydrolysis step by means of pre-treatment is also evaluated. Pre-treatment of high lignin content substrates, such as plant matter, is particularly important in order to promote co-digestion of manure with agriculture residues. Therefore, in **chapter 6**, three plant species, hay, straw and bracken, with varying lignin content, were thermo-chemically pre-treated with calcium hydroxide, ammonium carbonate and maleic acid and then anaerobically digested for 40 days. Improved hydrolysis and biodegradability was found for substrates with high lignin content, giving best results with maleic acid as dosing agent.

In **chapter 7**, the conclusions and discussion points from each chapter are integrated and suggestions are given for future research. A complete overview of this thesis is given in Figure 1.8.

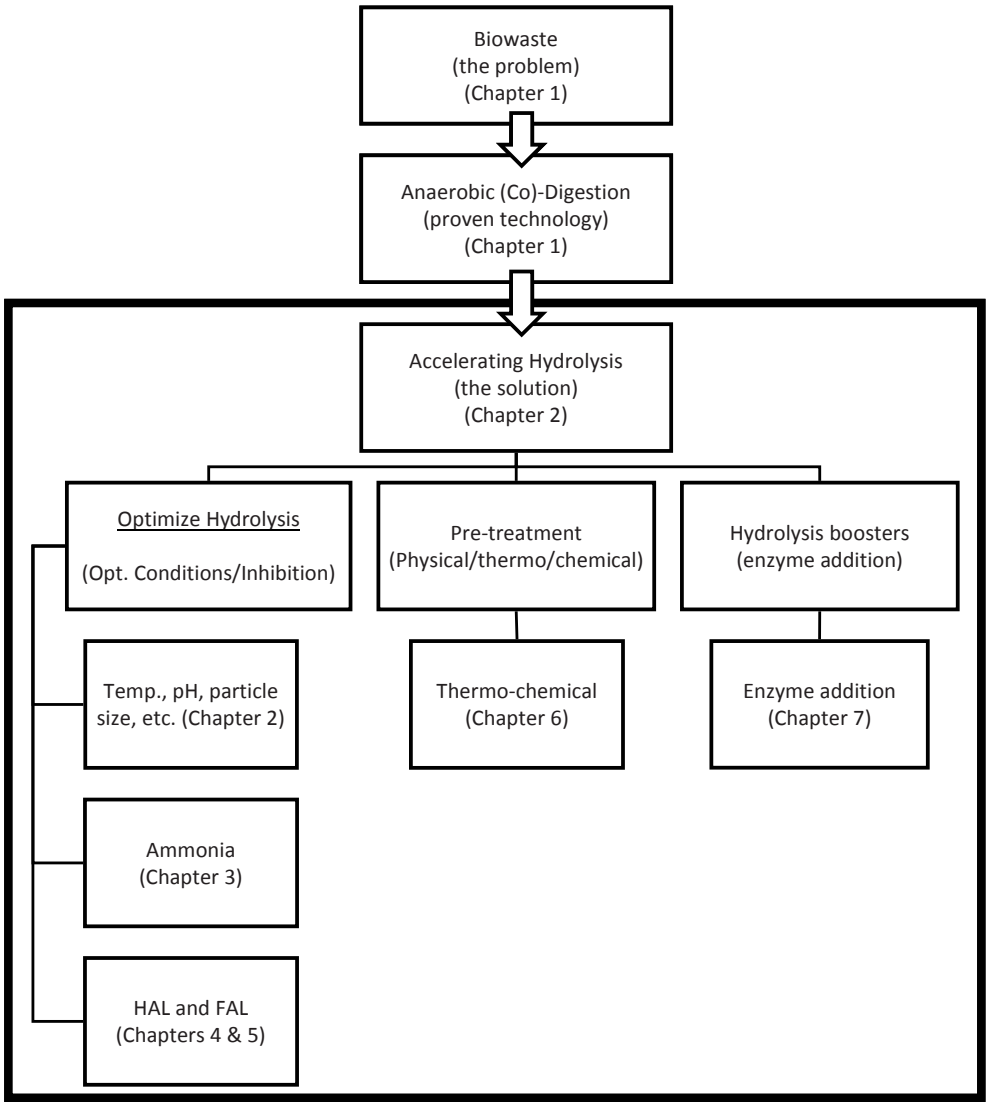


Figure 1.8. Overview of thesis.

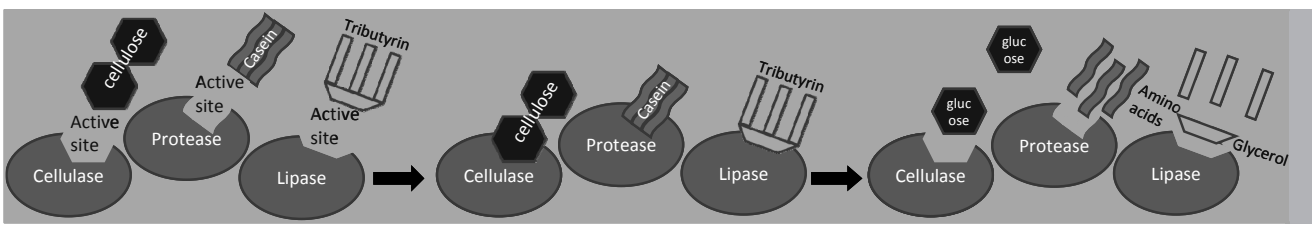
Nomenclature:

Biofuels	Includes: bioethanol, which is the ethanol produced from biomass and/or biodegradable fraction of waste; biodiesel, which is the diesel quality liquid fuel produced from biomass or used fried oils; biomethanol, which is the methanol produced from biomass and/or the biodegradable fraction of waste; and biodimethylether, which is the diesel quality fuel produced from biomass and/or the biodegradable fraction of waste (Eurostat, 2002).
Biogas	Gas composed principally of methane and carbon dioxide produced by anaerobic digestion of biomass. It includes: landfill gas, formed by the digestion of landfilled wastes; sewage sludge gas, produced from the anaerobic fermentation of sewage sludge; and biogas produced from the anaerobic fermentation of animal slurries and of wastes in abattoirs, breweries and other agro-food industries (Eurostat, 2002).
Biomass	Biodegradable part of products, waste and residues from biological origin from agriculture, forestry, industries including fisheries and aquaculture, and municipal and industrial waste (Renewable Energy Directive, 2009/28/EC)(2009/28/EC, 2009).
Fossil fuels	Primary energy source that includes: crude oil and natural gas liquids, petroleum products (refinery gas, ethane, kerosene, motor oil, lubricants, naphta, etc.), coal and coal products (hard coal, lignite, patent fuel, peat, etc.) and dry natural gas.
MSW	Municipal Solid Waste. For the renewable energy part, it includes waste produced by households, industry, hospitals and the tertiary sector which contains biodegradable materials which are incinerated at specific installations (Eurostat, 2002).

OECD	Organization for Economic Cooperation and Development. Members: Australia, Austria, Belgium, Canada, Czech Republic, Denmark, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Japan, Luxembourg, Mexico, Netherlands, New Zealand, Norway, Poland, Portugal, Slovakia, South Korea, Spain, Sweden, Switzerland, Turkey, United Kingdom, and United States.
Renewable energies	Primary energy sources that are infinite, it includes: biomass, hydro, geothermal, solar, wind, ocean thermal, wave action, and tidal action.
Waste	It includes: municipal solid waste, liquid acetonitrile waste, tall oil, waste alcohol, medical waste, paper pellets, sludge waste, solid byproducts, tires, agricultural byproducts, closed loop biomass, fish oil, and straw.
Wood, wood-waste	Purpose-grown energy crops (poplar, willow etc.), woody materials generated by industrial processes (wood/paper industry in particular) or provided directly by forestry and agriculture (firewood, wood chips, bark, sawdust, shavings, chips, black liquor etc.) as well as wastes such as straw, rice husks, nut shells, poultry litter, crushed grape dregs etc. Combustion is the preferred technology for these solid wastes (Eurostat, 2002).

Chapter 2

Anaerobic hydrolysis of complex wastes
- review -



Abstract

Hydrolysis is a crucial step in the anaerobic digestion of complex wastes; however, it has been poorly described. This review provides understanding on the mechanisms involved in hydrolysis and how it is affected by the environmental conditions, such as specific temperature and pH, generally applied in anaerobic digesters. The review also reveals the importance of specific substrates characteristics, such as particle type, size and structure and their impact on the hydrolysis rate. Furthermore, a detailed summary of the studies performed on the inhibiting effects of compounds present in anaerobic digesters, such as VFA, ammonia, LCFA, glucose and HAL and FAL is presented. Additionally, a literature review of the different hydrolysis kinetics, including an inventory of first order hydrolysis constants, and the modelling tools nowadays used, is described. Finally, a general overview on different pre-treatment methods and the most recent trend of enzyme addition for enhancing the anaerobic digestion process is evaluated. With this review we aim for an up to date overview and a better understanding of the anaerobic hydrolysis step, allowing for optimization of the overall anaerobic digestion process.

Tânia V. Fernandes, Grietje Zeeman, Jules B. van Lier

2.1 Introduction

Understanding and optimizing the hydrolysis step in order to improve the anaerobic digestion of biowaste is a necessity for maximising its energy potential.

Following the biochemical definition, hydrolysis is a chemical reaction involving the breaking of a chemical bond between two atoms with a water molecule as reactant (Bettelheim et al., 2010). This reaction is catalysed by enzymes (hydrolases) secreted by microorganisms (Bettelheim et al., 2010). In anaerobic hydrolysis, these microorganisms are anaerobic acidogenic bacteria (Gallert and Winter, 2005).

Hydrolysis - the first step of anaerobic digestion - is considered as the rate limiting step in the anaerobic digestion of complex waste, such as animal manure, agricultural residues, sewage sludge, etc. (Pavlostathis and Giraldo-Gomez, 1991). However, the hydrolytic process is still poorly described (Vavilin et al., 2008), which results in non-optimized digester volumes. Therefore, to properly design anaerobic digesters, not only the maximum bio-methane potential (BMP) of the complex waste needs to be determined, but also the hydrolysis rate needs to be ascertained to determine the biogas production rate (El-Mashad, 2003).

The objective of this review is to report the current knowledge on anaerobic hydrolysis of complex wastes. Firstly, the hydrolysis step is biochemically described, with emphasis on anaerobic conditions. This is followed by a literature review on the most important factors affecting anaerobic hydrolysis, such as, environmental factors, substrate related factors, and inhibitors. Moreover, an overview of the used kinetics and models to describe hydrolysis are presented, and the appropriate methodology to determine the hydrolysis kinetic constants. Finally, ways of enhancing anaerobic hydrolysis, either by pre-treatment or enzyme addition, are discussed.

2.2 Enzymatic hydrolysis - Process description

Hydrolysis is a chemical reaction involving the breaking of a chemical bond between two atoms using water as reactant. One fraction of the split molecule gains a hydrogen ion and the other fraction gains a hydroxyl group (Bettelheim et al., 2010).

In the anaerobic digestion of complex organic matter, hydrolysis is the first step. During hydrolysis, biopolymers, such as carbohydrates, proteins, and lipids, are converted into monomeric compounds, such as sugars, amino acids, fatty acids, and glycerol (Sanders, 2001). This conversion of biopolymers is catalysed by

exoenzymes (hydrolases) secreted by facultative and obligate anaerobic acidogenic bacteria (Gallert and Winter, 2005). The enzymes cellulases and amylases hydrolyse carbohydrates, proteases hydrolyse proteins and lipases and phospholipases hydrolyse lipids (Gallert and Winter, 2005). As each exoenzyme converts only a specific substrate or group of substrates, diverse and numerous acidogenic bacteria are necessary in anaerobic digesters to which different substrates are added.

The exoenzymes (hydrolases) are secreted by different genera of acidogenic bacteria, including Clostridium, Acetivibrio, Bacteroides, Selenomonas and Ruminococcus (Insam et al., 2010).

Enzymes are proteinaceous molecules that catalyse biochemical reactions and can be divided in two types, endoenzymes (function within the cell) and exoenzymes (function outside the cell) (Gerardi, 2003). Their catalytic mechanism was first described by Emil Fisher in 1840 with the lock-and-key model (Fischer, 1894), which proposed that the substrate molecule fitted perfectly with the active site of the enzyme enabling the chemical reaction. This theory was later replaced by the induced-fit model (Koshland, 1958) which added that a correct substrate for a certain enzyme can induce a conformational change on the active site of the enzyme, therefore enabling the catalysis. This later model, which includes enzyme's properties such as plasticity, flexibility and allostery, is still the base of presently developed models for explaining the protein-ligand binding processes (Benkovic and Hammes-Schiffer, 2003; Teilum et al., 2009).

In anaerobic digestion, less attention has been given to the catalytic mechanism of enzyme active sites with the substrate, as described above, but more to the mechanisms involved in the release of the enzymes by the acidogenic bacteria and its further attachment to the substrate. In literature two models have been described (Batstone et al., 2000): 1) the acidogenic bacteria secrete the exoenzymes to the bulk liquid, which subsequently adsorb to the substrate that is present either in the particulate or soluble form (Jain et al., 1992); 2) the acidogenic bacteria colonise the substrate and then secrete exoenzymes (Vavilin et al., 1996). The latter model has been illustrated by SEM images, showing that the microorganisms fully colonise the substrate's surface (Song et al., 2005). Irrespective of the used model, it is postulated that acidogenic bacteria always produce an excess of enzymes, so full colonisation of all available surface of the substrate is achieved (Hobson, 1987). As a consequence, a higher acidogenic bacteria concentration will not increase the hydrolysis rate, which explains why hydrolyses can be generally described by first order kinetics (San Pedro et al., 1994; Song et al., 2005; Tong et al., 1990).

After hydrolysis three more steps take place in anaerobic digestion: acidogenesis, acetogenesis and methanogenesis. The monomeric compounds released during hydrolysis are then small enough to enter the acidogenic cell membrane to be subsequently converted to VFA, alcohols, NH_3, lactic acid, H_2, CO_2 and H_2S. These products are then excreted from the acidogenic bacteria and mainly oxidised to acetate, formate, H_2 and CO_2 by acetogenic bacteria. Finally, the strictly anaerobic methanogenic archaea reduce the CO_2 using H_2 as electron donor and anaerobically oxidises the acetate to CH_4 and CO_2 (Insam et al., 2010; van Lier et al., 2008).

2.2.1 Enzymatic hydrolysis of carbohydrates

Carbohydrates are homo- or heteropolymers of hexoses, pentoses, or sugar derivates, which are mainly insoluble in water (Gallert and Winter, 2005). They are macromolecules of polymers that contain several sugar monomers. When including many chains of sugar monomers bound by glycosidic bounds, they are referred to as polysaccharides, and when including only one sugar monomer, as monosaccharides, or simple sugars (Gerardi, 2003). Starch and cellulose are the most common compounds studied in anaerobic hydrolysis. Starch is hydrolysed by amylases, that break the α-1,4 and α-1,6 glycosidic bonds, to produce glucose. Cellulose, an unbranched polymer of glucose residues joined by β-1,4 glycosidic bonds, is hydrolysed by cellulases to produce glucose (Sanders, 2001), as shown in Figure 2.1.

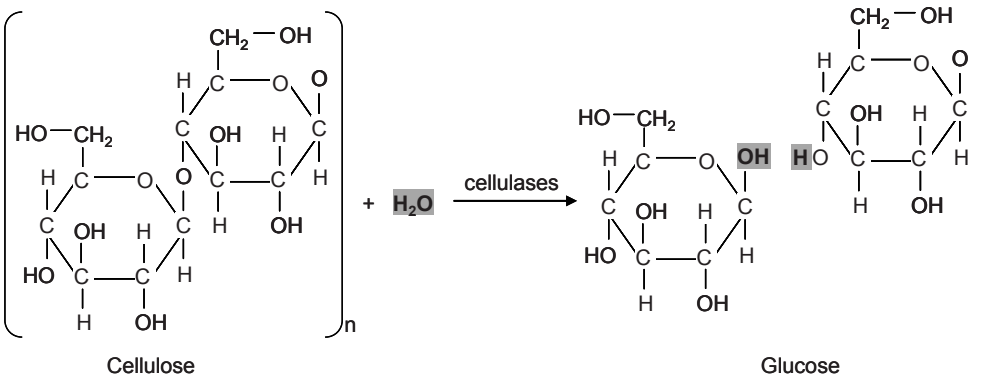


Figure 2.1. Hydrolysis of cellulose (adapted from Bettelheim et al. (2010))

Lignocellulosic biomass consists of lignin, cellulose and hemicellulose, which quantitatively and qualitatively vary according to the plant material. Unlike cellulose and hemicellulose, lignin is a cross linked network hydrophobic polymer consisting of aromatic rings and quinone moieties (Hatfield and Fukushima, 2005) that is fairly resistant to anaerobic degradation (Gallert and Winter, 2005). On the other hand, hemicellulose, an heterogeneous branched polymer of pentoses, hexoses and acetylated sugars, is easily hydrolysed by hemicellulases to produce pentoses, hexoses and acetylated sugars (Chandra et al., 2007).

Cellulose, together with lignin are the main structural components of plants and the most abundant biopolymers on earth (Gallert and Winter, 2005). It is also the most abundant polysaccharide in complex organic waste (Sanders, 2001). Due to its abundance and energy potential, research on cellulose enzymatic hydrolysis has significantly increased in the last decades (Zhang and Lynd, 2004), not only for anaerobic digestion in order to produce biogas but also to produce bioethanol.

Waste(water) with high carbohydrate content originates from carbohydrate food processing, brewery and starch (Batstone et al., 2002). Lignocellulosic biomass originates from plant and wood residues, such as crop and forest waste (Deublein and Steinhauser, 2008). Cow manure is also high in lignocellulosic biomass.

2.2.2 Enzymatic hydrolysis of lipids

Lipids are natural occurring hydrophobic molecules usually found in animal and plant tissues (Gerardi, 2003). There are many types of lipids, but the most commonly found in anaerobic digestion are fats and oils (Gerardi, 2003). Triglycerides are the chemical form in which most fats exists and they are composed by a glycerol ester with three long chain fatty acids (Sanders, 2001). Triglycerides are hydrolysed by lipases by breaking the fatty acid-ester bonds, therefore releasing the corresponding fatty acids and glycerol, as exampled in Figure 2.2, showing the hydrolysis of tributyrin into glycerol and butyric acid.

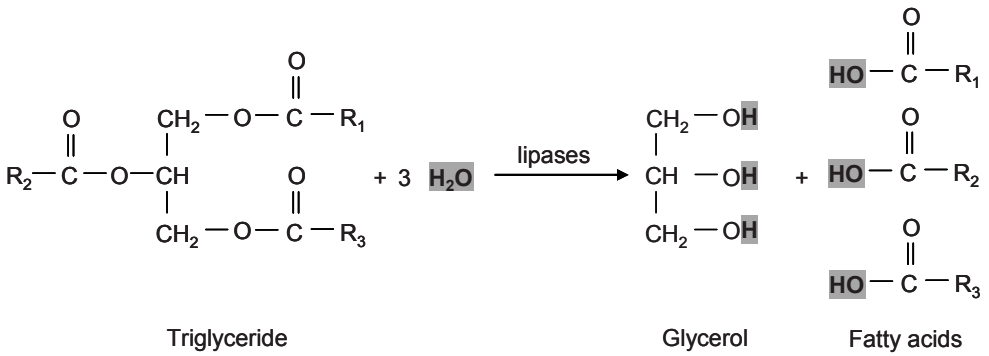


Figure 2.2. Hydrolysis of triglycerides (adapted from Bettelheim et al. (2010))

Due to their hydrophobicity, triglycerides will stay in the original emulsion or adsorb to the sludge during the digestion, so it is suggested that the hydrolysis of triglycerides only takes place at the lipid-water surface (Sanders, 2001).

Waste(water) with high lipids content originates from animal or food-processing (Batstone et al., 2002), such as waste oil from gastronomy, hotels and canteens (Deublein and Steinhauser, 2008), but also from dairies and slaughterhouses (Cammarota and Freire, 2006) and fish processing (González et al., 2005).

2.2.3 Enzymatic hydrolysis of proteins

Proteins are complex, high molecular-weight biomacromolecules and they can be divided into globular or fibrous (Sanders, 2001). Fibrous proteins, such as keratin, are very important because they make up most of the animal tissue. These proteins are insoluble in water and resistant to temperature and pH variations. Globular proteins, such as gelatine, are soluble in water or form colloidal suspension. These proteins are more sensitive to temperature and pH variations (Sanders, 2001). Proteins consist of amino acids, with an aliphatic or cyclic structure, containing an amino group and a carboxylic group. The amino acids, summing 20 different kinds, are joined by peptide bonds (Gerardi, 2003). During proteins hydrolysis, these peptide bonds are broken by proteases and peptidases, together known as proteinases, therefore releasing individual amino acids (Sanders, 2001), as shown in Figure 2.3.

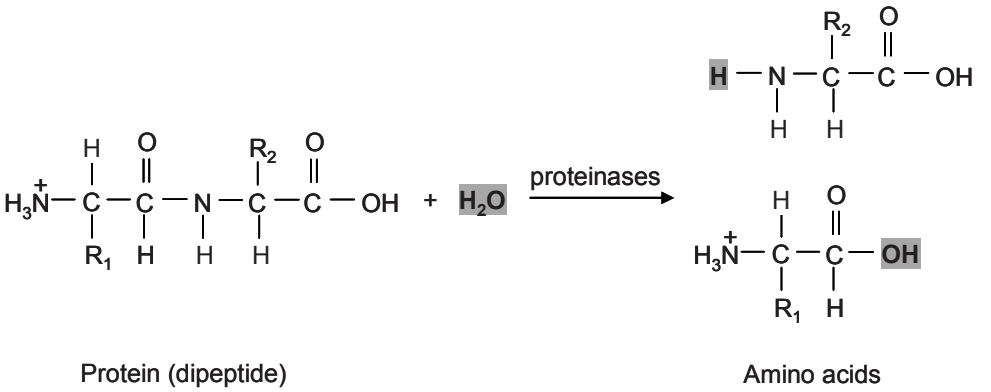


Figure 2.3. Hydrolysis of casein (adapted from Bettelheim et al. (2010))

Conjugated proteins, as glycoproteins and lipoproteins, might also be found in anaerobic digesters. Glycoproteins contain, besides amino acids, carbohydrates, and lipoproteins contain lipids (Gerardi, 2003). Hydrolysis of simple or conjugated proteins during anaerobic digestion has not been as extensively studied as the other complex substrates.

Similar to lipids, waste(water) with high proteins content originates from animal or food-processing (Batstone et al., 2002), such as fish industry (González et al., 2005), but also from dairies and slaughterhouses (Cammarota and Freire, 2006).

2.3 Factors affecting anaerobic hydrolysis

Many factors have been shown to affect anaerobic digestion, however not so much is known in terms of the anaerobic hydrolysis step (Vavilin et al., 2008). This gap in knowledge is mostly due to difficulties in measuring the effect on hydrolysis alone independent from the subsequent steps of anaerobic digestion, such as acidogenesis, which occurs simultaneously. Another reason is the difficulty in analysing the affecting factors independently.

The factors that affect the anaerobic hydrolysis should, therefore, be analysed as factors that 1) prevent the enzymes from catalysing the substrate, and 2) prevent or reduce the secretion of enzymes by the acidogenic bacteria.

2.3.1 Environmental factors

2.3.1.1 Temperature

Hydrolases have an optimum temperature generally between 40 and 60°C, however, enzymes exhibiting optimum temperatures outside this range can also be found in exceptionally cold or warm environments (Jonke and Michal, 2007). The relation between temperature and enzymatic activity is commonly described by the Arrhenius equation 2.1:

$$k = A \cdot e^{\left[\frac{-Ea}{R \cdot T}\right]} \tag{2.1}$$

where k is the rate constant, A is the pre-exponential factor, Ea is the Activation energy, R is the gas constant and T the temperature. Basically, this equation indicates that as temperature increases, so does the kinetic energy of the reactants, which indicates that the increase in temperature will increase the reaction rate up to its optimum. However, once the optimum of the temperature is reached, further increase will change the tertiary structure of the enzyme, resulting in thermal denaturation, therefore decreasing and eventually inactivating the enzyme (Bettelheim et al., 2010; Jonke and Michal, 2007).

Veeken and Hamelers (1999) anaerobically digested biowaste, consisting of whole-wheat bread, leaves, orange peels, bark, straw and grass, at temperatures between 20 and 40°C. They successfully related the first order hydrolysis rate constant with the temperature by the Arrhenius equation. The Arrhenius equation has also been used in the ADM1 for describing the influence of temperature on hydrolysis. After the optimum temperature, the rate follows a rapid drop to zero (Batstone et al., 2002).

2.3.1.2 pH

The hydrolases optimum pH is mostly dependent on the substrates and enzymes present, and lies somewhere between the pKa values of two or more catalytic amino acids, as a decrease or increase in pH outside the optimum can respectively protonate or ionize the side chains of its amino acids (Whiteley and Lee, 2006). Most enzymes have an optimum pH between 5 and 7, however, this is also dependent on the ionic strength (Jonke and Michal, 2007). When exposed to

conditions outside the optimum pH, hydrolases activity will reduce or even stop (Whiteley and Lee, 2006).

Many authors have studied the influence of pH on hydrolysis and, even though they were not able to quantify the inhibiting mechanism, they have concluded that a neutral pH is preferred for anaerobic hydrolysis (Dinamarca et al., 2003; Hu et al., 2005; Lü et al., 2006; Veeken et al., 2000a). According to the overview of Gallert and Winter (2005), hydrolysis of carbohydrates is favoured at slightly acidic pH, proteins and lipids hydrolysis are stimulated at neutral or even slightly alkaline pH. Wu and Tsai (2004) studied the effect of pH on hydrolysis of tributyrin with lipases from *Pseudomonas fluorescenes* CCRC-17015, using pH ranges from 6 to 8, and concluded that the optimum pH values for lipase specific activity were between 7.5 and 8. When anaerobically digesting kitchen wastes, which has high protein and lipids content, at pH of 5, 7, 9 and 11, Zhang et al. (2005) concluded that adjusting pH to 7 improves the hydrolysis rates. However, at the applied pH ranges other than 7, methanogenesis is also inhibited, which has been reported to negatively affect lipid hydrolysis (Palenzuela-Rollón, 1999). Hu et al. (2005) studied the effect of pH on hydrolysis of cellulose by rumen microbes at ranges from 5.5 to 7.5 and concluded that the pH should be kept above 6, most likely because the pH found in rumen is around neutrality. He et al. (2006) reported that a pH of 7 to 8 favoured protein hydrolysis when anaerobically digesting vegetable waste. In the ADM1 (Batstone et al., 2002) it was mentioned that pH could inhibit hydrolysis, however this inhibition was not included in the rate equations, because its inhibiting effect has still not been quantified. One of the main reasons why it so difficult to quantify pH inhibition is because a drop in pH can be a result of VFA increase, which are released during acidogenesis. It is, therefore, difficult to isolate either of these factors. The studies on pH and VFA effect on hydrolysis are discussed under VFA.

2.3.2 Substrate related factors

2.3.2.1 Particle size

Many authors have shown that particle size reduction of the substrate accelerates hydrolysis of complex substrates, particularly carbohydrates, since it increases its surface area, therefore increasing the number of adsorption sites of the substrate available for the enzymes (Hobson, 1987; Sanders, 2001; Sun and Cheng, 2002; Wen et al., 2004; Zhang and Lynd, 2004). Recently, Wen et al. (2004) reported an enhancement of glucose yield by 29% when reducing the particle size of acid-pre-

treated dairy manure from 840 – 590 to 590 – 350 µm. Their experiments were performed at 50°C (pH 4.8) by addition of commercial enzyme solutions. However, at particle sizes below 350 µm there where almost no changes on the glucose yield, suggesting that particle size was not limiting anymore (Wen et al., 2004). Masse et al. (2002) reported that particle size from 60 to 450 µm had no significant effect on the hydrolysis of neutral fat from pork fat at 25°C. According to the authors, this was probably due to the change in shape with the fat particle size. The small particle size had spherical shape whereas big particle sizes had a plate-like shape, which seemed to be better accessible to the enzymes than the spheres, due to its filamentous nature (Masse et al., 2002). The findings by Masse et al. (2002), however, contradict the findings of Hobson (1987), who showed that spherical particles are faster hydrolysed than flat particles. In the review on enzymatic cellulose hydrolysis by Zhang and Lynd (2004), it is mentioned that even though the external surface area is closely related to particle shape and size, this area only accounts for a small fraction of the overall surface area. The internal surface area is likely much bigger. Therefore, the increase in cellulose hydrolysis with decreasing particle size and shape might not only be due to increased external area, but also due to a decrease in mass transfer resistance (Zhang and Lynd, 2004).

Overall, the decrease in the particle size by mechanically cutting or grinding biowaste before anaerobic digestion is a recommended procedure in biogas plants (Deublein and Steinhauser, 2008). Benefits of decreasing particle size and increasing surface area have shown advantages on hydrolysis, therefore improving the overall anaerobic digestion process, resulting in higher biogas yields (Deublein and Steinhauser, 2008; Hu et al., 2005; Zhang and Lynd, 2004).

2.3.2.2 Substrate accessibility and intrinsic characteristics

The biopolymers, such as carbohydrates, proteins and lipids that are present in organic matter are not always accessible to the hydrolases, therefore delaying or even preventing the hydrolysis step. Lignocellulosic matter is the most common example for exemplifying limited substrate accessibility, because cellulose is then encrusted in lignin structures, therefore preventing the cellulases from accessing the cellulose (Cammarota and Freire, 2006; Palonen and Viikari, 2004). This is also found for hemicellulose. Therefore, enzymatic hydrolysis of hemicellulose is essential for higher yields of cellulose hydrolysis (Chandra et al., 2007). But cellulose intrinsic characteristics, such as crystallinity and degree of polymerization can also influence its availability to cellulases (Zhang and Lynd,

2004). Crystallinity is one of the most controversial factors involving the hydrolysis rate of cellulose due to contradictory results, but also due to uncertainty in the analysis methodology (Chandra et al., 2007; Mansfield et al., 1999; Zhang and Lynd, 2004). Recent studies, with identical and improved methodology concluded that increasing crystallinity reduces the hydrolysis rate of cellulose (Hall et al., 2010; Yoshida et al., 2008; Zhang et al., 2006). Degree of polymerization, i.e. number of glucosyl residues per cellulose chain, has been shown to limit hydrolysis. However, its influence is thought to be directly related to crystallinity (Mansfield et al., 1999; Zhang et al., 2006). Starch is another common carbohydrate found in biowaste, however it can be easily hydrolysed due its branching and helical structure (Gallert and Winter, 2005).

The degree of lipids hydrophobicity makes them less accessible for the enzymes, therefore increasing its emulsification, for example by increasing the operational temperature or by enhanced biogas production (Sanders, 2001), has been reported to accelerate hydrolysis (Cammarota and Freire, 2006; Mendes et al., 2006).

Jurado et al. (2008) reported that tributyrin hydrolysis proceeds in two partially overlapping stages. The first and fastest stage is the hydrolysis of an outer ester group of the surface molecules of tributyrin, which leads to the formation of glycerol dibutyrin. As the concentration of surface tributyrin molecules decreases, the hydrolysis of the remaining ester groups of the dibutyrin molecules takes place. This second stage is much slower because it needs an appropriate arrangement of the dibutyrin molecules surface (Jurado et al., 2008). On the other hand, Sanders (2001) did not find any intermediates when anaerobically batch digesting neutral lipids.

For proteins, the structure and the type of end group on the amino acids, can determine how accessible proteins are for proteases, therefore influencing the hydrolysis rate (McInerney, 1988). It has also been suggested that higher protein solubility results in faster hydrolysis (González et al., 2005), therefore, soluble globular proteins hydrolyse faster than fibrous proteins, because in the former only part of the substrate is accessible to the enzymes (Sanders, 2001). However, this is not always verified, as has been shown that ovalbumin (soluble) was slower hydrolysed than casein (insoluble) (McInerney, 1988).

2.3.3 Inhibitors

Many compounds have been reported to inhibit the anaerobic digestion process, such as ammonia, sulphide, salts, (earth)alkali metal ions (Na, K, Mg, Ca and Al),

heavy metals and organic compounds, which include chlorophenols, halogenated aliphatics, N-substituted aromatics, LCFAs and lignins and lignin related compounds (Chen et al., 2008b). However most of them have focused only on acetoclastic methanogenesis, and acetogenesis and not on hydrolysis (Vavilin et al., 2008).

2.3.3.1 Inhibition mechanisms

Hydrolysis inhibition is the result of hydrolases activity reduction. This reduction is mostly due to compounds that bind to the enzymes, therefore decreasing its activity.

There are mostly two types of enzymatic inhibition: competitive and non-competitive, as shown in Figure 2.4.

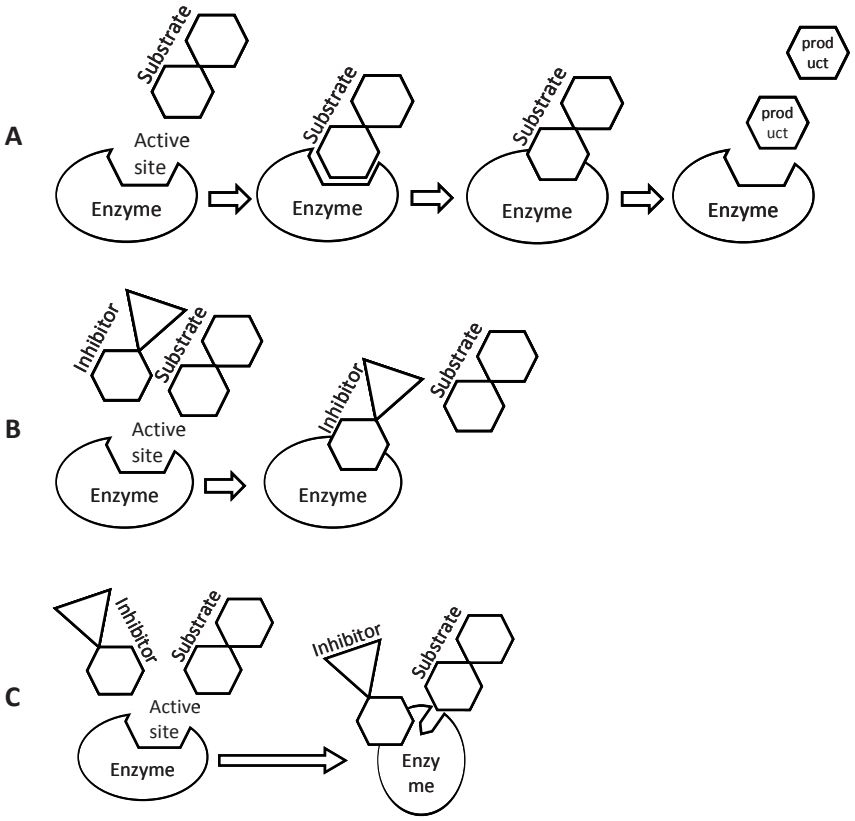


Figure 2.4. No inhibition (A), competitive inhibition (B) and non-competitive inhibition (C) illustration.

When there is no hydrolysis inhibition, all the accessible substrate gets converted by the hydrolases at its maximum rate (**A**). When an inhibiting compound is present there are two types of inhibition possible: **Competitive**, where the hydrolysis will be slower because the inhibitor and the substrate compete for the same active site. At higher substrate concentrations the inhibitor is then out-competed (**B**); and **non-competitive**, where the inhibiting compound attaches to an inactive site of the enzyme changing its conformation and, therefore, active site. This type of inhibition can only be solved when the inhibitor is removed (**C**) (Bettelheim et al., 2010).

2.3.3.2 VFA

Controversial conclusions on the effect of VFA on hydrolysis can be found in hydrolysis literature as it is difficult to distinguish between pH and VFA inhibition. Veeken et al. (2000a) studied the effect of pH and VFA (both total and undissociated) separately on the hydrolysis of organic solid waste. They were able to conclude that hydrolysis was pH dependent at pH between 5 and 7, but not VFA dependent (both total and undissociated), reaching concentrations up to 30 g COD·l^{-1}. On the other hand Romsaiyud et al. (2009) concluded that at a pH other than 7 and acetate > 1.8 g·l^{-1}, hydrolysis of cellulose was inhibited. He et al. (2007) suggested that mesophilic hydrolysis of potato, containing 87% of carbohydrates and 12% of proteins, is inhibited by pH and VFA, however pH had a stronger influence than VFA (20 g·l^{-1}), especially at acidic pH. On the other hand González et al. (2005) reported that hydrolysis of soluble protein, under mesophilic and saline (24 g·l^{-1} NaCl) conditions, is inhibited by acetic acid concentrations of 1.25 g·l^{-1} and higher. They further concluded that hydrolysis was more sensitive to acetic acid than pH, that varied from 5 to 7 (González et al., 2005).

For substrates high in ammonia, such as manure, VFA can accumulate in the anaerobic digester due to methanogenesis inhibition, therefore making it difficult to distinguish if hydrolysis is inhibited by VFA or ammonia (Zeeman, 1991). El-Mashad (2003) reported that hydrolysis was not inhibited by VFA (up to 5 g·l^{-1}) when anaerobically digesting cow manure under thermophilic conditions. On the other hand, Angelidaki et al. (1999) reported VFA inhibition on hydrolysis at concentrations of 3 g·l^{-1}, when anaerobically digesting cattle manure under thermophilic conditions. Process conditions like temperature and pH may impact the degree of inhibition by affecting the equilibrium constants of ionisable inhibitors such as VFA, NH_3 and H_2S. Only the non-ionised (non-charged) forms of the mentioned examples cause inhibition.

2.3.3.3 Ammonia

According to van Velsen (1981), who studied the anaerobic digestion of pig manure at mesophilic conditions, and Zeeman (1991), who studied the anaerobic digestion of cattle manure at mesophilic and psychrophilic conditions, not only methanogenesis but also the hydrolysis is inhibited by ammonia, although this relationship could not be quantified. El-Mashad (2003) found a linear relationship between the first order hydrolysis rate constant and the free ammonia concentration during the batch digestion of liquid cow manure at 50°C and 1 to 3.7 g NH_4^+-N·l^{-1}. The experiments of El-Mashad (2003) were conducted with sludge that was adapted to 1 g NH_4^+-N·l^{-1}. However, in our research (chapter 3), we did not find such inhibiting relation when anaerobically hydrolysing tributyrin and cellulose at mesophilic conditions and 2.4 to 7.8 g NH_4^+-N·l^{-1} (283 to 957 mg NH_3-N·l^{-1}). In our batch experiments, adapted sludge to 4.8 g NH_4^+-N·l^{-1}, from a pig manure digester was used. The reason for such contradictory conclusion might be due to the strong influence of pH and temperature on the free ammonia concentrations, therefore making difficult to distinguish if the hydrolysis is inhibited by pH or ammonia. Moreover, from the previous studies it was shown that together with the increase in ammonia, the inert fraction of the soluble COD also increased, indicating that there might have been other compounds, present in this soluble fraction that were inhibiting the hydrolysis (Zeeman, 1991). Such compounds, might have been HAL and FAL, which have been reported to be present in animal manure (chapter 4; Riffaldi et al., 1983).

2.3.3.4 Humic acid-like and fulvic acid-like

Humic acid-like (HAL) and fulvic acid-like (FAL) were first reported to inhibit the hydrolysis of proteins by Brons et al. (1985). Later, Jahnel and Frimmer (1994) reported the binding of Pronase E to humic matter, extracted from water, sediments and soils, when hydrolysing L-leucine-4-nitroanilide (Leupa). They found EC50 values of humic matter of 153 mg·l^{-1} for soil and 6360 mg·l^{-1} for surface water (Jahnel and Frimmel, 1994). Our present research (chapter 6) shows that the hydrolysis of cellulose and tributyrin (lipid) is inhibited by HAL and FAL extracted from cow manure and maize. From our results we concluded that cellulose hydrolysis was inhibited at concentrations of HAL and FAL as low as 0.5 g·l^{-1}. The reason for such inhibition is most likely due to the binding of the enzymes to the reactive functional groups present on humic matter, which has

also been reported for lignin (Hall et al., 2010; Palonen and Viikari, 2004; Pan, 2008) and for the low weight soluble phenolics (Ximenes et al., 2010).

Addition of a cation, such as calcium, has been shown to reverse the inhibitory effect of humate (Brons et al., 1985), since the calcium competes with the enzymes for cation-exchange on the humic and fulvic acids (Ladd and Butler, 1971). However, this is difficult to evaluate, since calcium, in the presence of carbonate and phosphate, which are found in anaerobic digesters, tends to precipitate as calcite and hydroxyapatite, respectively.

2.3.3.5 Hydrolysis products

2.3.3.5.1 LCFA

Long chain fatty acids (LCFA), which are commonly found in anaerobic digestion of lipid-rich waste(waters), have been reported to inhibit acetogenesis and methanogenesis (Angelidaki and Ahring, 1992; Hanaki et al., 1981), however its effect on hydrolysis is not clear. Sanders (2001) did not find inhibition of hydrolysis when digesting tripalmitin with addition of palmitic acid at initial concentrations of 0.2 to 1.2 g $COD \cdot l^{-1}$, under mesophilic methanogenic conditions. Hanaki (1981) reported that neutral fats are easily hydrolysed into LCFA, which results in accumulation of LCFA for high lipids concentrations, and further inhibition of the β-oxidation performed by the acidogenic bacteria (Keisuke Hanaki, 1981). This accumulation of free LCFA, which prefer to stay at the lipid-water interface, has been reported to cause inactivation of lipases by physical–chemical changes of the lipid water interface, such as the surface tension (Verger, 1980). Yet, this effect is not clear under anaerobic conditions (Sanders, 2001). Angelidaki and Ahring (1992) reported that oleate, one of the most inhibitory LCFA, and stearate inhibited thermophilic hydrolysis of cattle manure at concentrations of 0.2 and 0.5 $g \cdot l^{-1}$, respectively. Such inhibition due to oleate was also found during milk fat hydrolysis (Perle et al., 1995).

Palenzuela-Rollón (Palenzuela-Rollón, 1999) suggested that lipid hydrolysis into LCFA, during anaerobic treatment of fish wastewaters, is enhanced when acidogenesis and methanogenesis are stimulated. This was latter confirmed by Miron et al. (Miron et al., 2000) for primary sludge, and Sanders (2001) for neutral lipids. Likely, diffuse biogas bubbles enhance emulsification of the lipids, making them more accessible for enzymatic attack (Sanders, 2001).

2.3.3.5.2 Glucose

Glucose accumulation has been reported to inhibit hydrolysis. When glucose concentrations are too high it might repress cellulase activity (Gallert and Winter, 2005) known as feedback inhibition. On the other hand, low glucose concentrations might stimulate cellulase activity (Sanders, 2001). Glucose feedback inhibiting effects have also been observed for other sugars, such as mannose, xylose and galactose, however, glucose was the only sugar that inhibited both β-glucosidae and cellulase activities (Xiao et al., 2004). They reported approximately 50% reduction of enzymatic hydrolysis of cellobiose at glucose supplementation concentrations up to 100 $g \cdot l^{-1}$ (Xiao et al., 2004). Oh et al. (2000) reported a decrease of β-glucosidae activity of 80% at glucose concentrations of 5 $g \cdot l^{-1}$ and almost complete inactivation at glucose concentrations of 20 $g \cdot l^{-1}$. On the other hand, Kadam et al. (2004) reported that at 30 $g \cdot l^{-1}$ glucose, β-glucosidae activity was indeed reduced, but cellobiose conversion still occurred. In the just mentioned studies, β-glucosidae were added to cellulobiose, an intermediate of enzymatic hydrolysis. Therefore, the results depend also on the substrate and enzyme loading rates. Unfortunately, no studies could be found on the effect of glucose during anaerobic hydrolysis without enzyme addition. In anaerobic digestion of complex wastes, glucose is generally not detected because it is immediately consumed by the acidogenic bacteria and further converted to biogas. Inhibition of hydrolysis by glucose and cellobiose is of concern for the ethanol production industry. Therefore, reduction of inhibition by addition of enzymes and removal of sugars, by simultaneous saccharification and fermentation (SSF), is nowadays applied (Sun and Cheng, 2002).

2.3.3.5.3 Amino acids

Perle et al. (1995) observed that 91-93% casein was solubilised within 10 minutes during anaerobic batch digestion inoculated with a casein acclimated culture. The 1.94 g $COD \cdot g\ VSS^{-1}$ added amino acids did not inhibit the anaerobic hydrolysis. On the other hand, Örlygsson et al. (1994) found amino acid inhibition on hydrolysis when anaerobically digesting protein-rich slaughterhouse waste, but only when also methanogenesis was inhibited, which was evidenced by H_2 accumulation. No hydrolysis inhibition was found when methanogenesis conditions were guaranteed.

2.3.3.5.4 Glycerol

The only study found on the effect of glycerol on the anaerobic hydrolysis has been conducted by Perle et al. (1995) who found no inhibition on milk fat hydrolysis from dairy wastewaters to which glycerol was added at a concentration of 1.94 g COD·g VSS^{-1}.

2.4 Hydrolysis kinetics

The way to mathematically describe the hydrolysis step has been recently reviewed by Vavilin et al. (2008) and He et al. (2007), therefore only a small overview will be given here.

Commonly, anaerobic hydrolysis is described by first-order kinetics (Eastman and Ferguson, 1981), which tend to fit most experimental data quite accurately (Batstone et al., 2002; Sanders, 2001; Vavilin et al., 2008). However, in some particular cases first-order does not seem to describe the hydrolysis step so well, probably due to the many unknowns in relation to how this step proceeds, therefore different kinetics, such as Contois and surface-related two-phase kinetics, are also successfully being used (Vavilin et al., 2008).

Surface based kinetics (SBK), as proposed by Hills and Nakano (1984) and Hobson (1987), assumes that the bacteria cover the whole substrate particles' surface, thus enzyme availability is not limiting and the hydrolysis rate per unit area available for hydrolysis is constant. Based on the SBK model, Sanders (2001) concluded that the amount of available surface significantly determines the hydrolysis rate. First order kinetics can still be applied, but hydrolysis constants are only comparable when both substrate composition and particle size distribution of particulate substrate are known. This also implies that for dissolved substrates, where the available surface is directly corresponded to the substrate concentration, enzyme activity determines the hydrolysis rate (Sanders, 2001). However, as for the anaerobic digestion of complex wastes it is not possible to determine the size and shape of the particles of the substrate, first order hydrolysis seems to be the simplest way to describe the hydrolysis step (Sanders, 2001; Vavilin et al., 2008).

The models that are usually used to describe anaerobic digestion of complex waste(waters) are: ADM1 (Batstone et al., 2002), Siegrist model (Siegrist et al., 2002) and complex substrate anaerobic bioconversion model (Angelidaki et al., 1999). For describing the hydrolysis step they all apply first-order kinetics because it has been found to be a good simplification of this complex step

(Batstone, 2006). In ADM1, unlike for the other two models, one step precedes hydrolysis, that is disintegration, which was included to facilitate the modelling of waste-activated and primary sludge digestion, as disintegration represents the lysis of whole cells and separation of composites, therefore enabling the recycling of dead anaerobic biomass. Hydrolysis proceeds then as a first-order reaction and no inhibition is accounted for (Batstone et al., 2002). The Siegrist model (Siegrist et al., 2002), which is commonly used for anaerobic digestion of sewage sludge, is similar to ADM1 on the hydrolysis step (first-order and no inhibition), however, it includes different hydrolysis rate constants, which were determined experimentally. For the complex substrate anaerobic bioconversion model, presented by Angelidaki et al. (1999), dead cell matter is assumed to slowly decay into carbohydrates and proteins, therefore becoming new substrate. In this model, lipids hydrolysis is also not considered, only carbohydrates and proteins hydrolysis are included, and described by first order kinetics. VFA was included as having non-competitive reversible inhibition on hydrolysis, yet its quantification only accounted for the apparent loss of biogas observed in inhibited reactors, indicating that this inhibition might have been on the acidogenic step and not on the hydrolytic step. While the model proposed by Angelidaki et al. (1999) was tested and validated with co-digestion substrates, namely manure, proteinous wastewater and lipid-rich waste, ADM1 (Batstone et al., 2002) was developed for describing anaerobic digestion of domestic wastewater treatment. Therefore, ADM1 has been further improved to simulate anaerobic digestion of different types of substrates, such as industrial wastewater and agricultural wastes (Fezzani and Cheikh, 2009; Galí et al., 2009). But yet, no improvement has been done in terms of adding inhibition kinetics or adapting the ADM1 in relation to the hydrolysis step (Batstone et al., 2006). Only the hydrolysis rate constants have been changed to the different substrates, according to experimental data (Derbal et al., 2009). The only reported change in the hydrolysis step has been presented by Ramirez et al. (Ramirez et al., 2009), who successfully modelled hydrolysis according to Contois kinetics. However, the validation of the model was done with thermally pre-treated waste activated sludge, of which the structure might have changed during the pre-treatment step, making it less complex for anaerobic hydrolysis and, therefore, not comparable to untreated complex substrates. Moreover, no inhibition on hydrolysis was taken into account (Ramirez et al., 2009).

The models usually used to predict inhibition are non-competitive, Luong and Levenspiel (Aspé et al., 2001; González et al., 2005; He et al., 2007). However they are most commonly used in other steps of the anaerobic digestion process and not on the hydrolysis.

2.4.1 Hydrolysis rates

The first order hydrolysis rate constant of several complex substrates is shown in Table 2.1. The large numerical variation is due to the type and accessibility of the substrate, as discussed before.

Table 2.1. First-order hydrolysis rate constant (updated from Vavilin et al. (2008))

Substrate	Experimental set-up	Temperature (°C)	pH	k_h (d^{-1})	Reference
Cellulose	continuous	37	≈ 7	1.18	(Pavlostathis et al., 1988)
Corn stover	semi-continuous	35	-	0.076 – 0.18	(Pavlostathis and Giraldo-Gomez, 1991)
Casein	-	-	-	0.35	(Pavlostathis and Giraldo-Gomez, 1991)
Gelatin	-	-	-	0.60	(Pavlostathis and Giraldo-Gomez, 1991)
Zein (maize protein)	-	-	-	0.04	(Pavlostathis and Giraldo-Gomez, 1991)
Primary sludge	continuous	35	≈ 5	3.0	(Eastman and Ferguson, 1981)
Raw sewage sludge	-	-	-	0.4 – 1.2	(O'Rourke, 1968)
Cellulose	continuous	15 - 25	-	0.14 – 0.38	(O'Rourke, 1968) (Sanders, 2001)
Proteins	continuous	15 - 35	-	0.14 – 0.67	(O'Rourke, 1968) (Sanders, 2001)
Proteins	continuous	30	-	6.2	(Palenzuela-Rollón, 1999)
Carbohydrates	continuous	30	-	7.0	(Palenzuela-Rollón, 1999)
Fish processing wastewater	batch	30	6 - 8	0.11 – 0.18	(Palenzuela-Rollón, 1999)
Starch	batch	30	-	0.96 – 2.16	(Sanders, 2001)
Tripalmitin	batch	30	-	0.03 – 0.04	(Sanders, 2001)
Lipids	-	55	-	0.005 – 0.010	(Christ et al., 2000)
Proteins	-	55	-	0.015 – 0.075	(Christ et al., 2000)
Carbohydrates	-	55	-	0.025 – 0.2	(Christ et al., 2000)
Sewage sludge	continuous	35	≈ 7	0.25	(Siegrist et al., 2002)
Sewage sludge	continuous	55	≈ 7	0.4	(Siegrist et al., 2002)
Activated sludge	batch	35	-	0.15	(Pavlostathis and Gossett, 1986)

Table 2.1. First-order hydrolysis rate constant (updated from Vavilin et al. (2008)) (continued)

Substrate	Experimental set-up	Temperature (°C)	pH	k_h (d^{-1})	Reference
Lipids	-	-	-	0.08 – 1.7	(Gujer and Zehnder, 1983)
Proteins	-	-	-	0.02 – 0.03	(Gujer and Zehnder, 1983)
Cellulose	-	-	-	0.04 – 0.13	(Gujer and Zehnder, 1983)
Hemicellulose	-	-	-	0.54	(Gujer and Zehnder, 1983)
Carbohydrates and Proteins	-	55	-	1	(Angelidaki et al., 1999)
Cellulose	batch	37	-	0.75	(Myint and Nirmalakhandan, 2006)
Hemicellulose	batch	37	-	1.0	(Myint and Nirmalakhandan, 2006)
Starch	batch	20	≈ 7	2.87 – 3.2	(San Pedro et al., 1994)
Wholewheat bread	batch	30	-	0.195	(Veeken and Hamelers, 1999)
Leaves	batch	20 - 40	-	0.068 – 0.386	(Veeken and Hamelers, 1999)
Bark	batch	30 - 40	-	0.076 -0.24	(Veeken and Hamelers, 1999)
Straw	batch	20 - 40	-	0.024 – 0.14	(Veeken and Hamelers, 1999)
Orange peelings	batch	20 - 40	-	0.145 – 0.474	(Veeken and Hamelers, 1999)
Grass	batch	20 - 40	-	0.035 – 0.266	(Veeken and Hamelers, 1999)
Solid waste	continuous	28	≈ 7	0.108 – 0.245	(Veeken et al., 2000a)

2.5 Methodology for hydrolysis assessment

Hydrolysis is measured by substrate depletion or products formation. The latter is usually preferred, but it should be guaranteed that this product is not metabolised or physico-chemically converted or removed from the water matrix (Whiteley and Lee, 2006). Moreover, when adding inoculum, it is only possible to measure hydrolysis by product formation, since it is impossible to determine which carbohydrate, lipid or protein fraction belongs to the substrate and which to the inoculum.

When acidogenic, acetogenic and methanogenic conditions are guaranteed and hydrolysis is the rate-limiting step, then hydrolysis can be measured by final products formation, such as methane (Sanders, 2001).

The methodology for performing hydrolysis, and also biodegradability experiments has been described by Angelidaki and Sanders (2004). Basically, hydrolysis efficiency and rate are mostly assessed by batch and continuous set-ups. A known concentration of a complex substrate is anaerobically digested at 35°C under completely mixed conditions, brought about by mixing or shaking. For batch experiments, an adapted inoculum, meaning an inoculum acclimatised to the substrate and operational conditions, is added to the substrate, as well as an appropriate medium. The addition of a medium is not necessary when the inoculum already has the required macro-nutrients and trace-elements.

For both batch and CSTR experiments, total hydrolysis efficiency is determined according to Zeeman (1991):

$$H\,(\%) = \frac{COD_{CH_4,t=x} + COD_{diss,t=x}}{COD_{total,t=0}} \times 100 \qquad (2.2)$$

Where, *H* is the hydrolysis efficiency (%); $COD_{CH4,t=xl}$ is the concentration of methane, in COD, at t=x; $COD_{diss,t=x}$ is the concentration of dissolved COD at t=x; and $COD_{total,t=0}$ is the concentration of total substrate added, in COD, at t=0 (biodegradable + non-biodegradable).

In order to remove the already solubilised substrate, therefore accounting only for the hydrolysis efficiency of the particulate substrate, hydrolysis is determined according to Zeeman (1991) by:

$$Hr\,(\%) = H - \frac{COD_{diss,t=0}}{COD_{total,t=0}} \times 100 \qquad (2.3)$$

Where, *Hr* is the hydrolysis efficiency that occurs in the reactor (%) which excludes the already dissolved COD present at the beginning; $COD_{diss,t=0}$ is the concentration of dissolved COD at t=0.

To assess the hydrolysis rate, first order kinetics are usually used. For batch experiments, first order hydrolysis constant (k_h) is calculated according to Sanders (2001):

$$\ln \frac{COD_{SS,t=x} - COD_{SS,t=0} \cdot (1 - fh)}{COD_{SS,t=0} \cdot fh} = -k_h \cdot t \tag{2.4}$$

Where, $COD_{SS,t=x}$ is the concentration of total particulate substrate, in COD, at t=x (biodegradable + non-biodegradable)($g \cdot l^{-1}$); $COD_{SS,t=0}$ is the concentration of total particulate substrate, in COD, at t=0 (biodegradable + non-biodegradable)($g \cdot l^{-1}$); *fh* is the biodegradable fraction of total substrate added, ranging between 0 and 1; k_h is the first order hydrolysis constant (d^{-1}); and *t* is the digestion time of the experiment (d). Therefore, the biodegradable particulate COD at t=x is calculated as the biodegradable + non-biodegradable particulate COD at t=x minus the non-biodegradable particulate COD at t=0.

For CSTR experiments, first order hydrolysis constant (k_h) is calculated according to Sanders (2001):

$$HRT = fh \cdot COD_{SS,\text{inf}} \left(\frac{HRT}{COD_{SS,\text{inf}} - COD_{SS,eff}} \right) - \frac{1}{k_h} \tag{2.5}$$

Where, HRT is the hydraulic retention time of the reactor (d); $COD_{SS,inf}$ is the concentration of total particulate substrate, in COD, in the influent (biodegradable + non-biodegradable) ($g \cdot l^{-1}$); and $COD_{SS,eff}$ is the concentration of total particulate substrate, in COD, in the effluent (biodegradable + non-biodegradable) ($g \cdot l^{-1}$). The k_h is then determined by the intercept of the linear curve with the Y axis, of the plotted HRT against the HRT divided by the hydrolysed particulate COD (Sanders, 2001).

2.6 Hydrolysis enhancement

2.6.1 Pre-treatment

Pre-treatment of biowaste, such as lignocellulosic matter is becoming attractive due to an increase in biogas production and a shorter hydraulic retention time for the subsequent anaerobic digesters (Chen et al., 2008b). Many studies have been published on different pre-treatment methods, and many have been reviewed (Chandra et al., 2007; Chen et al., 2008b; Hendriks and Zeeman, 2009).

Physical pre-treatments of lignocellulosic matter, such as grinding, milling or shearing, are commonly applied at farm digesters, since it reduces particle size and increases surface area, therefore accelerating hydrolysis (Deublein and Steinhauser, 2008; Ladisch et al., 1983; Sanders, 2001). Other pre-treatments include chemical or solvent addition, thermal treatment, radiation, microbial treatment, micro-aeration, and the combination of some of them, such as thermo-chemical treatment (Deublein and Steinhauser, 2008; Hendriks and Zeeman, 2009; Johansen and Bakke, 2006).

Thermal pre-treatment (80°C) has been reported to accelerate pig slurry hydrolysis, but only when ammonia concentrations where low (1.3 g NH_4^+-N·l^{-1}), because at high ammonia concentrations (3.7 g NH_4^+-N·l^{-1}) methane formation was inhibited (Bonmatí et al., 2001). For lignocellulosic matter, acid or base hydrolysis pre-treatments are the most commonly applied since they have shown very high efficiencies in making complex structures more available for anaerobic microorganisms (Chen et al., 2008b). The combination of thermo-chemical pre-treatment of lignocellulosic biomass was reported to have a positive effect on hydrolysis and biodegradability of the plant material when the lignin content was high (185 g·kg VS^{-1}) and not so much when the lignin content was low (25 g·kg VS^{-1}) (Fernandes et al., 2009).

Johansen and Bakke (2006) reported enhanced hydrolysis of carbohydrates and proteins from primary sludge with micro-aeration of about 33.3 l air·kg TS^{-1}·d^{-1} prior to anaerobic digestion. This was, however, not verified for lipids hydrolysis (Johansen and Bakke, 2006). The improvement of anaerobic hydrolysis by micro-aeration has been confirmed and reviewew by Zhu et al. (2009).

Hendricks and Zeeman (2009) concluded, in their review on lignocellulosic pre-treatment, that steam, lime, LHW (liquid hot water) and ammonia pre-treatments have the highest potential according to economical and effectiveness criteria.

Ignoring cost considerations, choosing the appropriate pre-treatment is, however, a compromise between minimization of biodegradable substrate losses and the maximization of hydrolysis enhancement with regard to the less biodegradable substrates (Chandra et al., 2007). Moreover, the effectiveness of the chosen pre-treatment will not only affect up-stream with the selection of the biowaste, but also down-stream with the selection of the reactor design and operation conditions (Chandra et al., 2007). Finally it has to be taken into account that due to the high costs involved in pre-treatment, the applicability is generally limited to large scale biogas plants (Deublein and Steinhauser, 2008).

Lists of pre-treatments advised for the different types of substrates can be found in Deublein and Steinhauser (2008) and www.adnett.org/havrt-_codigestion_substrates.html

2.6.2 Enzyme addition

Lipases, which are mostly mesophilic, are becoming increasingly attractive for industrial applications due to their stability, selectivity and versatility (Cammarota and Freire, 2006; Wu and Tsai, 2004). They are also becoming a useful tool for hydrolysing fat particles in slaughterhouse wastewater (Cammarota and Freire, 2006) and dairy industries wastewater (Mendes et al., 2006). Moreover, the enzymatic hydrolysis of triglycerides occurs at ambient temperature, ambient pressure, and neutral pH, which represents substantial energy savings regarding pre-treatment possibilities (Jurado et al., 2006). We should realise, however, that addition of enzymes, beyond the concentration at which interface saturation occurs will not enhance hydrolysis but only increase the costs (Jurado et al., 2006).

Cellulase addition to anaerobic digestion is receiving increasing attention. The addition of cellulases to a certain extent, has been shown to accelerate hydrolysis, however it also significantly increases the costs involved in anaerobic digestion (Chen et al., 2008a; Sun and Cheng, 2002). A way to decrease these costs would be to produce cheaper and more efficient enzymes, and to be able to recycle them (Recktenwald et al., 2008; Sun and Cheng, 2002; Yang et al., 2010). Advances in molecular engineering will result in new enzymes, where enzymes' properties can be exploited to, for example, enlarge binding pockets and to change substrate specificity (Whiteley and Lee, 2006).

2.7 Final considerations

Hydrolysis of complex biowaste under anaerobic conditions is still not fully understood, not only in terms of the mechanisms involved in the enzymatic catalysis of the substrate, but also on the mechanisms of release of the enzymes from the acidogenic bacteria and adsorption to the substrate. Together with the incomplete knowledge on the complex intrinsic characteristics of the substrates and their effect on the activity of the hydrolases, it is, therefore, still difficult to determine which compounds and conditions inhibit anaerobic hydrolysis and to which extent.

Ideally, it would be valuable to relate the characteristics of substrate, such as amount of lignin present in lignocellulosic biomass, with the hydrolysis rate of that substrate, as was suggested by Tong et al. (1990). However, as Tong et al. (1990) reported, this relation is not yet verified, because the hydrolysis rate is not only dependent on the amount of lignin present, but also how the lignin is binding and encrusting the cellulose and the intrinsic characteristic of cellulose. Moreover, it is also necessary to determine how much hemicellulose has been hydrolysed during hydrolysis of lignocellulosic biomass, since it can also interfere with cellulose hydrolysis.

Due to all above mentioned reasons, a database with hydrolysis yields and rates for different types of biowaste at different operational conditions should be developed, like it has been presented by Deublein and Steinhauser (2008) for biodegradability of biomass.

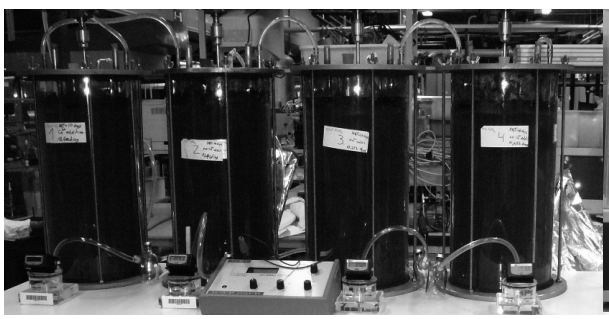
According to the reported literature, anaerobic hydrolysis is affected by pH, temperature, VFA, particle size and shape (surface area), substrate accessibility and its intrinsic characteristics, hydrolysis products, and HAL and FAL. However its extent is yet not fully understood. Therefore, further studies, only on hydrolysis inhibition should be performed, with identical methodology and ensuring that the effect of the compound is on the hydrolysis step and not on the acidogenic step or any other step of the anaerobic digestion process.

Modelling anaerobic digestion in order to improve the design of biogas plants is an increasing necessity, therefore inhibition parameters on hydrolysis should be included as well. Moreover, when the database of hydrolysis constants for different substrates at different environmental conditions is available, validation of the upgraded models, where inhibition is included, will be possible, therefore offering a good tool for predicting the potential rate of biogas production.

Accelerating hydrolysis by pre-treatment, such as chemical addition has to be well studied and balanced before implementation, since not only costs, but also environmentally correct solutions including life cycle analysis (LCA) should be taken into consideration.

Chapter 3

Effect of ammonia on the hydrolysis of cellulose and tributyrin



Abstract

Ammonia nitrogen is one of the most common inhibitors in the anaerobic digestion of complex biowaste containing high concentrations of ammonia like animal manures, blackwater and waste oil from gastronomy and olive oil production. The inhibiting effect of high ammonia concentrations on methanogenesis has been well established. In contrast, the knowledge on the effect of ammonia on the hydrolysis is rather limited. This study focused on evaluating the effect of ammonia nitrogen on the hydrolysis of carbohydrates and lipids. Batch digestion of tributyrin and cellulose at varying ammonia nitrogen concentrations were performed, using adapted biomass to 4.8 g NH_4^+-N·l^{-1}. From this experimental study it was concluded that total ammonia nitrogen in the range of 2.4 to 7.8 g NH_4^+-N·l^{-1} (283 to 957 mg NH_3-N·l^{-1}) does not inhibit the hydrolysis of tributyrin or cellulose. This result is further confirmed by mathematical analysis of the estimated variation of the first-order hydrolysis constant as a function of the total ammonia concentration.

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3.1 Introduction

Ammonia nitrogen is one of the most common inhibitors during anaerobic digestion of complex wastes containing high concentrations of ammonia and/or proteins, like animal manures, slaughterhouse wastewaters and fish industry wastewaters (Angelidaki and Ahring, 1994; Sanders, 2001; Zeeman, 1991). Therefore, ammonia is frequently being referred to as the primary cause of digester failure (El-Mashad, 2003). Total ammonia is the ammonium ion (NH_4^+) plus the free ammonia (NH_3), which form depends on the pH and temperature of the solution. For anaerobic digesters operated at pH 7 and 35°C less than 1% is in the free ammonia form, however, at the same temperature, but pH 8 the free ammonia increases to 10%, according to the Figure 3.1.

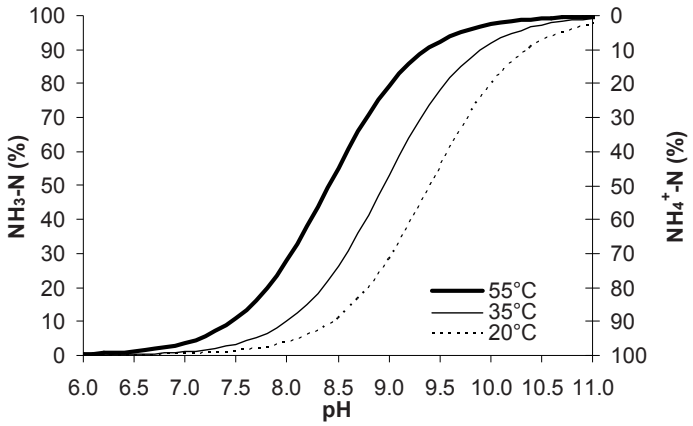


Figure 3.1. Free ammonia and ammonium percentages present in solution at 20, 35 and 55°C and varying pH.

This un-dissociated ammonia form, unlike the ammonium ion, is able to diffuse through cell membrane, which has been reported to inhibit cell functioning by disrupting the proton and potassium balance inside the cell (Kayhanian, 1999), therefore making the free ammonia as the main inhibitory form for methanogenesis (Van Velsen, 1981). The effect of high ammonia concentrations on methanogenesis has been well established. It is known that its inhibition will cause accumulation of intermediate digestion products like volatile fatty acids (VFA) (Van Velsen, 1981; Zeeman et al., 1985), unless the hydraulic retention time (HRT) is increased. In the anaerobic digestion literature, it is common to find total ammonia inhibitory concentrations between 1.2 and 4.0 mg NH_4^+-N·l^{-1} (Angelidaki and Ahring, 1994; Braun et al., 1981; Kayhanian, 1999;

Koster and Lettinga, 1984; Krylova et al., 1997). Variation in the inhibitory concentration was shown to be strongly dependent on the adaptation level of the biomass (Van Velsen, 1979). In contrast to methanogenesis, knowledge on the effect of ammonia on the hydrolysis is rather limited (El-Mashad, 2003). This is of major importance since hydrolysis is considered as the rate limiting step in the anaerobic digestion of complex waste, such as animal manure and agricultural residues (Pavlostathis and Giraldo-Gomez, 1991). Van Velsen (1981) and Zeeman (1991) showed that the hydrolysis of complex substrates is inhibited at high ammonia concentrations, however other compounds present in animal manure, that were diluted at the same rate, could have also caused the inhibition. Lu et al. (2008) investigated the influence of 0-16 g ammonia-N·l^{-1}, at neutral pH, on mesophilic acidogenesis of protein-rich fish waste and concluded that up to 8 g ammonia-N·l^{-1} reduced the acidogenic efficiency slightly, while 16 g ammonia-N·l^{-1} resulted in accumulation of intermediates, such as lactic and formic acid. El-Mashad (2003) found a linear relationship between the hydrolysis rate and the free ammonia concentration during the batch digestion of liquid cow manure at 50°C and 60°C and 1 to 3.7 g NH_4^+-N·l^{-1}. The experiments of El-Mashad (2003) were only conducted with sludge that was adapted to 1 g NH_4^+-N·l^{-1}. He verified that enzyme production and not enzyme activity was affected by the high ammonia concentrations. It was however unclear if, similar to methanogenic biomass (Van Velsen, 1981), the acidogenic biomass that produces the enzymes for hydrolysis could adapt to high ammonia concentrations. According to van Velsen (1979) and Angelidaki et al. (2003) the degree of ammonia inhibition depends greatly on the degree of adaptation of the inoculum to the ammonia. Hansen et al. (1998) and Zeeman (1991) showed that the interaction between free ammonia, VFA and pH can lead to an “inhibited steady state”, which is a condition where the process is running stable over an extensive period of time, but with a lower methane yield and high VFA concentrations in the effluent.

As most researches on ammonia inhibition of hydrolysis were done with animal manure diluted to different concentrations, the effect of ammonia alone on hydrolysis could not be determined.

Once the estimation of the hydrolysis rate has been ascertained, based on the experimental data, it is also important to determine the reliability of the estimates. For these types of experiments, the data set is usually rather small. Hence, for these limited data sets, a so-called set-membership estimation procedure is more appropriate than a statistical procedure. During the last two decades a growing amount of literature on set-membership estimation or bounded error approaches has become available (see Walter, 1990; Norton, 1994, 1995; Milanese *et al*, 1996; Keesman, 2003 for overviews). The key problem in

set-membership parameter estimation is not to find a single vector with optimal parameter estimates, but a set of feasible parameter vectors that are consistent with a given model structure and bounded-error data. This feasible parameter set (FPS) directly reflects the uncertainty in the estimates. A bounded error characterization, as opposed to a statistical characterization in terms of mean, variances or probability distributions, is favoured when the central limit theorem is inapplicable, such as in situations with small data sets or with heavily structured (modelling) errors.

This chapter focused on determining the possible inhibiting effect of ammonia on the anaerobic hydrolysis of carbohydrates and lipids. To elucidate the effect of ammonia, sludge adapted to high total ammonia concentrations of 4.8 g NH_4^+-N·l^{-1}, and particulate substrates with identical particle size were used in the inhibition batch trials. Crystalline cellulose was the carbohydrate substrate and tributyrin was the lipid substrate. Carbohydrates are the main substrate during animal manure and agricultural residues digestion. Lipids are often considered for co-digestion substrates. Hydrolysis efficiencies and rates where used for assessment of the process.

3.2 Materials and methods

3.2.1 Experimental set-up

Fourteen trials were performed in batch bottles at 35 ± 2˚C and anaerobic conditions. Seven batch trials had 1 g COD·l^{-1} tributyrin (Fluka, ≥ 98%) as substrate and the other seven batch trials had 1 g COD·l^{-1} cellulose (Sigmacell® type 50), with an average particle size of 50 µm, as substrate. Each experiment was performed in duplicate including two controls with no substrate addition, but having the corresponding ammonia concentration in order to correct for the volatile fatty acids and methane produced by the control. All test vials were inoculated with 100 ml of supernatant (creamy consistency with no fibrous material) of digested pig slurry from a pig manure co-digester operated at mesophilic conditions (Sterksel, The Netherlands). The inoculum had 75.0 ± 0.2 g TS·kg^{-1}, 45.8 ± 1.9 g VS·kg^{-1} and 4.89 g NH_4-N·l^{-1}. In all test vials 100 ml of 1.14 M of ammonium bicarbonate or 1.14 M of sodium bicarbonate solution was added according to the final ammonia concentrations desired, therefore maintaining the same ionic strength and pH. The pH of all batch experiments was set to approximately 8 at the beginning of the test. Trace elements and macro nutrients

were not added since they were already present in the pig slurry. All bottles were placed in an incubator (Model 420, Thermo Electron Corporation) at 150 rpm. All trials were performed in 0.5 litre Scott bottles (Germany) with two sampling ports, closed with butyl rubber stoppers and aluminium clamps, one for liquid sample to determine the intermediate digestion products and another for gas samples to determine the gas composition. All bottles had a total working volume of 0.2 litres and were fitted with an Oxitop® head for gas pressure measurement, together with the gas composition, to assess the cumulative methane production.

3.2.2 Chemical analysis

Total solids (TS) and volatile solids (VS) were determined according to standard methods (APHA, 1998). pH was measured with a Multi 340i meter and SenTix P14 electrode (WTW, Germany). NH_4^+-N was determined at the beginning and end of the trials, according to the ammonium coloration method (APHA, 1998). Samples for VFA were centrifuged at 13000 rpm for 10 minutes in a Hermle Z300 centrifuge. After centrifuging, the supernatant was membrane filtered (0.45 µm, Schleicher & Schuell ME 25, Germany) and used for the analysis. VFA was analysed in a Hewlett Packard 5890A gas chromatogram equipped with a 2 m × 6 mm × 2 mm glass column packed with Supelco port, 100-120 mesh, coated with 10% Fluorad FC 431. The flow rate of the carrier gas, i.e. nitrogen saturated with formic acid, was 40 $ml \cdot min^{-1}$, and the column pressure was 3 bar. The temperatures of the column, the injector port, and the flame ionization detector were 130, 200, and 280°C, respectively. Biogas composition was analysed in a Fisions Instrument GC 8340 gas chromatogram equipped with a 30 m × 0.53 mm × 25 µm Molsieve column (Alltech 13940), and a 2 × 25 m × 0.53 mm × 10 µm PoraBond Q column (Varian 7354). The columns were connected in parallel. Helium was the carrier gas and its flow rate was 42.5 $ml \cdot min^{-1}$. The temperatures of the oven, the injection port, the thermal conductivity detector and the filament were 40, 110, 100 and 140°C, respectively.

3.2.3 Calculations

Free ammonia was calculated according to El-Mashad (2003):

$$NH_3 - N = NH_4^+ - N \cdot \left[1 + \frac{10^{-pH}}{10^{-\left(0.1075 + \frac{272.5}{T}\right)}} \right]^{-1} \quad (3.1)$$

To determine the hydrolysis efficiency (%), as shown in equation 3.2, the digestion intermediates were measured during each trial:

$$Hydrolysis(\%) = \frac{\sum COD_{hydrolysis\ products,t=x}}{COD_{total,t=0}} \cdot 100 \quad (3.2)$$

Where the $COD_{hydrolysis\ products,t=x}$ is the amount of VFA plus CH_4 produced during the cellulose and tributyrin hydrolysis, at t=x, expressed as mg COD·l^{-1}. The $COD_{total\ t=0}$ is the amount of cellulose or tributyrin added at the beginning of the trial, t = 0, also expressed as mg COD·l^{-1}.

The first order hydrolysis constant (k_h) of the cellulose and tributyrin was calculated according to Angelidaki and Sanders (2004) and is here presented as equation 3.3.

$$\frac{COD_{total,t=0} \cdot f_h - \sum COD_{hydrolysis\ products,t=x}}{COD_{total,t=0} \cdot f_h} = e^{-k_h t} \quad (3.3)$$

Where *fh* is the maximum biodegradable fraction of total substrate added (cellulose or tributyrin), that is 1 for completely biodegradable substrates.

3.2.4 Data analysis

In a first step of the data analysis, the k_h values were estimated from batch data at different ammonia concentrations. Next, the possible relationship between the k_h values and the ammonia concentration, including the uncertainty in this relationship, was investigated. Since the resulting small data set does not allow a

proper statistical analysis, in what follows a so-called set-membership approach to the estimation problem is applied.

In some experiments a lag phase in the cellulose hydrolysis was observed. This behaviour was analysed with a time-varying first-order hydrolysis model.

3.2.4.1 Set-membership parameter estimation

Given the limited data set of k_h-values and corresponding ammonia concentrations, the set-membership approach was used and is here shortly summarized. Consider here the following general non-linear regression type of model in vector form,

$$\mathbf{y} = \mathbf{F}(\vartheta) + \mathbf{e} \tag{3.4}$$

where **y** is an *N*–dimensional vector with observed output data, **F**(ϑ) is a non-linear vector function mapping the unknown *m*-dimensional parameter vector ϑ into a noise-free model output. The error or information uncertainty vector **e** is assumed to be bounded. More specifically, in what follows, it is assumed that each individual error e_k, for $k = 1, \ldots, N$ is bounded such that:

$$\|\mathbf{e}\|_\infty \leq \varepsilon \tag{3.5}$$

where $\|\mathbf{e}\|_\infty = \max_k |e_k|$ and ε is a fixed positive number. Hence, a measurement uncertainty set (MUS), containing all possible output measurement vectors consistent with the observed output data and uncertainty characterization, is defined as:

$$\Omega_y := \{\tilde{\mathbf{y}} \in \mathbb{R}^N : ||\, \mathbf{y} - \tilde{\mathbf{y}} \,||_\infty \leq \varepsilon\} \tag{3.6}$$

This set is a hypercube in $\mathbb{R}^N$, the *N* – dimensional real vector space. Let the set

$$\Omega_\vartheta := \{\vartheta \in \mathbb{R}^m : ||\, \mathbf{y} - \mathbf{F}(\vartheta) \,||_\infty \leq \varepsilon\} \tag{3.7}$$

define the feasible parameter set. Then, the set-membership estimation problem is to characterize this feasible parameter set (FPS), which is consistent with the model (3.4), the data (**y**) and uncertainty characterization (equations 3.5 and 3.6).

Hence, instead of trying to find the optimal parameter vector as in an ordinary least-squares approach, the goal now is to find the set with feasible parameter vectors that are consistent with the model and the data with related error bounds. Therefore, we will not consider the measurements as such, but define intervals for each measurement. This approach avoids the distortion of the original probability density function of the error after some non-linear transformation, because only bounds are considered.

3.2.4.2 Mathematical model

The time-varying first-order hydrolysis model, used for further analysis of the trials showing a lag phase, is given by:

$$\begin{aligned} &\frac{dP}{dt} = \kappa P, P(0) = P_0 \\ &\frac{d\kappa}{dt} = r\kappa(K - \kappa), \kappa(0) = \kappa_0 \end{aligned} \tag{3.8}$$

Where *P* is the dimensionless depletion of cellulose, κ the time-varying first order hydrolysis constant, *r* is the rate parameter, and *K* the upper limit on κ, which can be interpreted as $k_{h,max}$. Typically, κ(*t*) is an *S*-curve that could, for instance, represent the colonization of enzymes on substrate's surface, therefore, stimulating the hydrolysis products formation. For the reversed curve, which becomes relevant in case a possible inhibitor is present and needs time to be inactive, we define $\tilde{\kappa} = (K - \kappa)$.This transformation gives exactly the same response for *P* for given values of *r, K* and κ_0. Consequently, $\frac{d\tilde{\kappa}}{dt} = -\frac{d\kappa}{dt}$ and $\tilde{\kappa}(0) = \left(K - \kappa(0)\right)$ and thus equation 3.8 can also be written as:

$$\begin{aligned} &\frac{dP}{dt} = (K - \tilde{\kappa})P, \quad P(0) = P_0 \\ &-\frac{d\tilde{\kappa}}{dt} = r(K - \tilde{\kappa})\tilde{\kappa}, \quad \tilde{\kappa}(0) = K - \kappa_0 \end{aligned} \tag{3.9}$$

Hence, instead of estimating only k_h, two parameters are now estimated, i.e. r and K. Given the time series of P, both parameters were estimated using a nonlinear least-squares estimation procedure, as Matlab®'s *lsqnonlin*.

3.3 Results and Discussion

The ammonia and pH ranges applied in this study and presented in Tables 3.1 and 3.2 are commonly found in animal manures such as cattle and pig (El-Mashad, 2003), which are, together with chicken manure, the main sources of manure for anaerobic digestion in Europe (Weiland, 2006).

3.3.1 Cellulose hydrolysis

Most of cellulose hydrolysis occurred in the first 11 days, as can be seen by Figure 3.2, which is commonly found for this type of crystalline cellulose (O'Sullivan et al., 2008; Song et al., 2005).

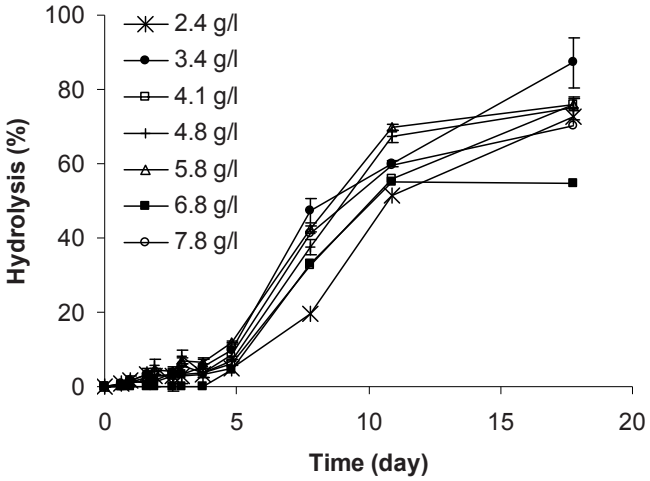


Figure 3.2. Hydrolysis as a function of time at batch digestion of cellulose at varying total ammonia concentrations, with inoculum sludge adapted to 4.8 gNH_4^+-N·l^{-1}.

What can also be seen in Figure 3.2 is that for all trials the hydrolysis of cellulose had a lag phase that, for most trials took up to 4 days. The reason for such lag phase is not completely clear. Song et al. (2005) suggested this lag phase

is the time needed for the bacteria to occupy the crystalline surface of the cellulose. Other authors point at the degree of accessibility and/or crystallinity of the cellulose to the hydrolysing enzymes (Zhang and Lynd, 2004). Yet, no relation is known between the lag phase and the suggested factors.

In chapter 5 no lag phase was observed when adding enzymes for hydrolysing crystalline cellulose at mesophilic conditions and neutral pH. In these experiments no leachate or animal manure was added, as it was in the case of Song et al. (2005) and O'Sullivan et al. (2008) studies. Indicating, as suggested by Van Velsen (1981) and Zeeman (1991), that there are compounds, other than ammonia, present in animal manure that could cause hydrolysis inhibition. This was indeed verified by the experiments performed in chapter 5, where crystalline cellulose hydrolysis was inhibited by addition of HAL and FAL, extracted from cow manure. The reason for the lag phase observed in the present research could then be that produced hydrolytic enzymes, secreted by the acidogenic bacteria, would first be adsorbed to the inhibiting compound and only when all the adsorbing sites were taken, the excess of enzymes, would hydrolyse the cellulose. As the lag phases observed in this study are identical for all trials, independently of the varying ammonia concentrations, it can be concluded that this inhibiting compound is not ammonia. The model to describe this lag phase was developed in this study (equations 3.8 and 3.9) and its results are presented below.

The suggested mathematical model (equation 3.8) showed a good fit with the experimental data from all trials, as can be seen in Figure 3.3 by the example of cellulose hydrolysis trial at 4.8 g NH_4^+-N·l^{-1}.

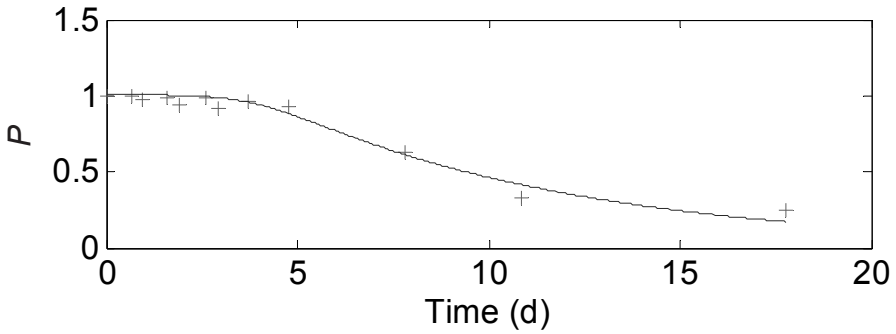


Figure 3.3. Depletion of cellulose as a function of time at batch digestion of cellulose at total ammonia concentrations of 4.8 g NH_4^+-N·l^{-1}. Symbol (+) refers to experimental data and solid line to model predictions.

Notice from Figure 3.3 that the model (equation 3.8) is able to predict the depletion of cellulose. In this example, $K = k_{h,max} = 0.17 \pm 0.03$ d^{-1} and $r = 3.25 \pm$

1.01. As mentioned before and confirmed by the mathematical model, the lag phase was similar for all cellulose trials, independent of the ammonia concentrations, indicating that ammonia did not influence the availability and hydrolysis of cellulose to the hydrolytic enzymes.

Since during the lag phase there is negligible cellulose hydrolysis, the data for estimating the hydrolysis rate constant, k_h, of cellulose should not include the lag phase. The hydrolysis rate constants for cellulose hydrolysis, where the lag phase is excluded, are presented in Table 3.1.

Table 3.1. pH, NH_3-N and k_h for cellulose hydrolysis trials. Trial name based on their NH_4^+-N concentrations. Data expressed as mean (standard deviation). k_h values include the last 5 sampling points and exclude the lag phase.

Trial	pH	NH_3-N ($mg.l^{-1}$)	k_h (d^{-1})	R^2
2.4 g/l	8.16 (0.04)	333 (27)	0.095	0.98
3.4 g/l	8.17 (0.04)	471 (34)	0.144	0.99
4.1 g/l	8.16 (0.05)	546 (60)	0.103	0.99
4.8 g/l	8.16 (0.04)	621 (56)	0.104	0.91
5.8 g/l	8.16 (0.04)	729 (62)	0.104	0.90
6.8 g/l	8.16 (0.04)	842 (65)	0.061	0.80
7.8 g/l	8.16 (0.04)	957 (54)	0.088	0.94

Hydrolysis of cellulose was well described by first-order kinetics, as can be seen by the high correlation factors found in all constants and presented in Table 3.1.

First order kinetics are commonly used to describe the hydrolysis of degradable particulate polymeric substrates (Pavlostathis and Giraldo-Gomez, 1991; Sanders, 2001).

According to the poor linear relation between the first order hydrolysis constants of the trials and their free ammonia concentrations, shown in Figure 3.4, the hydrolysis of cellulose was not inhibited by free ammonia. In order to confirm such poor correlation k_h data analysis was performed and the results are presented in section 3.3.3.1.

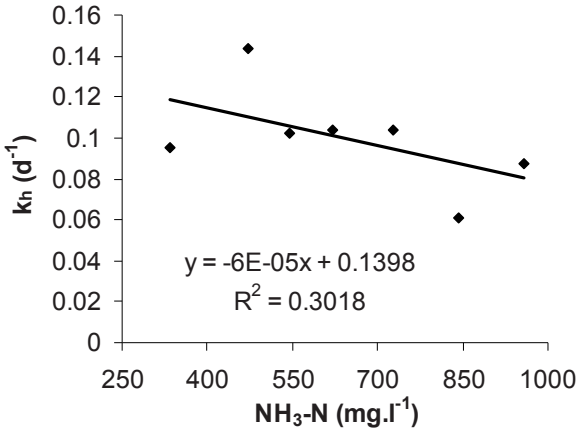


Figure 3.4. Hydrolysis rate constant (k_h) of cellulose as a function of free ammonia.

Hydrolysis rate constants of carbohydrates have been reported to vary between 0.025 and 2.0 d^{-1} (Pavlostathis et al., 1988; Sanders, 2001; Vavilin et al., 2008; Veeken and Hamelers, 2000). O'Sullivan et al. (2008) reported first order hydrolysis rates between 0.015 and 0.5 d^{-1} by anaerobically hydrolysing crystalline cellulose (same used in this study) using leachate or rumen content as inoculum and having free ammonia concentrations greater than 300 $mg.l^{-1}$. Most of their k_h values were between 0.6 and 0.14 d^{-1}, therefore, similar to the ones found in this study.

The pH variation between samples and controls and throughout the trials was negligible (Table 3.1), guaranteeing that the pH did not affect the k_h values of the different trials. Moreover, it kept the free ammonia concentrations at the desired ranges throughout the trial, which is important as the free ammonia range of these trials stood at the exponential part of the NH_4^+-N/NH_3-N balance curve (shown in Figure 3.1).

VFA in these trials remained very low, less than 5% from the initial added cellulose, indicating that hydrolysis was indeed the rate limiting step, as proposed by Angelidaki and Sanders (2004) in there batch test set-up. When this is the case, and therefore methanogenic conditions are guaranteed, hydrolysis efficiency and rate can be determined by methane formation (Sanders, 2001). The low VFA also indicates that the methanogenic biomass was adapted to the high ammonia concentrations, as there was no accumulation of VFA, and thus no inhibition of methanogenesis.

3.3.2 Tributyrin hydrolysis

In all experiments, almost all of the tributyrin was converted within the first day of the run, as is shown in Figure 3.5. This fast hydrolysis is in accordance with literature (Sanders, 2001; Wu and Tsai, 2004).

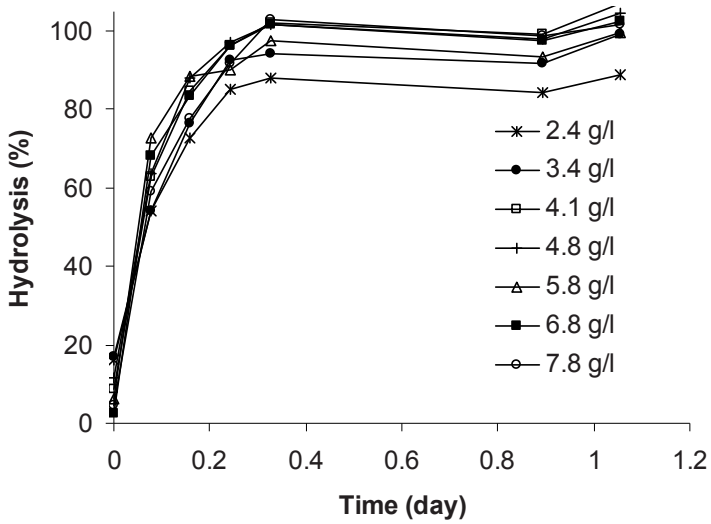


Figure 3.5. Hydrolysis as a function of time at batch digestion of tributyrin at varying total ammonia concentrations, with inoculum sludge adapted to 4.8 g NH_4^+-N·l^{-1}.

Unlike for the cellulose trials, no lag phase was observed for the tributyrin trials. This might indicate that the inhibiting compounds, such as HAL and FAL, are probably able to form complexes with the lipid, therefore making it still available for enzymatic hydrolysis, as explained in chapter 5.

Independently of the ammonia concentrations, tributyrin digestion described similar curves, and in all trials tributyrin was fully hydrolysed within two days. The similar behaviour resulted in similar hydrolysis rates, as shown in Table 3.2. Tributyrin hydrolysis was well described by first order kinetics, as can be seen by the high correlation value (Table 3.2). For determining the k_h values, only the first five points were used, where 88 to 100% of the tributyrin was hydrolysed. The remaining points were not taken into account because they entail possible errors due to the low amount of substrate left, as calculated by Sanders (2001). According to Sanders (2001), at very low biodegradable levels, a small error on the hydrolysis products concentration has a big impact on the k_h, therefore, the k_h values from where the substrate concentration approaches its minimum should not be included for k_h determination.

Table 3.2. pH and NH_3-N and k_h for tributyrin hydrolysis trials. Trial name based on their NH_4^+-N concentrations. Data expressed as mean (standard deviation). k_h values include the first 5 sampling points.

Trial	pH	NH_3-N ($mg.l^{-1}$)	k_h (d^{-1})	R^2
2.4 g/l	8.06 (0.10)	299 (96)	4.86	0.96
3.4 g/l	8.10 (0.12)	444 (146)	4.73	0.96
4.1 g/l	8.06 (0.10)	506 (154)	4.64	0.96
4.8 g/l	8.05 (0.10)	577 (158)	4.53	0.93
5.8 g/l	8.06 (0.10)	713 (213)	4.54	0.85
6.8 g/l	8.05 (0.09)	809 (237)	3.58	0.90
7.8 g/l	8.04 (0.09)	876 (205)	4.70	0.98

The ammonia and pH ranges presented in Table 3.2 are slightly lower than the ones from the cellulose hydrolysis trials (Table 3.1).

Hydrolysis rate constants of lipids have been reported to vary from 0.005 to 7.8 d^{-1} (Batstone et al., 2002; Pavlostathis et al., 1988; Sanders, 2001; Vavilin et al., 2008; Veeken and Hamelers, 2000). The hydrolysis rate constants found in this study were within the above reported range. However, this range includes different lipid sources, which are, in a lot of cases, slower to hydrolyse than the tributyrin here studied. Sanders (Sanders, 2001) compared lipases activity during digestion of palm oil and tributyrin and concluded that the activity of the latter was much faster than the former due to the steric hindrance of the LCFA in the triglycerides. Moreover, it has also been reported that tributyrin is easily hydrolysed into butyric acid (Wu and Tsai, 2004), as was verified in this study.

According to the poor linear relation between the first order hydrolysis constants of the tributyrin trials and their free ammonia concentrations, shown in Figure 3.6, the hydrolysis of tributyrin was not inhibited by free ammonia.

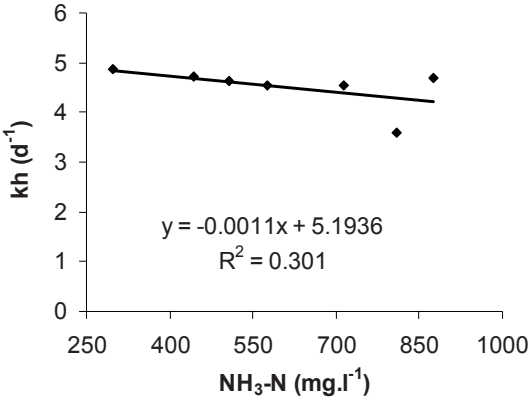


Figure 3.6. Hydrolysis rate constant (k_h) of tributyrin as a function of free ammonia.

The pH chosen for these experiments was approximately 8, because the sludge was already adapted to this pH, which is commonly found in manure anaerobic digesters. Moreover, Wu and Tsai (2004) studied the effect of pH on hydrolysis of tributyrin with lipases produced by Pseudomonas fluorescenes CCRC-17015, using pH ranges from 6 to 8 and concluded that the optimum pH values for lipase specific activity were between 7.5 and 8.

3.3.3 Data analysis

3.3.3.1 Set-membership parameter estimation

To determine the accuracy of the relationship between ammonia and k_h, a set-membership estimation procedure was implemented.

For the cellulose trials (see Table 3.1, Figure 3.4), in order to obtain a non-empty FPS, a relatively large error bound of 0.0291 (d^{-1}) had to be specified. This minimum error bound for the whole data set, together with the intercept and slope of a linear relationship between ammonia and k_h, was found after solving a min-max optimization problem, as described in Keesman and van Straten (1989). After considering trial 3.4 g/l as unreliable, and thus removing the corresponding data point from the data set, a smaller minimum error bound of 0.0177 (d^{-1}) was needed. Removing the estimated k_h value for trail 6.8 g/l even further decreased the minimum error bound to 0.0067 (d^{-1}) (Table 3.3). Notice, furthermore, that even after removing data points from the data set, the resulting slope in the linear

ammonia - k_h relationship remains close to zero. Only the intercept with the Y axes, as can be seen by the values presented in Table 3.3, significantly changes.

Table 3.3. Error bound (ε), potential outlier, intercept and slope of the linear trend for cellulose hydrolysis.

Iteration nr.	Nr of data points	Error bound	Potential outlier	Intercept	Slope
1	7	0.0291	471	0.1464	-0.0001
2	6	0.0177	842	0.1350	-0.0001
3	5	0.0067	333	0.1055	-0.0000
4	4	0.0038	-	0.1268	-0.0000

Using an error bound of 0.06 (d^{-1}), which is approximately twice the minimum error bound for all trials, resulted in a FPS as indicated by the shaded area in the left graph of Figure 3.7. Notice that the slope (including its uncertainty) is close to zero and that the FPS contains zero slope values. This indicates that the variation of each trial's k_h on the slope is small, therefore, confirming that ammonia does not inhibit the hydrolysis of cellulose. The right panel of Figure 3.7 with the bounded linear trend (bold lines), as a result of the presumed bounded error properties of the data, confirms this finding. Notice that each of the lines in the right panel of Figure 3.7 is related to a vertex of the FPS, as presented in the left panel of Figure 3.7.

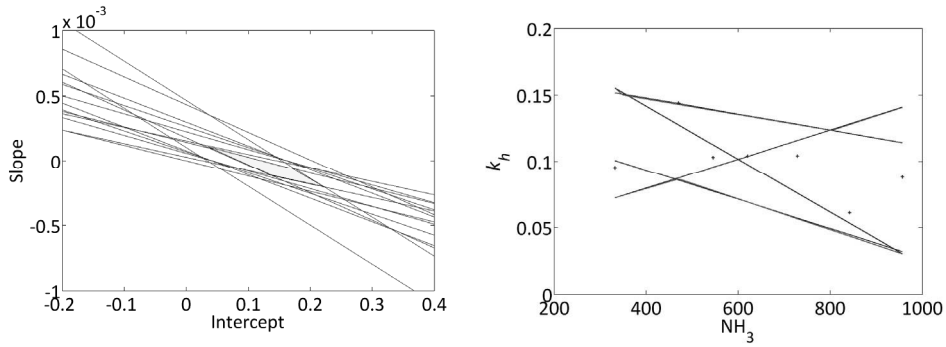


Figure 3.7. Feasible Parameter Set (FPS) for all cellulose hydrolysis trials (left), as a result of the 7 (number of data points) intersecting strips. Bounded linear trend (bold lines) showing the uncertain and independent relationship between ammonia and k_h (right).

From the set-membership literature, it is well-known that for the linear regression case, the FPS is a polytope found from the intersection of *N* (number of data points) strips in the parameter space, as in the left graph of Figure 3.7. It can even be shown that the well-known weighted least-squares techniques can be

used to solve the bounded linear regression problem (Milanese, 1995; Keesman, 1997). But, in general, the FPS can be a complex, and even unconnected, set (see Keesman, 2003, for details and possible solutions).

For the tributyrin trials (see Table 3.2, Figure 3.6), the large error bound of 0.6527 (d^{-1}) was due to the large variation in the estimated values of k_h, that is from 3.58-4.86 d^{-1}. After considering trial 6.8 g/l as unreliable, and thus removing the corresponding data point from the data set, a smaller minimum error bound of 0.1265 (d^{-1}) was needed. Removing the estimated k_h value for trail 7.8 g/l even further decreased the minimum error bound to 0.0576 (d^{-1}) (Table 3.4). From this analysis, we concluded that the k_h - value related to the 6.8 g/l trial is an outlier. Consequently, for further analysis we removed this data point from the data set. Hence, after removing this outlier a minimum error bound of 0.1265 (d^{-1}) is required (Table 3.4).

Table 3.4. Error bound (ε), potential outlier, intercept and slope of the linear trend for tributyrin hydrolysis.

Iteration nr.	Nr of data points	Error bound	Potential outlier	Intercept	Slope
1	7	0.6527	809	4.9763	-0.0010
2	6	0.1265	876	4.8165	-0.0003
3	5	0.0576	-	5.0336	-0.0008

Using an error bound of 0.25 (d^{-1}), which is approximately twice the minimum error bound for the remaining six data points, resulted in an FPS as indicated by the shaded area in the left graph of Figure 3.8. Notice that again the slope (including its uncertainty) is close to zero and that the FPS encloses a zero value of the slope. This indicates that the variation of each trial's k_h on the slope is small, therefore, confirming that ammonia does not inhibit the hydrolysis of tributyrin. The right panel of Figure 3.8 with the bounded linear trend (bold lines), as a result of the presumed bounded error properties of the data, again confirms this finding, as it contains negative and positive slopes.

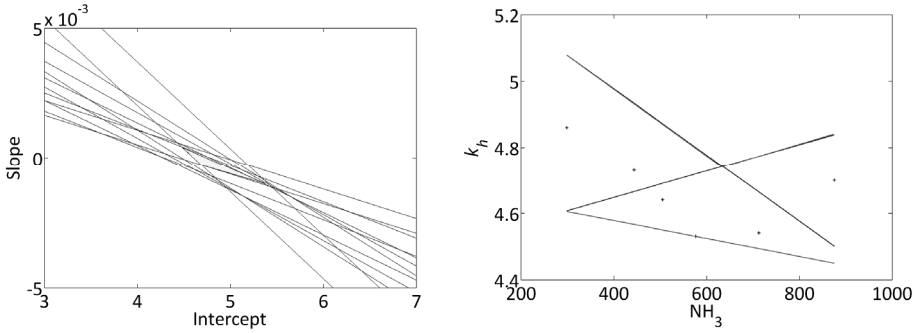


Figure 3.8. Feasible Parameter Set (FPS) for all tributyrin hydrolysis trials (left), as a result of the 6 (number of data points) intersecting strips. Bounded linear trend (bold lines) showing the uncertain and independent relationship between ammonia and k_h (right).

3.4 Conclusions

Ammonia nitrogen in the range of 2.4 to 7.8 g NH_4^+-N.l^{-1} (283 to 908 mg NH_3-N.l^{-1}) does not effect hydrolysis of tributyrin. This was also verified for the hydrolysis of cellulose where ammonia nitrogen in the range of 2.4 to 7.8 g NH_4^+-N.l^{-1} (333 to 957 mg NH_3-N.l^{-1}) was applied. Both conclusions were confirmed by mathematical analysis.

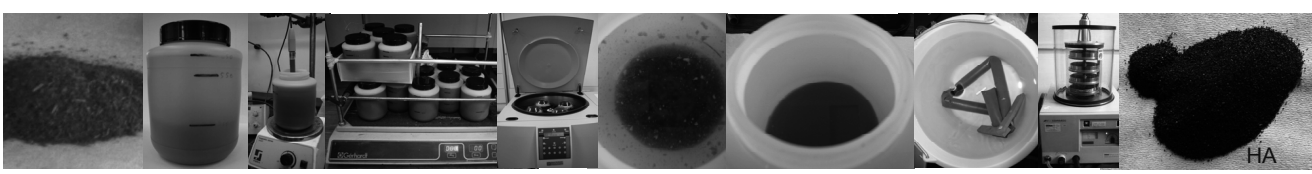
Ammonia nitrogen in the range of 2.4 to 7.8 g NH_4^+-N.l^{-1} (333 to 957 mg NH_3-N.l^{-1}) does not effect the lag phase observed for cellulose hydrolysis.

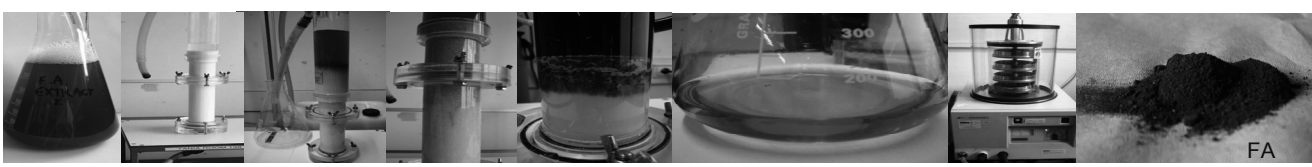
Acknowledgements

The authors would like to acknowledge Katja Grolle for her technical support.

Chapter 4

Characterization of humic acid-like and fulvic acid-like isolated from fresh cow manure and silage maize





Abstract

Humic acid-like (HAL) and fulvic acid-like (FAL) extracted from fresh cow manure and silage maize were characterized by elemental analysis, ^{13}C CPMAS NMR, Pyrolysis GC/MS and direct titration, resulting in a detailed chemical description, in terms of elemental composition, biomacromolecules, pyrolysis products and functional groups, of the humic matter (HM) present in this type of agricultural wastes. HAL and FAL from silage maize and fresh cow manure showed to have similar carbon contents, however higher oxygen for the FAL than for the HAL. Total acidity was higher for the FAL than for the HAL, and higher for the fresh cow manure than the silage maize. HAL from silage maize had higher polysaccharide content than HAL from fresh cow manure. On the other hand, HAL from fresh cow manure were richer in lignin than HAL from silage maize. HAL retained more of the recalcitrant plant-derived fraction, while FAL contained a larger amount of microbially derived proteinaceous material. HM from fresh cow manure was more degraded than HM from silage maize, but all these lignocellulosic biomass samples showed to have more newly formed HM, than soil. Such detailed chemical characterization of HM extracted from lignocellulosic biomass, enables a better understanding of the possible mechanisms involved in the inhibitory effect of HAL and FAL matter on the anaerobic hydrolysis of agriculture organic wastes.

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4.1 Introduction

Natural organic matter (NOM) has been studied for many years, especially within soil sciences. Due to their wide spread in nature and high concentrations in water, NOM is nowadays studied in many other fields, as is the case for environmental sciences. NOM can be divided into humic and non-humic matter. Residues resulting from human activity are known as Anthropogenic organic matter (AntOM) (Tipping, 2002) and Anthropogenic Humic Matter (AntHM) is known as the humic matter derived from agricultural, industrial and domestic wastes (Tan, 2003). Humic matter can be extracted from soils and sediments, but also from lignocellulosic biomass, such as animal manure and plant material, due to their solubility and adsorption properties (Tipping, 2002). Humic acids are soluble above pH 3,5 and fulvic acids are soluble at all pH values (Chen and Wang, 2007). Even though the extraction method is identical, nomenclature of fractions changes according to the source, therefore, HA and FA for soil matter, and HAL and FAL for AntOM.

Humic and fulvic acids are products of the biological decay of biota residues and behave like weak acid polyelectrolytes (Stevenson, 1994; Veeken, 1998). They contain organic acids of low and high molecular weight, mono, oligo and polysaccharides, proteins, peptides, amino acids, lipids, waxes, polycyclic hydrocarbons and lignin fragments (Saiz-Jimenez, 1992). The two main classes of acid groups in humic matter are aliphatic and phenolic, both having –OH which yield protons by dissociation (Tipping, 2002). Even though extensive literature has been published on humic matter, there are still many unknowns relating to their formation, composition and reactivity.

There is a negligible amount of literature on the characterization of HAL and FAL from animal manure and plant material. Most of the literature that characterizes the HM in these types of organic waste is related to composting (Huang et al., 2006; Ouatmane et al., 2000; Veeken et al., 2000b; Fuentes et al., 2007; Ait Baddi et al., 2004). Because the term ‘compost’ includes processed biowaste from many different sources, there is not a general chemistry that can be taken as a basis. This is clearly illustrated by Dignac et al. (2005). Moreover, the only reports on the characterization of HAL from animal manure (Deiana et al., 1990) and maize silage (Adani et al., 2006) lack detail in terms of the organic matter origin or/and a full characterization, not only with Elemental Analysis and ^{13}C CPMAS NMR, but also Pyrolysis GC/MS, and acid direct titrations, which determines the carboxyl (COOH) and phenolic (-OH) content of the HM.

Anaerobic digestion of agricultural wastes, which are high in lignocellulosic biomass, has been applied at a farm scale since the 1980s, especially in northern

European countries. The present increasing demand for renewable energy sources is encouraging further application and optimization of anaerobic farm digesters for solid waste treatment, where this waste is converted into bioenergy in the form of biogas.

For the anaerobic digestion of lignocellulosic biomass, the hydrolysis, which is the enzymatic conversion of complex polymers, such as carbohydrates, lipids and proteins, into soluble monomers, is considered to be the rate limiting step (Pavlostathis and Giraldo-Gomez, 1991). Consequently, biogas production depends not only on the biodegradability of the biomass, i.e., how much methane a certain biomass can potentially produce, but also the hydrolysis rate (El-Mashad, 2003). Humic and fulvic acids have been reported to have a scavenger effect on enzymes, inhibiting anaerobic hydrolysis (Brons et al., 1985). Therefore, a complete characterization of HAL and FAL extracted from lignocellulosic biomass is essential to understand the mechanisms involved in the inhibition of anaerobic hydrolysis.

In the present research HAL and FAL extracted from fresh cow manure and silage maize are characterized by Elemental Analysis, ^{13}C CPMAS NMR, and Pyrolysis GC/MS. The presented characterization gives a better insight in the elemental composition, biomacromolecules and pyrolysis products of these agricultural wastes. Elucidation of the carboxylic and phenolic functional groups of the HAL and FAL is also given in order to, in future work, predict their reactivity during anaerobic hydrolysis.

4.2 Materials and methods

4.2.1 Manure and maize samples

Fresh cow manure and silage maize were collected at different times and different farms, as shown in Table 4.1. From the harvest time to the collection date, the silage maize was stored in a bunker silo, after being pressed and covered airtight. After collection, the fresh cow manure and silage maize were air-dried at 40°C to a constant weight, and ground to particles smaller than 2 mm by a cutting mill (Retsch Muhle; type sm1)

4.2.2 Extraction and purification of HAL and FAL

The air-dried and ground fresh cow manure and silage maize were used for HAL and FAL extraction. The HAL and FAL extractions were performed at different times, according to collection of the original material. Detailed information on the extraction dates can be found in Table 4.1.

Table 4.1. Origin, processing dates, yields and ash content of HAL and FAL from manure and maize, 1st and 2nd extraction.

Sample	Original matter	Origin	Harvest date	Collection date	Extraction date	Extraction yield *	Ash (%)
HALman1	Manure[1]	A	N.A.	Sep. 2006	May 2007	6.83	-
HALman2	Manure[1]	A	N.A.	Aug. 2008	Aug. 2008	7.27	0.42
HALmai1	Maize[2]	B	Oct. 2006	May 2007	Nov. 2007	26.24	-
HALmai2	Maize[2]	C	Oct. 2007	Aug. 2008	Aug. 2008	11.54	0.75
FALman1	Manure[1]	A	N.A.	Sep. 2006	Jul. 2007	1.77	1.35
FALman2	Manure[1]	A	N.A.	Aug. 2008	Nov. 2008	1.11	21.1
FALmai1	Maize[2]	B	Oct. 2006	May 2007	Jul. 2007	2.41	0.5
FALmai2	Maize[2]	C	Oct. 2007	Aug. 2008	Nov. 2008	6.07	20.4

[1] Fresh cow manure
[2] Silage maize
A: Slurry pit at experimental farm Nij Bosma Zathe, The Netherlands.
B: Silo at maize producing farm in Nieuweroord, The Netherlands.
C: Silo at experimental farm Nij Bosma Zathe, The Netherlands.
N.A. = not applicable.
* g HAL or FAL·kg^{-1} of fresh manure or maize calculated on ash-free basis.

HAL and FAL extractions were performed according to the methods developed by the International Humic Substances Societyb (IHSS) (IHSS, 2009) with small alterations due to the nature of the original material. The method is here shortly explained. Air-dried and ground material was mixed with 0.1 M HCl at a solid-liquid ratio of 1:25 in polyethylene centrifuge bottles. The pH was then further decreased to 1. HAL were sequentially extracted with HCl and NaOH, under nitrogen atmosphere, for reaching pH values of 1 and 7 respectively. After the last acidification the HAL precipitate was dialyzed (Spectra/Por, MWCO 1000) against distilled water until the conductivity was below 5 mS, and finally freeze-dried. Due to the organic nature of the original material, use of HF for removal of silicates was not necessary. FAL were adsorbed onto XAD-8 resin, purified with H^+ saturated cation exchange resin (Bio-Rad AG-MP-50) and finally freeze dried. All extracted humic matter was stored in a exsiccator until use.

4.2.3 Elemental Analysis

C, H, N, S and O of extracted HAL and FAL from maize and manure were analysed in duplicate on an Interscience Elemental Analyser EA1110 (ThermoQuest CE Instruments). The results are presented in mass percentages, and were calculated on an ash-free basis.

4.2.4 Functional groups

Direct titrations were performed in a Metrohm 702 SM Titrino titrator (Metrohm, Switzerland) using a pH 340i pH meter (WTW, Germany). The Sentix 21 glass electrode (WTW, Germany) was calibrated using standardized freshly prepared buffer solutions of pH 4.0 and 7.0. In order to fit the calibration line, a buffer of pH 10 was used as last. A 50 ml stock solution of each HAL and FAL extracted from cow manure and silage maize sample was prepared. Each stock solution had 0,66±0,05 $g \cdot l^{-1}$ of HAL or FAL extracted from cow manure and silage maize in 0.1 M $NaNO_3$, which was the background electrolyte. NaOH was added to the stock solutions to a pH of approximately 11, in order to dissolve the samples. A 10 ml aliquot of each stock solution was transferred into a 25 ml Teflon vessel. Before titration, enough HCl was added to each sample to neutralize the NaOH initially added. Samples were then stirred and equilibrated at 25.00 ± 0.02°C (Heidolph MR 3001K) during 30 min to ensure the heat homogeneity of the solution. Ten minutes prior to the onset, and during each titration, water-saturated atmosphere of nitrogen was created inside the Teflon vessel so as to avoid the evaporation of the sample. The titrant (carbonate-free 0.1 M NaOH) was added in increments of 2 to 6 µl using a calibrated Gilmont micro burette and pH was recorded 15 seconds after each addition. At least 30 seconds were needed to record the pH values when the titration curve was approaching the inflection point. All the samples were titrated from and initial pH of 2.68 to 3.24 to a maximum pH of 10.50 to 10.52. The humic and fulvic charge (Q) was calculated according to López et al. (2008). Carboxyl content was estimated as the value of Q at pH 8, and the phenolic hydrolxyl content as two times the change in Q between pH 8 and 10 (Ritchie and Perdue, 2003).

4.2.5 ^{13}C CPMAS NMR

Solid-state ^{13}C Nuclear Magnetic Resonance (NMR) spectra were recorded using Cross Polarization Magic Angle Spinning (CPMAS) on a wide-bore AMX 300 spectrometer (Brucker, Karlsruhe, Germany) operating at 75.48 MHz of ^{13}C frequency. The spinning speed was 5 kHz, the acquisition time was 34 ms, the ^{13}C 90° pulse length was 5 µs, the contact time was 1 ms, the recycle delay was 2 s and the line broadening was 50 Hz. For each spectrum, 1000 scans were recorded. The resulting spectra were analyzed quantitatively according to the equations developed by Veeken et al. (2001) and Nelson and Baldock (2005), where the NMR spectrum is divided into four types of carbon: alkyl (0-50 ppm), O-Alkyl (50-110 ppm), aromatic (110-160 ppm) and Carbonyl (160-220 ppm). From the relative areas of the four types of carbon, four bio-macromolecules were calculated: aliphatics (lipids, biopolymers and fatty acids), polysaccharides (cellulose and hemicellulose), proteins and lignins. The results were presented in g bio-macromolecule per kg of volatile solids (VS). VS is the weight loss between ignition at 105°C and 550°C and can be considered as organic matter.

4.2.6 Pyrolysis-GC/MS

All samples were analyzed using a Curie-Point pyrolyser (Curie temperature 600°C) connected to a Carlo Erba GC8000 gas chromatograph. The pyrolysis products were separated on a fused silica column (Chrompack 25 m, 0.25 mm i.d.) coated with CP-Sil 51 b (film thickness 0.40 µm), with He as carrier gas. The initial oven temperature was 40°C and the heating rate 7°C·min^{-1}. The final temperature, 320°C, was maintained for 20 min. The GC column was connected to a Fisons MD800 mass spectrometer (m/z 45 - 650, cycle time 1 s). Pyrolysis products were identified using the internal NIST library and using spectra from literature, such as Ralph and Hatfield (1991), van Bergen et al. (1998), etc.

All pyrograms were interpreted in detail, resulting in 275 different pyrolysis products. These products were subdivided into chemical groups, as follows: acids, alkanes and alkenes, aromatics (excluding lignin), cyclo-alkanes, esters, fatty acids, lactones, lignins, N-compounds, phenols, polyaromatics, polysaccharides, sterols, sulphur compounds, terpenoids, and unknown. Based on frequency and abundance 157 products were chosen for quantification. These were quantified for all pyrograms using the two main fragment ions for each pyrolysis product (Appendix, page 93). All quantifications were checked manually.

4.3 RESULTS AND DISCUSSION

4.3.1 Yields of HAL and FAL from manure and maize

The extraction yields of all samples are shown in Table 4.1. Maize had higher HAL and FAL content than manure, given that manure extraction yields were less than half on the maize ones. The difference of yields between the manure samples was as low as 6% for the HAL, and 37% for the FAL. On the other hand for the maize samples the difference was more than double in both HAL and FAL. This shows that the different origin of the maize silage might have had an influence on the yield. The manure was collected from the same slurry pit, but in different years, indicating that the composition of the manure might suffer little change throughout the years in terms of yields.

4.3.2 Elemental composition

The ash content of most samples was below 1%, except for the second set of FAL samples, i.e., FALman2 and FALmai2, which contained approximately 20% each, as shown in Table 4.1. This unusually high ash content of the second FAL samples might be due to leakage of inorganic matter (XAD-8 or H^+ resins) from one of the FAL extraction columns. All elemental analyses are given on an ash-free basis (Table 4.2).

Table 4.2, shows the elemental composition of the HAL and FAL extracted from manure and maize, for both extracts.

Table 4.2. Elemental analysis, as %, calculated on ash-free basis, and atomic ratios of HAL and FAL from manure and maize, 1st and 2nd extraction. Data expressed as mean (standard deviation).

Sample	C	H	O	N	S	C/H	O/C	C/N
HALman1	55.08	6.10	30.36	5.95	0.34	0.76	0.41	10.79
	(0.08)	(0.04)	(0.28)	(0.03)	(0.15)	(0.01)	(0.00)	(0.04)
HALman2	57.45	6.57	26.81	6.23	1.15	0.73	0.35	10.76
	(0.21)	(0.01)	(0.00)	(0.06)	(0.15)	(0.00)	(0.00)	(0.06)
HALmai1	54.51	6.97	31.73	4.63	0.00	0.66	0.44	13.72
	(1.62)	(0.08)	(0.03)	(0.02)	(0.00)	(0.01)	(0.01)	(0.34)
HALmai2	57.55	7.38	30.05	4.07	0.57	0.65	0.39	16.49
	(0.71)	(0.04)	(0.07)	(0.02)	(0.01)	(0.01)	(0.00)	(0.29)
FALman1	59.01	5.85	33.28	2.43	0.65	0.85	0.42	28.32
	(0.19)	(0.02)	(0.54)	(0.04)	(0.13)	(0.00)	(0.01)	(0.56)
FALman2	54.17	6.32	34.61	2.59	4.13	0.72	0.48	24.36
	(0.42)	(0.07)	(0.49)	(0.01)	(0.04)	(0.02)	(0.00)	(0.08)
FALmai1	59.55	5.90	33.99	1.72	0.00	0.85	0.43	40.59
	(0.31)	(0.05)	(0.19)	(0.11)	(0.00)	(0.00)	(0.00)	(2.85)
FALmai2	58.74	6.73	34.69	2.68	0.33	0.73	0.44	25.56
	(1.48)	(0.09)	(0.42)	(0.09)	(0.01)	(0.04)	(0.02)	(1.94)

When looking at the elemental composition of HAL and FAL, two common differences are reported in literature: HA have higher C than FA, and FA have higher O than HA (Stevenson, 1994). In this study this was not the case, since the C was similar for all samples, and the O was only slightly higher for FAL than HAL, as is shown in Table 4.2. The N content was found to be larger in the manure samples than in the maize samples, indicating that manure has higher organic N-compounds concentrations. This is later confirmed by NMR and pyrolysis results.

In Figure 4.1 the C, O, H, N and S content from this study were compared to literature results. These literature references were chosen on the similarity of the HAL and FAL extraction methodology and origin of the AntHM. Oxygen shows large differences when comparing the results from this study with the ones from literature. The O content in the HAL samples from this study is within the range of the values found in literature. For the FAL, the O contents are clearly lower than those found in literature. The C content in the HAL samples from this study is within the range of the values found in literature. However this is not the case for the FAL, where literature values are lower than the ones from this study. The N contents of the maize samples from this study were higher than those reported in literature, probably due to differences in maturity state of the plant material used. H contents were similar, both between all the samples of this study, and for

literature data. The S content found in the manure-HAL was similar to that reported in literature, but high contents as found in FALman2 were not found in literature. In the maize samples, S contents were negligible, as also reported in the references used.

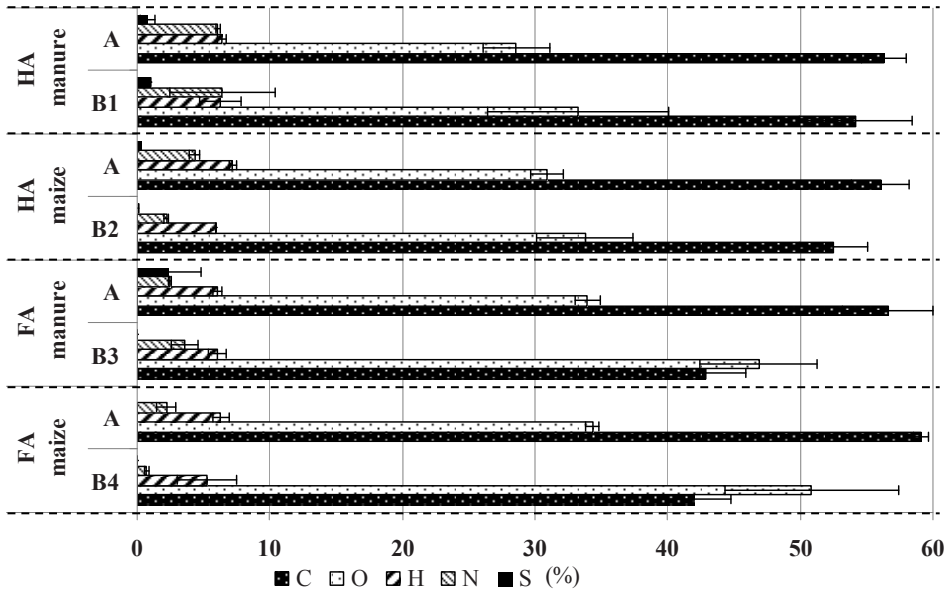

Figure 4.1. Average elemental composition of HAL and FAL from manure and maize, from this study (A) and from literature (B). B1 - (Relan et al., 1984; Riffaldi et al., 1983; Prasad and Sinha, 1980; Deiana et al., 1990); B2 - (Adani et al., 2006; Riffaldi et al., 1983); B3 - (Riffaldi et al., 1983); B4 - (Riffaldi et al., 1983; Fuentes et al., 2007).

In Figure 4.2 the O/C, C/H and C/N ratios from this study were compared to the ones from literature. O/C ratio values for all samples varied from 0.35 to 0.48 The O/C ratios of HAL are within the range found in literature for HAL, however for FAL the ratios are lower than the literature values, specially for the FAL maize samples. The lower O/C ratio for the FAL samples from this study can be explained by the unusually high C content found in the FAL samples. In this study, C/H ratios were slightly higher for FAL than HAL, which indicates a higher presence of unsaturated structures in HAL over FAL (Huang et al., 2006). Comparing to the chosen literature, the HAL from manure and FAL from maize show similar ratios, while for the HAL from maize and FAL from manure the show different ratios. In this study, the C/N ratios for the FAL were more than double those of the HAL.

The C/N ratios from this study were different from the ones from literature, except for the HAL manure samples.

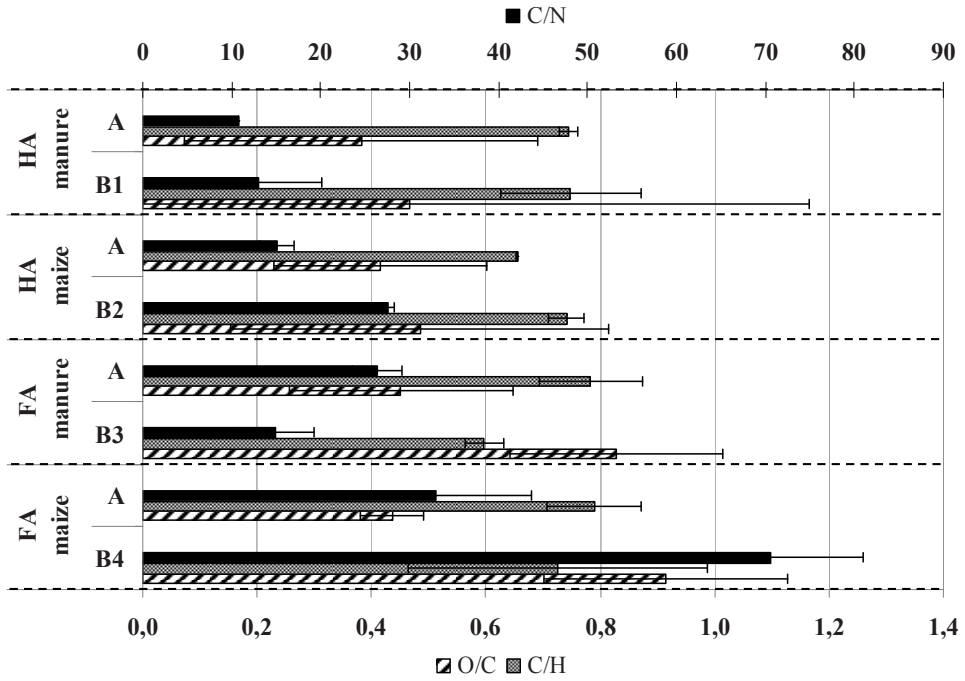

Figure 4.2. O/C, C/H and C/N ratios of HAL and FAL from manure and maize, from this study (A) and from literature (B). B1 - (Relan et al., 1984; Riffaldi et al., 1983; Prasad and Sinha, 1980; Deiana et al., 1990); B2 - (Adani et al., 2006; Riffaldi et al., 1983); B3 - (Riffaldi et al., 1983); B4 - (Riffaldi et al., 1983; Fuentes et al., 2007).

4.3.3 Functional groups

The titration curves from this study, shown in Figure 4.3, were similar to the ones reported by López et al. (2008), Ritchie and Perdue (2003) and Plaza et al. (2005), except for the HALman2 and FALmai2, which are the lowest curves, where probably a too high pH at the beginning of the titration, >3.1, resulted in a lower Q.

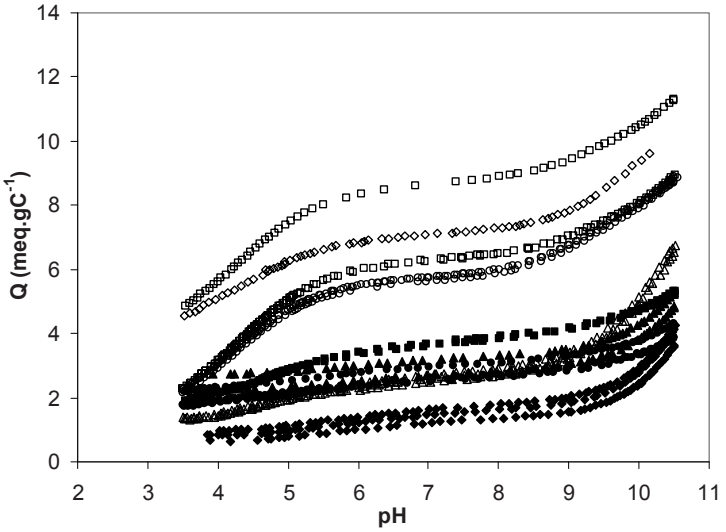

Figure 4.3. Charge curves from direct titration for HAL (filled symbols) and FAL (open symbols) from cow manure, extraction 1 (□) and 2 (◊), and silage maize, extraction 1 (o) and 2 (Δ).

Table 4.3, shows the acidic functional groups content of the HAL and FAL extracted from manure and maize, from both extractions.

Table 4.3. Acidic functional group contents of HAL and FAL extracted from cow manure and silage maize (meq.·g C^{-1}). Data expressed as mean (standard deviation).

Sample	Total acidity	Carboxyl	Phenolic hydroxyl
HALman1	5.53 (0.05)	3.81 (0.06)	1.71 (0.11)
HALman2	3.85 (0.30)	1.57 (0.22)	2.28 (0.11)
HALmai1	3.94 (0.07)	2.67 (0.06)	1.27 (0.05)
HALmai2	4.45 (0.92)	2.64 (0.77)	1.81 (0.15)
FALman1	10.35 (1.44)	7.26 (1.36)	3.08 (0.09)
FALman2	11.15 (0.00)	7.28 (0.00)	3.86 (0.00)
FALmai1	9.87 (0.21)	5.92 (0.07)	3.95 (0.22)
FALmai2	7.14 (0.21)	2.80 (0.12)	4.34 (0.17)

The total acidity was higher for the FAL than for the HAL, and higher for the manure samples than the maize ones. All samples, except for the HALman2 and FALmai2, showed higher carboxyl than phenolic hydroxyl content, as is usually reported for HM from soil literature (Ritchie and Perdue, 2003). The reported acidic functional groups data from similar source of HM, namely from poultry

litter (Prasad and Sinha, 1980), poultry manure, pig slurry and farm yard manure (Riffaldi et al., 1983) are variable. For poultry litter and pig slurry, the Carboxyl content is lower than the phenolic hydroxyl, but for the remaining samples it is the opposite. Their data was, however gathered by indirect titrations, which gives, according to Ritchie and Perdue (2003), less comparable and consistent estimates of carboxylic and phenolic groups than direct titrations. Due to a lack of literature on the determination of acidic functional groups from animal manure and plant matter by direct titration, and since the methodology can influence so much the outcome, carboxylic and phenolic contents from our samples were compared to soil literature. Carboxylic groups constituted 60 to 70% of the total acidity for FAL and 59 to 69% for HAL, which is lower than usually 78 to 90% found in soil samples (Ritchie and Perdue, 2003). The lower content of acidic groups in our samples in comparison to soil samples, indicate that our HM are less decomposed (Riffaldi et al., 1983).

The corresponding ratio carboxyl HAL/carboxyl FAL for the manure samples was 0.52 and for the maize samples 0.45, which is lower than the reported ratios of 0.65 (López et al., 2008), 0.59 (Milne et al., 2001) and 0.66 (Ritchie and Perdue, 2003). These lower ratios, which indicate a higher content of phenolic hydroxyl than the usually reported soil samples, might be due to the fact that our HM were formed in systems rich in water or poorly aerated (Ritchie and Perdue, 2003) as they originated from silage maize and animal manure. In both manure and maize samples, the phenolic contents of FAL were higher than of the HAL. The corresponding ratio phenolic HAL/phenolic FAL for the manure samples was 0.49 and for the maize samples 0.39, which is lower than the reported ratios of 1.36 (López et al., 2008) and 1.00 (Ritchie and Perdue, 2003).

4.3.4 ^{13}C CPMAS NMR

The amount of literature where ^{13}C CPMAS NMR spectroscopy is used for carbon analysis is abundant, however, it is mostly related to soil and sediment HM and not manure and plant HM, making it difficult to find comparable results.

In general, the four spectra of HAL, as shown in Figure 4.4, have larger peak area differences than the four spectra of FAL, shown in Figure 4.5. However, there are larger differences in several parts of the spectra, between the 1st and the 2nd samples of the FAL than the HAL, as explained below.

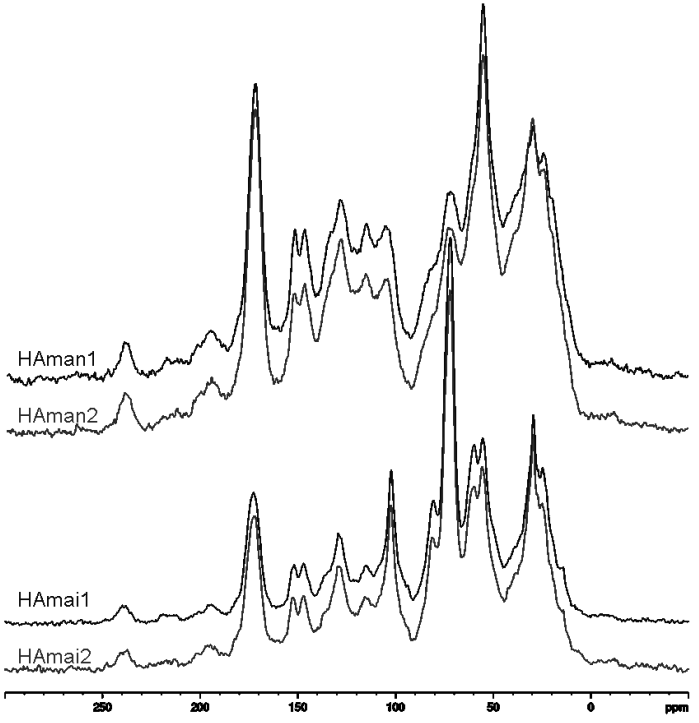

Figure 4.4. ^{13}C CPMAS NMR spectra of HAL from manure (top two) and maize (bottom two), 1st and 2nd extraction.

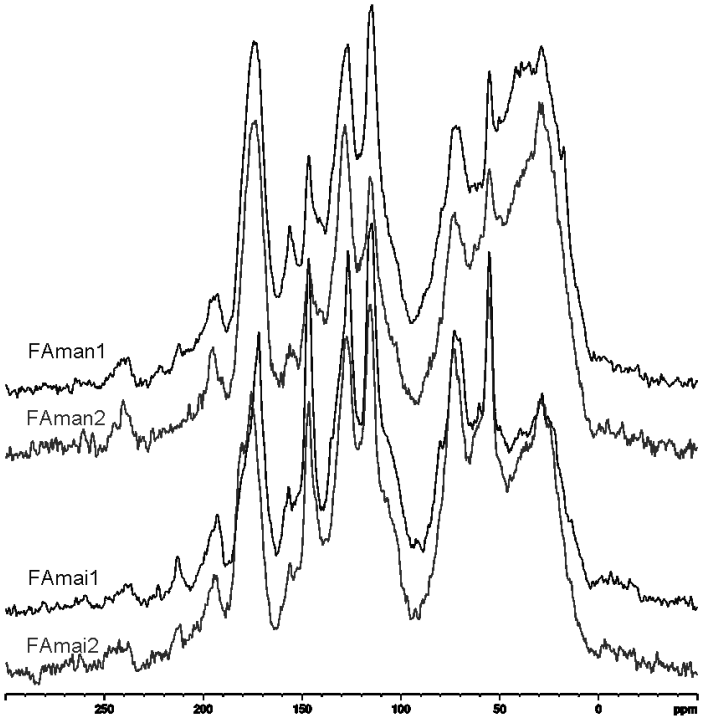

Figure 4.5. ^{13}C CPMAS NMR spectra of FAL from manure (top two) and maize (bottom two), 1st and 2nd extraction.

Of the two higher peaks found in the Alkyl carbon region (50 – 0 ppm) , the one around **22** ppm is assigned to terminal methyl groups, like acetyl groups attached to hemicelluloses (Golchin et al., 1996; Wikberg and Liisa Maunu, 2004), while the peak at **30** ppm is assigned to methylene in aliphatic rings and chains (Golchin et al., 1996; Tang et al., 2006; Veeken et al., 2001). These structures are most likely lipids, waxes and aliphatic bio-polyesters such as cutin and suberin (Rumpel et al., 2005). Both peaks are clear in the HAL samples, but higher for the HAL manure than for the HAL maize. In the FAL samples the alkyl area shows less distinct peaks.

In the O-Alkyl carbon region (110 – 50 ppm) two pronounced peaks are found in all spectra: **55** and **72** ppm. The signal at **55** ppm is assigned to methoxyl carbon associated with lignin (phenolmethoxyl of coniferyl and sinapyl moieties) and with hemicellulose (glucuronic acid in xylan) (Haw et al., 1984; Veeken et al., 2001). This characteristic lignin peak (Hatcher, 1987) is much lower in the HAL maize than the remaining samples, which indicates that the maize used does not have such large lignin content. The peak at **60** ppm is assigned to aliphatic C-O carbons other than methoxyl, and it is often a shoulder of the methoxyl peak (Hatcher, 1987), as is clearly seen in the HAL maize sample due to the reduced size of the 55 ppm peak. This shoulder region (**58 – 60** ppm) has also been reported as carbon in amino acids, peptides and/or proteins (Breitmaier and Voelter, 1987).

The **72** ppm peak is assigned to C2, C3 and C5 atoms of polysaccharides, mainly cellulose, (Hatcher, 1987; Veeken et al., 2001; Tang et al., 2006) but also to C–OH bonds in ester-bound structures of cutin and suberin (Rumpel et al., 2005). This cellulose peak is considerably higher in maize than in manure, especially in the HALs. Therefore HAL maize has higher polysaccharide content, while HAL manure has more lignin. The duplicates of the FAL samples are not as similar as the ones from HAL, and therefore such detailed conclusions cannot be attained. However looking at the FALmaize2 and FALmanure2 this peak height difference becomes clearer in relation to the **72** ppm peak. The remaining relevant peaks of the O-Alkyl carbon region are only found in the HAL samples and are at around **82** ppm and around **104** ppm. The peak at around **82** ppm is much clearer in the HAL maize than the HAL manure, but its chemical assignment is unclear (possibly β-O-5 carbon structures (Vane et al., 2001) or overlapping resonances of C2, C3 and C5 carbons in the pyranoside structure of cellulose and hemicellulose (Spaccini and Piccolo, 2008). This region (around **82** ppm) is, however often reported as a shoulder peak assigned to aliphatic C-O carbons, other than methoxyl derived from lignin (Hatcher, 1987). The region around **104** ppm is assigned to di-oxygenated anomeric C1 carbon of cellulose and hemicellulose in fresh plant material (Rumpel et al., 2005). HAL maize shows a more distinct and slightly

higher 104 ppm peak than HAL manure, confirming the presence of fresh matter in the former, even though it has been ensiled for over a year.

In the Aromatic carbon region (160 – 110 ppm) all spectra showed four distinctive peaks: **115, 130**, around **147** and around **153** ppm. The signal at **115** ppm is assigned to unsubstituted, *ortho* or *para*, and substituted aromatic carbons in lignin structures (Veeken et al., 2001; Spaccini and Piccolo, 2008). This signal was much stronger for FAL than for HAL. All four FAL spectra showed a similar peak intensity, except for FALmanure2 which was notably lower than the others, even though still much higher than in HAL spectra. The 115 ppm peak intensity in HAL manure is much higher than in HAL maize. This large difference between HAL manure and maize remains throughout the whole aromatic region, indicating that the manure HAL are more aromatic, and probably have higher lignin contents, than maize HAL. The peak at **130** ppm indicates the presence of C-substituted and/or protonated aromatic carbon which may originate from lignin, and unsubstituted carbon resonances of p-hydroxy phenyl rings of cinnamic units in both lignin and suberin biopolymers (Rumpel et al., 2005; Spaccini and Piccolo, 2008). The signal at **147** ppm is assigned to O-substituted aromatic carbon of guaiacol in lignin structures (Veeken et al., 2001). Tannins and tannin-like structures may also be found in this region (Rumpel et al., 2005). Signals around **153** ppm are due to aromatic carbons connected to methoxy groups in syringol units derived from lignin (Veeken et al., 2001; Spaccini and Piccolo, 2008). In the FAL manure spectra the signal at 147 ppm was at least twice as high as the signal at 153 ppm, and in the FAL maize this difference was much larger, indicating that maize has more methoxyphenols than FAL manure.

In the Carbonyl carbon region (220 – 160 ppm) all the spectra showed two distinctive peaks: a very high peak at **172** ppm and a much smaller peak around **195** ppm. The **172** ppm peak is assigned to carboxyl carbons of acetyl groups attached to hemicellulose, amide carbon present in proteins and COO of esters (Wikberg and Liisa Maunu, 2004; Rumpel et al., 2005). This peak was not very clear for the FAL maize spectra, since FALmaize2 seems to have not only a broader peak, but also a slightly higher chemical shift (176 ppm) with a shoulder peak at 180 ppm, than FALmaize1. The reason for this difference is not understood, due to a lack of references on the 176 to 180 ppm region. Of the HAL spectra, manure samples show a much higher 172 ppm peak than maize samples. The signal at **195** ppm is assigned to ketone and aldehyde groups (Deiana et al., 1990; Kaal et al., 2007) and is larger for the FAL than the HAL spectra.

The peak at around 240 ppm is a result of the spinning sidebands and is not included in the carbon analysis.

Table 4.4 shows the integrated relative areas of the four chemical shift regions described above and their contribution in terms of the four biomacromolecules as calculated according to Veeken et al. (2001).

Table 4.4. Relative areas of peaks and its composition as biomacromolecules by ^{13}C CPMAS NMR of HAL and FAL from manure and maize, 1st and 2nd extraction.

	HAL				FAL			
	man1	**man2**	**mai1**	**mai2**	**man1**	**man2**	**mai1**	**mai2**
Alkyl [a]	27.5	28.9	25.4	25.3	28.3	29.7	20.8	22.8
O-alkyl [a]	33.9	33.0	48.0	45.3	26.1	26.1	31.4	31.7
Aromatic [a]	23.4	22.8	15.6	17.3	28.1	25.8	32.4	29.3
Carbonyl [a]	15.2	15.6	11.0	12.1	17.5	18.4	15.4	16.2
Aliphatics [b]	19	28	73	50	0	0	0	0
Proteins [b]	458	476	313	356	533	582	399	455
Polysaccharides [b]	170	160	401	354	33	44	81	99
Lignins [b]	353	336	212	240	434	374	519	446

(a) % of total peak area
(b) $g.kgVS^{-1}$

The remarks made in the spectra interpretation according to the four main carbon regions can be compared and confirmed with the data from Table 4.4. Alkyl carbon for the manure samples is always slightly higher than for the maize ones. On the other hand, the O-Alkyl carbon shows the opposite. The Aromatic carbons do not seem to follow any tendency. The Carbonyl carbon shows the same tendency as the Alkyl carbon, thus the manure has slightly higher contents than the maize.

Because results of 1st and 2nd extractions are similar, they will be discussed together for both FAL and HAL.

HAL manure consists mostly of O-Alkyl, followed by Alkyl, then Aromatic and finally Carbonyl carbons. The HAL from swine manure-based compost spectra reported by Chang Chien et al. (2007) shows a high similarity with the HAL manure spectra obtained in this study. On the other hand, Deiana et al. (1990) reported a higher Aromatic than O-Alkyl carbon content for HAL extracted from manure, but the type of manure was not defined. The type of manure is an important aspect, since different animals and even different diets result in different predominant carbon groups (Mao et al., 2008). The tendency observed for HAL manure samples was also found for the HAL maize samples, even though the O-Alkyl C contents were somewhat higher. Adani et al. (2006) had similar results for the HAL from maize plant residue, where the O-Alkyl carbons represented 45% of the total integrated carbon area, and Carbonyl carbon had the lowest percentage (11.65%).

However, the Aromatic and the Alkyl carbons had opposite contents to the ones found in this study for HAL maize, i.e. 25.2% for Aromatic and 16% for Alkyl carbons (Adani et al., 2006), indicating a higher amount of aliphatics in their maize samples. For the FAL manure the Alkyl carbons show the highest percentage, followed by the similar percentages of Aromatic and O-Alkyl carbons and, once again, the Carbonyl shows the lowest percentage. Chang Chien et al. (2007) reported a predominant and much higher O-alkyl percentage (41.8%) for FAL from swine manure-based compost (MW>1000) than the FAL manure of this study, indicating a higher amount of methoxyl carbons in their samples. FAL maize showed similar O-Alkyl and Aromatic carbons, followed by Alkyl and finally Carbonyl.

HAL manure had higher lignin content than HAL maize. HAL manure samples were more aromatic, showing higher amount of functional groups derived from lignins, while HAL maize samples were richer in carbohydrates, especially polysaccharides, suggesting that HAL manure is more degraded than HAL maize (Stevenson, 1994). The aliphatics were low for both HAL, maize and manure, although almost 3 times higher for the former. The proteins were higher for HAL manure than HAL maize. The higher protein content for manure than maize is also verified on the FAL samples, where the proteins represented most of the carbon for the manure samples and not for maize samples. In the FAL maize most of the carbon was in the lignin form. Even though, the lignin content of the FAL manure was not as high as for the FAL maize, it still represented a major part of the carbon.

4.3.5 Pyrolysis-GC/MS

4.3.5.1 General chemistry

This part is based on the total number of identified pyrolysis products. The products that have been included in the quantification are indicated with a code that is listed in the Appendix (page 93).

The group of **acids** contained 36 different compounds. Acids are due to partial oxidation of polysaccharides and lignins, and breaking up of wax esters. They were largely restricted to the FAL fractions. Both aliphatic and aromatic acids were abundant. The first group contained mainly propenoic (Ac1) and presumably lignin-derived methylated butanoic acids. The second groups contained a large number of lignin-derived acids, such as benzoic acid, p-hydroxy benzoic acid, vanillic acid, syringic acid. Although there is probably a difference in kind of acids

encountered in manure and maize FAL, the number of samples was too low to draw definite conclusions. One acid, 9,12-octadecadienoic acid, was restricted to the maize HAL fraction. Bull et al. (2000b) found this acid as a common compound in a grass (*Holcus lanatus* L) and in the underlying soil.

The group of **alkanes, alkenes and alcohols** contained many compounds but did not show all the *n*-alkanes and *n*-alkenes that are usually found in soil organic matter. Alkanes and alkenes in general were better represented in the humic acid fractions. Especially HALmai1 contained a significant amount of the C21 alkene. Pristene (a chlorophyll pyrolysis product) and the C_{29}-C_{31}-C_{33} alkanes were most common, together with the C_{19} and C_{21} alkenes. C_{16}-C_{26} alkanes and alkenes are usually pyrolysis products of biopolymers such as cutan and suberan (Kögel-Knabner et al., 1992; Nierop, 1998). The C_{29}-C_{31} members are derived from protective waxes on leaves and roots.

The **aromatic** group contained 21 compounds, of which benzene, toluene and C2-benzenes were the most common ones. C3-benzenes were common, but present in low amounts. Benzene, toluene and alkylbenzenes may originate from proteins (e.g. Chiavari and Galletti, 1992), but possibly also from lignins (Saiz-Jimenez and de Leeuw, 1986). In strongly decomposed systems, however, toluene is usually associated with N compounds and represents microbial proteins (Buurman et al., 2007).

Cyclo-alkanes were found in all samples, but always as minor compounds. Two were included in the quantifications. Their significance is not clear, but cyclopropyl units are probably pyrolysis products and not natural compounds.

Esters, except the lignin pyrolysis product guaiacyl acetic acid methyl ester (E1), occur in minor amounts. E1 is an indication of relatively fresh lignin. Aliphatic esters are probably pyrolysis products of waxes.

Fatty acids are straight-chain aliphatic carboxylic acids. In plant tissues they have predominantly even numbers of C atoms. Members of 12-30 C atoms are common, and usually the C_{16} member predominates (in soils). According to Bull et al. (2000a), this fatty acid, while dominant in soil organic matter, is not a dominant compound in farmyard manure. Fatty acids were mainly represented by the plant-derived C_{16} and C_{18} members, which were found in both FAL and HAL, although more abundant in the latter. The FAL fraction contained some short-chain fatty acids, such as the C_5 and C_6 members. These were not found in the HAL and are probably of microbial origin. Iso- and ante-iso C15 fatty acid, which are usually also ascribed to microbial origin, were common in the HAL fractions.

Two '**lactones**' were identified, but the identification of these compounds is doubtful because the identification is based on a single molecular mass.

28 **Lignin** products were identified. Details on lignin structures as investigated by pyrolysis-GC/MS are reported by, e.g., Saiz-Jimenez and de Leeuw (1986) and Ralph and Hatfield (1991). These lignin products contained many guaiacyl and syringyl products with C3 side chains and some lignin dimers, indicating a relatively low decomposition of the lignin fraction.

A large number (18) of **sterols** was encountered, mainly restricted to the HAL fraction. Sterols are mainly tetracyclic or pentacyclic triterpenoids that are found in cell membranes and resins (Killops and Killops, 2005). Pyrolysis-GC/MS does not allow a good identification of sterols. Sterols tend to decay rapidly in aerobic soils (and possibly in manures) and their abundance may therefore indicate the grade of decomposition.

The number of **N-compounds** (23) is normal for systems with low C/N ratios. N-compounds are derived from amino-acids, DNA, RNA, and aminosugar polymers such as chitin (Chiavari and Galletti, 1992; Stankiewicz and Van Bergen, 1998) of both plant and microbial origin. Compounds that are more common in soils than in litter are probably (partially) derived from microbial tissues. To this group may belong pyrroles, pyridines, indole, and benzonitriles (Van Bergen et al., 1998). Dominant compounds were different in HAL and FAL fractions.

Polyaromatics (11) were virtually restricted to the FAL fraction. Although polyaromatics are frequently associated with burnt organic matter (char, e.g. Kaal et al., 2008), this is unlikely in the present case. Because identification of various members of this group is problematic, clear conclusions cannot be drawn.

Phenols (26), which can be pyrolysis products of degraded lignins and of proteins (Chiavari and Galletti, 1992), were common in all samples. In the FAL, the group contained a number of predominant diphenyl compounds, which might be lignin degradation products but are not usually found in soils. Catechols are probably pyrolysis products of tannins.

The main **polysaccharides** (carbohydrates) in plant tissue are cellulose (primary cell wall), and hemicellulose (secondary cell wall). Both are readily broken down in the herbivore gut. Polysaccharide compounds were common in both HAL and FAL. Levoglucosan, which usually represents relatively fresh lignocellulosic material, was relatively scarce except in maize HAL. Furan compounds, which are ascribed to microbial input, are rather common in the FAL fractions. Acetic acid, which in large abundances denotes strongly decomposed organic matter (e.g. Buurman et al., 2005), is most abundant in the FAL fraction. Because this acid is not necessarily present in the original material, but can be formed as a pyrolysis product, it was not included in the acid group.

Three **sulphur**-containing compounds were found in the FAL fractions. They were not included in the quantification. S is present in amino acids cisteine and methionine (Killops and Killops, 2005).

Terpenoids (7) were restricted to the HAL fraction. Because there is a large number of terpenoids in plant tissue, e.g. in chlorophyll, resins and oils, and the ones present in the investigated samples could not be identified with pyrolysis-GC/MS, conclusions cannot be drawn from these compounds. In the present samples, they cluster with the lignin moieties (see below), and they might be associated with woody structures.

Compounds that could not be identified were found in all samples. Only rarely these products had a significant contribution to the total spectrum. Two have been included in the quantification, but they do not contribute significantly to the differentiation of the samples.

4.3.5.2 Quantification of compounds

This part is based on the quantified pyrolysis products alone (see Appendix, page 93). For each sample, the sum of all pyrolysis products was set at 100% and relative amounts were calculated with respect to this sum. The resulting quantification gives the relative abundance of each pyrolysis product. This quantification is essentially different from wt%, because (i) MS response factors of pyrolysis products vary and (ii) molecular masses of the pyrolysis products are widely different. Changes in relative abundance of pyrolysis products can be interpreted in the same way as data quantified otherwise. The quantified data were analyzed with factor analysis using Statistica ® Version 6 (Statsoft Inc, Tulsa).

Table 4.5 gives a general impression of the larger differences between the samples. Within each chemical group, the sum consists of a large number of dissimilar compounds, so that the values cannot be used for detailed interpretation.

Table 4.5. Abundances of compounds by chemical group. Percentage of total abundance of quantified fragments. Compounds with abundances of <0.1% included.

	HAL				FAL			
	man1	**man2**	**mai1**	**mai2**	**man1**	**man2**	**mai1**	**mai2**
Acids	0.7	0.0	17.5[7]	4.4	0.2	74.5[3]	34.1[1]	27.0[2]
Aliphatics	3.4	1.6	12.9[6]	1.2	0.1	0.4	2.7	0.6
Aromatics	7.1	8.0	4.0	4.1	6.3	6.9	1.8	2.6
Cyclic compounds	0.1	0.2	0.3	0.6	0.2	0.1	0.2	0.1
Esters	2.6	2.8	0.1	0.0	0.1	0.0	3.7	2.2
Fatty acids	3.0	4.2	6.6	4.0	0.1	0.1	0.1	0.2
Lactones	0.3	0.6	0.7	1.0	0.0	0.0	0.6	0.0
Lignins[4]	60.6	61.7	22.8	36.9	75.4	3.6	44.2	40.0
Sterols	0.9	0.3	0.4	0.1	0.1	0.0	0.2	0.0
N-compounds	3.6	4.5	1.8	1.4	1.1	5.7	0.5	4.2
P-compounds	0.0	0.0	0.0	0.0	0.01	0.0	0.2	0.0
poly-aromatics	0.3	0.2	0.0	0.0	0.0	0.0	0.1	0.3
Phenols	15.0	11.0	9.0	6.2	15.5	6.8	8.3	19.0
Polysaccharides	1.6	3.3	22.6[5]	39.1[5]	0.7	1.5	3.3	3.1
Terpenoids	0.8	1.7	1.3	0.9	0.1	0.1	0.0	0.1
Unknown	0.1	0.0	0.0	0.1	0.0	0.2	0.1	0.5
Total	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

Dominant compounds indicated as follows:
[1] 4-hydroxy benzene propanoic acid and vanillic acid
[2] benzene acetic acid + 4-hydroxy benzoic acid
[3] benzene acetic acid, cyclohexane carboxylic acid, benzoic acid and benzene propanoic acid
[4] 4-vinyl phenol and 4-vinyl guaiacol
[5] levoglucosan
[6] C_{21} alkene
[7] 9,12-octadecadienoic acid

Table 4.5 indicates that the acid group is especially important in the FAL, even though one sample shows a low abundance (see discussion of acids, above). Aliphatics are unimportant in general, with exception of one maize HAL. Aromatics do not stand out either, and differences between samples are minor. Cyclic compounds, sterols and lactones are unimportant and show little variation between samples. Esters are variable without specific pattern. Fatty acids are better represented in the HAL than in FAL.

Lignin is the predominant group in all samples except in the manure FAL, which is dominated by acids. Most samples contain fair amounts of phenols. Only the maize HAL has a fair amount of polysaccharides. Terpenoids are more abundant in HAL than FAL.

4.3.5.3 Factor analysis of quantified compounds

Because compounds that were found in only one sample are not suitable for factor analysis, the number of compounds was reduced to 141. The first two factors explain, respectively, 35.0 and 17.3% of the total variation of chemical compounds. Figure 4.6 gives the factor loadings, i.e. the contribution of each compound to the first two factors, and Figure 4.7, the factor scores, or the projection of the composition of each sample in the same factor space. In the following, compounds with loadings smaller than 0.5 will not be discussed, because they contribute little to the differentiation between the samples.

Figure 4.6 shows that the acids form two clusters, one in the upper right quadrant and one in the lower right. The former cluster contains the benzene acids (Ac3-5) and cyclohexane carboxylic acid (Ac2). The maize-derived octadecadienoic acid (Ac14) plots close to the centre of the diagram, while the other acids plot in the lower right hand corner.

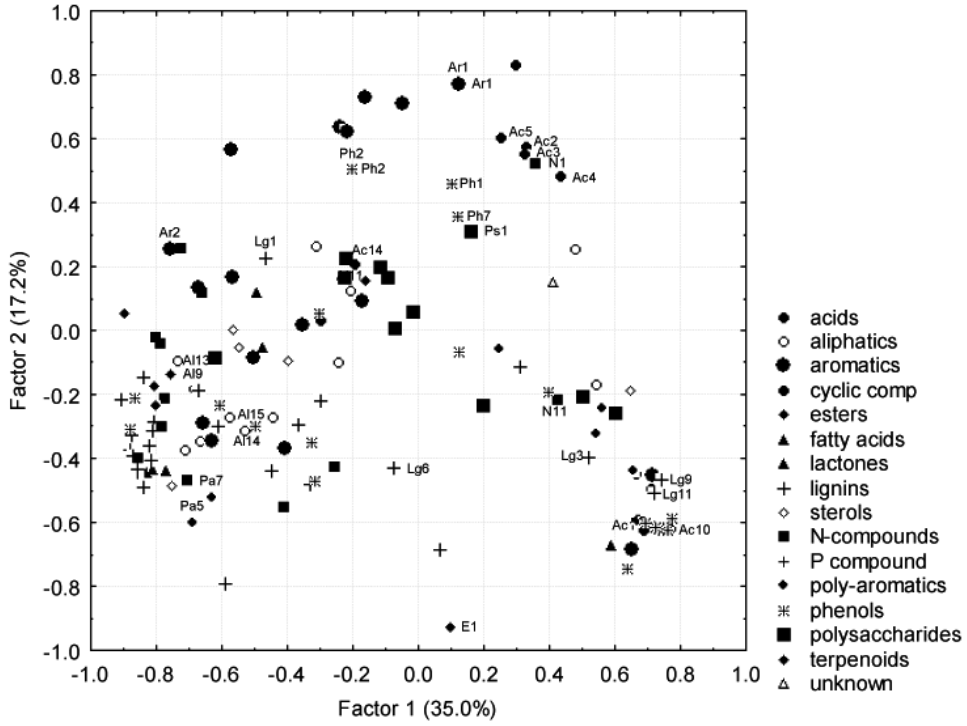

Figure 4.6. Loadings of pyrolytic compounds on Factor 1 and Factor 2.

The aliphatic compounds plot largely in the lower left quadrant. The maize-marker C_{21} alkene (Al11) plots close to Ac14 (see above), while the long-chain alkanes (Al13-15) and pristene (Al9) plot in the lower left quadrant, close to a lignin cluster.

The aromatics are split up into three groups. A number of benzene compounds, including benzene (Ar1) and xylenes, plot at top centre. Toluene (Ar2) plots in the upper left quadrant, while most other compounds plot with the lignins in the lower left quadrant. Two compounds plot in the lower right corner. The polyaromatics fall apart into two clusters, one with the acids in the lower right quadrant, while those of uncertain identity (Pa5, 7) plot in the lower left quadrant.

Lignins plot largely in two clusters, one in the lower left quadrant and one in the lower right quadrant. The lignin dimers (Lg27, 28) and the lignin ester (E1) plot toward bottom centre of the diagram and indicate relatively fresh lignin. The most degraded lignin compound, guaiacol (Lg1) plots in the upper left quadrant. There is no clear separation of the guaiacyl and syringyl units, but the phenol-

based lignin moieties (Lg3, 6, 11) plot more to the right of the diagram. Vanillin (Lg9) plots close to vanillic acid (Ac10) in the lower right quadrant.

The phenols plot in three broad areas. Phenol itself (Ph1), together with Ph2 and Ph7 plot at top centre, below the benzene cluster. A number of unidentified diphenyl and phenol compounds plots in the lower right quadrant, while most of the remainder plot in the lower left quadrant with the lignin cluster.

N compounds, except for pyridine (N1) and an aminopropanoic acid (N11) cluster largely in the left hand part of the diagram, while sterols are scattered.

Polysaccharides, finally, are largely concentrated in the centre of the diagram, terpenoids and sterols in the left hand part.

The factor scores of Figure 4.7 clearly separate the FAL, in the right half of the diagram from the HAL in the left half. While the repetitions of the HAL samples plot very close together in factor space, this is less the case for the FAL, especially those of maize. This suggests that FAL vary more over time than HAL do. Some of the variability can be understood by considering the structural formulas of the acid and lignin compounds. The FALman1 contains 75% of lignin fragments while the FALman2 contains 75% of acids. The dominant acids in this sample are all lignin-derived. Degradation of the acids would lead to intermediary degradation compounds such as vinyl phenol and vinyl guaiacol. This discrepancy would disappear when the lignin-derived acids were grouped with the lignins, but because the acid fraction is very important in these samples, we chose differently. Ralph and Hatfield (1991) mention that lignin-derived acids are not always visible in repeated pyrograms of the same material, and this may also be a reason for variation between samples that were measured a year apart. The fact that both manure FAL plot in the same area in Figure 4.7 indicates that they still are considerably similar.

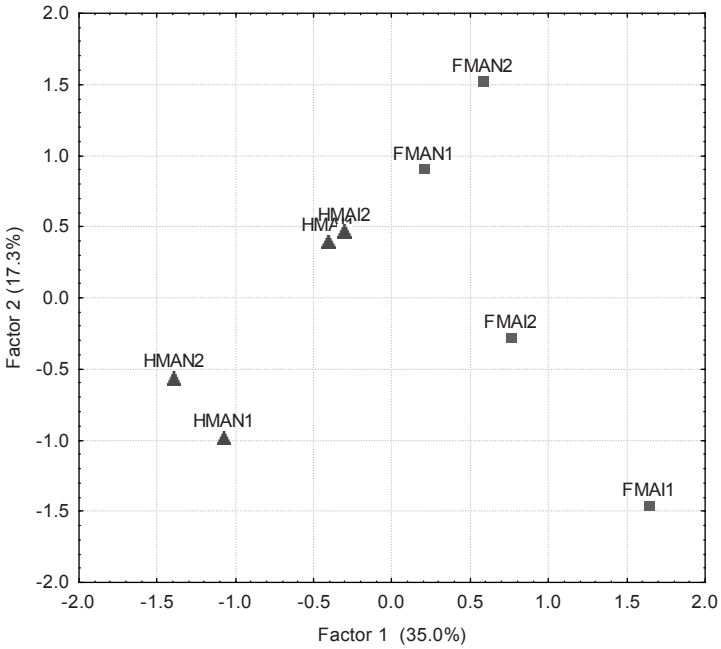

Figure 4.7. Scores of HAL (squares) and FAL (triangles) samples in F1-F2 factor space.

Combining the information of Figures 4.6 and 4.7, we conclude that HAL samples in general have larger amounts of aliphatics, N-compounds and lignin than FAL samples. The HAL from maize contains more polysaccharides and maize-specific compounds (Ac14, Al11) than that from manure, which is richer in lignin, N-compounds, and aliphatics. This suggests that the maize-HAL is less decomposed than manure-HAL. Upon decomposition, plant-derived polysaccharides are consumed and (microbial) N-compounds are enriched. The fact that toluene (Ar2) plots close to the N-compounds suggests that both are derived from microbial proteins. These results are somewhat contradictory to the lignin contents of Table 4.4. One should keep in mind, however, that the calculations of Table 4.4 are based on the assumption that all organic matter is plant-derived and contains cellulose and lignin. This causes errors in samples that have a large component of microbial organic matter.

FAL from manure are characterized by acid moieties of the upper right quadrant: cyclohexane carboxylic acid, benzoic acid, benzene acetic acid and benzene propanoic acid. The maize FAL are dominated by acid of the lower right quadrant: crotonic acid (Ac1), and lignin-derived acids with an OH group. Because the source of both groups, excepting the crotonic acid, is the same, the acids of

the upper right quadrant, lacking the phenolic OH group, should be considered more degraded.

FAL have a higher abundance of acid compounds than HAL, but the composition of FAL does appear to vary according to the season in which it is sampled or, as mentioned before, the pyrolytic yield of acids has been different for the two sets of samples.

The top of Figure 4.6 appears to indicate more strongly decomposed material, both by the presence of Acetic acid (Ps1), benzene (Ar1), and phenol (Ph1). This suggests that the manure FAL, plotting at the top of the diagram, have lost part of their acidity, together with lignin in general, lignin dimers, and diphenyl compounds.

The main difference between the HAL and FAL is in the high contents of organic acids of the latter. Both the HAL and the FAL of maize and manure are essentially different in chemical composition. The HAL retain more of the recalcitrant plant-derived fraction, while the FAL contain a larger amount of microbially derived proteinaceous material. Both the HAL and FAL of the manure are more degraded than their maize-derived equivalents.

4.3.6 Comparison of Pyrolysis-GC/MS with 13C CPMAS NMR

To compare results of pyrolysis-GC/MS with those of ^{13}C CPMAS NMR, a number of data transformations have to be carried out (e.g. Nelson and Baldock, 2005; Kaal et al., 2007). Although the results of both methods do show a close agreement, the limitations of both methods strongly restrict direct comparison of results. Nevertheless, some conclusions can be drawn.

Both pyrolysis-GC/MS and ^{13}C CPMAS NMR showed that HAL from maize has larger polysaccharide contents than that from manure. Similarly, the HAL from manure is richer in lignin than that from maize. The amount of carboxyl groups as determined by ^{13}C CPMAS NMR is not consistently reflected in the pyrolysis data. In three FAL samples, the abundance of acidic compounds is in fair agreement with the NMR data, but not in the fourth sample. A possible reason for this has been mentioned in the foregoing. In HAL samples, the abundance of acidic compounds as indicated by pyrolysis-GC/MS is systematically lower than indicated by NMR. As mentioned above, pyrolysis-GC/MS (without methylation or silylation) may not always give consistent information about acids, while Ritchie and Perdue (2008) mentioned that acidity determined by titration only addresses about 70% of the carboxylic groups indicated by NMR.

4.4 Conclusions

The silage maize, used in this study, has more HAL and FAL than the fresh cow manure. The maize yields might vary with harvest time and maize growth location.

HAL and FAL from silage maize and fresh cow manure have similar carbon and hydrogen contents, whereas the oxygen contents for the FAL are slightly higher than for the HAL. Nitrogen in manure samples was higher than in maize ones, due to the higher protein content of manure.

FAL from silage maize and fresh cow manure are more acidic than HAL from the same source. Moreover, HAL and FAL from fresh cow manure are more acidic than HAL and FAL from silage maize. FAL, from manure and maize, have more phenolic groups than HAL. This is also verified for the carboxylic groups.

HAL from silage maize have higher polysaccharides than HAL from fresh cow manure. On the other hand, HAL from fresh cow manure are richer in lignin than HAL from silage maize. These results were verified by both Pyrolysis-GC/MS and ^{13}C CPMAS NMR.

FAL from silage maize and fresh cow manure are high in lignin and acids, however the duplicates show bigger differences in chemical composition than HAL. These results were verified by Pyrolysis-GC/MS and ^{13}C CPMAS NMR.

HAL retain more of the recalcitrant plant-derived fraction, while FAL contain a larger amount of microbially derived proteinaceous material. Both HAL and FAL from fresh cow manure are more degraded than HAL and FAL from silage maize.

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Appendix

List of quantified pyrolytic compounds with codes and contents per sample.

Name	Grp	M[a]	M/z[b]	RT[c] (min)	FALmai1	FALmai2	FALman1	FALman2	HALmai1	HALmai2	HALman1	HALman2
Acids												
crotonic acid (prop-2-ene acid)	Ac1	86	68+86	6.392	1.44	0.07	0.00	0.00	0.03	0.00	0.00	0.00
cyclohexanecarboxylic acid	Ac2	128	55+73	13.643	0.00	5.83	0.00	18.70	0.06	0.00	0.00	0.00
Benzoic acic	Ac3	122	77+105	14.477	1.82	0.54	0.00	16.55	0.00	0.00	0.00	0.00
Benzeneacetic acid	Ac4	136	91+136	16.160	2.32	14.02	0.00	26.98	0.00	0.00	0.00	0.00
Benzenepropanoic acid	Ac5	150	91+104	17.637	0.18	0.41	0.00	12.32	0.08	0.00	0.09	0.00
benzenepropanoic acid, 4-hydroxy	Ac7	166	91+148	21.210	3.94	0.00	0.00	0.00	0.02	0.00	0.00	0.00
Benzoic acid, 4-hydroxy	Ac8	138	121+138	21.460	2.45	3.01	0.00	0.00	0.00	0.00	0.00	0.00
4-hydroxy-3methoxy benzoic acid (vanillic acid)	Ac10	168	153+168	22.621	4.45	1.10	0.03	0.00	0.06	0.00	0.00	0.00
benzenepropanoic acid, 4-hydroxy	Ac11	166	107+166	23.844	16.53	1.45	0.00	0.00	0.00	0.00	0.62	0.00
Syringic acid	Ac12	198	183+198	26.390	0.16	0.24	0.00	0.00	0.02	0.00	0.00	0.00
4-acetoxy-3-methoxycinnamic acid	Ac13	236	133+194	26.994	0.84	0.17	0.00	0.00	0.02	0.00	0.00	0.00
9-12-octadecadienoic acid	Ac14	280	67 81	30.927	0.02	0.04	0.00	0.00	16.78	4.45	0.00	0.00
9,12-octadecadienoic acid	Ac15	280	67+81	31.869	0.00	0.00	0.00	0.00	0.41	0.00	0.00	0.00
Benzoic acid, 2-(4-methylphenoxy)	Ac16	228	212+228	31.961	0.00	0.07	0.00	0.00	0.00	0.00	0.00	0.00
Alkanes, alkenes and alcohols												
1,3-butanediol	Al1	90	45+57	7.809	0.52	0.20	0.00	0.00	0.00	0.00	0.00	0.00

List of quantified pyrolytic compounds with codes and contents per sample (continued).

Name	Grp	M[a]	M/z[b]	RT[c] (min)	FALmai1	FALmai2	FALman1	FALman2	HALmai1	HALmai2	HALman1	HALman2
Alkene	Al2		55+69	8.204	0.02	0.02	0.01	0.02	0.01	0.00	0.03	0.00
ether/alcohol	Al3		57+85	8.587	0.39	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1-octanol, dimethyl	Al4		55+56	10.104	0.18	0.00	0.00	0.31	0.09	0.00	0.05	0.00
C9 alkene	Al5	126	55+69	12.482	0.02	0.00	0.00	0.00	0.13	0.00	0.06	0.00
1-octen-4-ol	Al6		69+87	13.446	1.47	0.00	0.00	0.00	0.00	0.00	0.00	0.00
alkene	Al7		55+69	22.752	0.00	0.07	0.00	0.00	0.04	0.03	0.06	0.21
alkyne	Al8		55+67	23.094	0.00	0.08	0.00	0.00	0.00	0.00	0.00	0.00
pristene	Al9		56+57	25.010	0.02	0.00	0.00	0.00	0.32	0.15	0.23	0.15
C 19 alkene	Al10		55+69	28.094	0.03	0.04	0.00	0.00	0.12	0.00	0.36	1.07
C21 alkene	Al11	280	55+69	30.978	0.04	0.08	0.00	0.00	11.33	0.44	1.05	0.03
branched alkene	Al12	280	83	33.444	0.00	0.00	0.02	0.01	0.23	0.19	0.02	0.03
C 29 alkane	Al13		57+71	37.544	0.00	0.01	0.03	0.02	0.11	0.07	0.13	0.05
C31 alkane	Al14		57+71	39.561	0.00	0.02	0.04	0.00	0.17	0.16	0.64	0.02
C33 alkane	Al15		57+71	41.587	0.00	0.01	0.02	0.01	0.25	0.19	0.57	0.04
alkane	Al16		57+71	44.029	0.00	0.01	0.00	0.03	0.08	0.00	0.22	0.00
Aromatics												
benzene	Ar1	78	77+78	3.326	0.06	0.30	0.32	0.94	0.26	0.20	0.08	0.32
Toluene	Ar2	92	91+92	4.955	0.24	0.98	4.01	3.99	1.68	2.71	4.65	5.46
ethylbenzene / xylene	Ar3	106	91+106	6.926	0.25	0.16	0.49	0.58	0.53	0.24	0.32	0.42
1,2/1,4 dimethylbenzene	Ar4	106	91+106	7.113	0.03	0.26	0.20	0.40	0.17	0.21	0.19	0.71
styrene	Ar5	104	78+104	7.543	0.15	0.15	0.70	0.53	0.29	0.21	0.26	0.46

List of quantified pyrolytic compounds with codes and contents per sample (continued).

Name	Grp	M[a]	M/z[b]	RT[c] (min)	FALmai1	FALmai2	FALman1	FALman2	HALmai1	HALmai2	HALman1	HALman2
ethylbenzene, p-xylene	Ar6		91+106	7.651	0.01	0.08	0.09	0.13	0.04	0.04	0.05	0.07
C3 benzene	Ar7		105+120	8.401	0.01	0.01	0.01	0.00	0.03	0.00	0.02	0.09
Benzaldehyde	Ar8	106	77+106	8.926	0.39	0.00	0.06	0.08	0.04	0.00	0.04	0.00
propylbenzene	Ar9	120	91+120	9.109	0.02	0.03	0.07	0.12	0.12	0.06	0.07	0.03
trimethylbenzene	Ar10		105+120	9.309	0.01	0.08	0.10	0.00	0.04	0.06	0.05	0.07
trimethylbenzene	Ar11		105+120	10.080	0.01	0.07	0.03	0.00	0.03	0.04	0.05	0.13
Benzene, 1 methoxy 4,3,2methyl	Ar12	122	77+122	10.543	0.01	0.03	0.10	0.02	0.06	0.06	0.10	0.05
trimethylbenzene	Ar13		105+120	10.776	0.50	0.09	0.06	0.00	0.04	0.04	0.09	0.03
benzenepropanol acetate	Ar14	178	117+118	10.782	0.01	0.03	0.09	0.10	0.05	0.05	0.08	0.08
benzeneacetaldehyde	Ar15	120	91+120	10.893	0.00	0.08	0.00	0.00	0.07	0.09	0.00	0.04
benzaldehyde, 2-hydroxy	Ar16	122	121+122	10.935	0.02	0.02	0.00	0.00	0.06	0.15	0.05	0.07
1,2/3/4/-benzenediol, 2/3/4-methyl	Ar17	124	78+124	16.778	0.04	0.22	0.00	0.00	0.47	0.00	0.97	0.00
Cyclic compounds												
cyclopropyl methyl carbinol	C1	86	58+71	3.119	0.13	0.04	0.04	0.03	0.26	0.53	0.08	0.19
cyclopentanol, 2/3 methyl	C2	100	56+57	5.422	0.04	0.02	0.13	0.11	0.07	0.10	0.00	0.00
Esters												
guaiacol acetic acid, methyl ester	E1	196	137+196	25.110	3.70	2.21	0.12	0.00	0.00	0.00	2.62	2.81
octadecenoic acid, methyl ester	E2	296	55+74	30.294	0.00	0.00	0.00	0.00	0.06	0.00	0.00	0.00

List of quantified pyrolytic compounds with codes and contents per sample (continued).

Name	Grp	M[a]	M/z[b]	RT[c] (min)	FALmai1	FALmai2	FALman1	FALman2	HALmai1	HALmai2	HALman1	HALman2
trienoic acid methyl ester / alkatrienol	E3		55+59	33.011	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Fatty acids												
FA (C14)	F14	228	60+73	25.311	0.09	0.02	0.01	0.04	0.11	0.16	0.29	0.55
C15 iso FA	F15i		60+73	26.319	0.00	0.02	0.00	0.00	0.05	0.02	0.14	0.20
C15 ante-iso FA	F15 a		60+73	26.465	0.00	0.01	0.00	0.00	0.05	0.02	0.21	0.30
C16 FA	F16	256	60+73	28.398	0.03	0.10	0.13	0.08	5.93	3.41	1.06	2.16
FA (C18)	F18	284	60+73	31.180	0.02	0.07	0.00	0.00	0.51	0.37	1.32	0.95
Lactones												
3-hydroxy-2-penteno-1,5-lactone	L1	114	58+114	9.789	0.22	0.04	0.00	0.00	0.74	0.98	0.23	0.57
Gamma dodecalactone	L2	198	55+85	30.044	0.34	0.00	0.00	0.00	0.00	0.00	0.07	0.00
Lignins												
2-methoxyphenol (guaiacol)	Lg1	124	109+124	12.093	0.84	1.14	8.20	1.34	2.24	3.44	3.99	4.59
4-methylguaiacol (2-methoxy-4-methylphenol)	Lg2	138	123+138	14.510	0.25	0.76	0.48	0.28	0.68	2.25	3.24	5.64
4-Vinylphenol	Lg3	120	91+120	15.126	23.57	23.16	25.03	0.48	6.29	9.23	11.30	7.33
4-Ethylguaiacol	Lg4	152	137+152	16.426	0.71	1.53	2.39	0.30	0.51	1.13	2.29	3.03
4-Vinylguaiacol	Lg5	150	135+150	17.181	14.91	8.79	36.10	0.77	7.58	12.20	23.90	21.30
4-(2-propenyl)phenol	Lg6	134	133+134	17.677	0.10	0.20	0.15	0.00	0.11	0.00	0.34	0.00
Syringol, (2,6-dimethoxyphenol)	Lg7	154	139+154	17.720	0.14	0.20	1.28	0.18	1.06	1.79	2.63	2.32

List of quantified pyrolytic compounds with codes and contents per sample (continued).

Name	Grp	M[a]	M/z[b]	RT[c] (min)	FALmai1	FALmai2	FALman1	FALman2	HALmai1	HALmai2	HALman1	HALman2
4-(1-Propenyl)guaiacol (eugenol)	Lg8	164	77+164	18.016	0.01	0.04	0.07	0.00	0.13	0.15	0.46	0.25
4-formylguaiacol, vanillin	Lg9	152	151+152	18.710	0.82	0.13	0.04	0.16	0.21	0.00	0.00	0.03
cis 4-(prop-1-enyl) guaiacol	Lg10		149 164	19.043	0.01	0.02	0.24	0.00	0.08	0.05	0.31	0.18
4-acetylphenol	Lg11	136	93+121	19.560	0.20	0.23	0.00	0.00	0.00	0.00	0.00	0.00
4-Methylsyringol	Lg12	168	153+168	19.656	0.04	0.11	0.15	0.04	0.27	1.32	1.22	2.61
trans 4-(prop-1-enyl) guaiacol	Lg13		149 164	19.848	0.05	0.09	0.11	0.00	0.45	0.67	2.08	1.88
4-Acetylguaiacol	Lg14	166	151+166	20.410	0.26	0.20	0.17	0.00	0.20	0.20	0.43	0.42
ethyl syringol	Lg14a	182	167+182	21.152	0.04	0.08	0.20	0.00	0.13	0.00	0.53	0.00
4-(propan-2-one)guaiacol, guaiacylacetone	Lg15	180	137+180	21.188	0.88	0.14	0.09	0.00	0.23	0.25	0.33	3.04
4-vinylsyringol	Lg16	180	165+180	21.858	0.19	0.23	0.47	0.00	0.84	1.85	2.12	3.14
4-(prop-2-enyl)syringol	Lg17		91+194	22.513	0.00	0.02	0.00	0.00	0.14	0.27	0.44	0.58
4-propylsyringol	Lg18	196	167+196	22.669	0.00	0.02	0.02	0.00	0.03	0.10	0.12	0.27
4-(prop-2-enyl)syringol	Lg20	194	91+194	23.367	0.00	0.05	0.00	0.00	0.09	0.13	0.37	0.47
4-(prop-1-enyl) syringol, cis	Lg21	194	91+194	24.202	0.00	0.09	0.12	0.02	0.57	1.06	2.41	3.26
4-(prop-1-enyl)syringol, trans	Lg22	194	91+194	24.231	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Acetylsyringone	Lg23	196	181+196	24.710	0.11	0.39	0.18	0.00	0.34	0.53	1.15	1.11
4-(3-hydroxy-1-propenyl)-guaiacol, coniferyl alcohol	Lg24	180	137+180	24.877	0.03	1.91	0.00	0.00	0.12	0.00	0.00	0.00
4-(propan-2-one)syringol	Lg25	210	167+210	25.181	0.01	0.09	0.08	0.00	0.18	0.24	0.19	0.20

List of quantified pyrolytic compounds with codes and contents per sample (continued).

Name	Grp	M[a]	M/z[b]	RT[c] (min)	FALmai1	FALmai2	FALman1	FALman2	HALmai1	HALmai2	HALman1	HALman2
prop-2-enyl syringol	Lg26	194	179+194	27.449	0.83	0.17	0.00	0.00	0.00	0.00	0.00	0.00
alpha-beta-diguaiacylacylethene	Lg27	272	211+272	37.211	0.08	0.10	0.00	0.00	0.16	0.00	0.53	0.00
guaiacyl, syringylethene	Lg28	302	302+303	39.845	0.09	0.10	0.00	0.00	0.07	0.00	0.16	0.00
Sterols												
lignin dimer / lipid (300 alone)	St1		161+300	36.095	0.09	0.03	0.09	0.00	0.00	0.00	0.00	0.00
161 253 285 300 lipid	St2		161+300	36.894	0.12	0.02	0.00	0.00	0.00	0.00	0.00	0.00
cholest-3-ene compound	St3		215+370	39.261	0.00	0.00	0.00	0.00	0.00	0.00	0.06	0.00
ergost-8(14)-ewne compound	St4	384	215+384	40.345	0.00	0.00	0.00	0.00	0.00	0.00	0.08	0.00
Lipid	St5		215+398	41.328	0.00	0.00	0.00	0.00	0.00	0.00	0.25	0.22
cholest-5-ene compound	St6		105+396	42.120	0.00	0.00	0.00	0.00	0.04	0.00	0.00	0.04
cholestan-3-ol compound	St7		215+388	42.311	0.00	0.00	0.00	0.00	0.00	0.00	0.13	0.00
cholest-5-ene compound	St8		147+396	42.584	0.00	0.00	0.00	0.00	0.20	0.12	0.11	0.05
Lipid	St9		55+215	43.761	0.00	0.00	0.00	0.00	0.00	0.00	0.23	0.00
ergost-5-en-3-ol comp	St10	400	213+400	44.254	0.00	0.00	0.00	0.00	0.03	0.00	0.02	0.00
stigmasterol	St11	412	55 83	44.678	0.00	0.00	0.00	0.00	0.08	0.00	0.00	0.00
N-compounds												
Pyridine	N1	79	52+79	4.480	0.39	2.85	0.46	5.05	0.26	0.47	0.27	0.70
1H-pyrrole, 2 methyl	N2		80 81	6.148	0.00	0.09	0.12	0.08	0.17	0.10	0.12	0.37
1H pyrrole, 3 methyl	N3		81 81	6.414	0.00	0.07	0.12	0.10	0.13	0.21	0.15	0.51
dimethylpyrrole	N4	95	94+95	8.065	0.00	0.03	0.11	0.00	0.04	0.07	0.05	0.10

List of quantified pyrolytic compounds with codes and contents per sample (continued).

Name	Grp	M[a]	M/z[b]	RT[c] (min)	FALmai1	FALmai2	FALman1	FALman2	HALmai1	HALmai2	HALman1	HALman2
pyridine, 2,x-dimethyl	N5	107	106+107	8.293	0.00	0.00	0.02	0.00	0.07	0.00	0.04	0.11
Benzeneacetonitrile	N6	117	90+117	12.818	0.00	0.04	0.10	0.35	0.35	0.20	0.40	0.29
indole	N7	117	90+117	16.613	0.00	0.14	0.16	0.09	0.46	0.29	1.52	1.97
2H-indol-2-one, 1,3-dihydro	N8	133	104+133	20.127	0.00	0.29	0.00	0.00	0.05	0.00	0.53	0.00
2,6-dimethylphenyl isocyanate	N9	147	118+147	20.477	0.03	0.24	0.00	0.00	0.05	0.05	0.23	0.17
diketodipyrrole	N10	186	93+186	24.469	0.01	0.03	0.00	0.00	0.08	0.00	0.16	0.08
N-(2-hydroxy-3,5-dimethylbenzyl)-beta-aminopropanoic acid	N11	223	134+163	27.569	0.06	0.37	0.00	0.00	0.00	0.00	0.00	0.00
alkylamide	N12		59+72	33.578	0.00	0.01	0.00	0.00	0.15	0.00	0.11	0.21
Phosphate												
chromium phosphate compound	P		176+300	36.000	0.16	0.03	0.00	0.00	0.00	0.00	0.00	0.00
Poly-aromatics												
polyaromatic	Pa1	164	107+122	18.943	0.00	0.03	0.00	0.00	0.01	0.00	0.00	0.00
1H-inden-1-one, 2,3,-dihydro-3,3,5,7-tetramethyl	Pa2	188	173+188	22.735	0.02	0.05	0.01	0.00	0.00	0.00	0.00	0.00
1-indanone, 3,3,4,5,7-pentamethyl	Pa3	202	187+202	24.027	0.02	0.06	0.00	0.00	0.00	0.00	0.00	0.00
1,4-naphthalenediol	Pa4	160	104+160	26.577	0.06	0.09	0.00	0.00	0.00	0.00	0.00	0.00
polyaromatic	Pa5	260	229 260	32.522	0.02	0.02	0.00	0.00	0.02	0.00	0.11	0.10
2-phenanthrylamine, 9,10-dihydro-3-nitro	Pa6	240	165+240	33.644	0.00	0.07	0.00	0.00	0.00	0.00	0.00	0.00

List of quantified pyrolytic compounds with codes and contents per sample (continued).

Name	Grp	M[a]	M/z[b]	RT[c] (min)	FALmai1	FALmai2	FALman1	FALman2	HALmai1	HALmai2	HALman1	HALman2
trimetroprim	Pa7	290	259+290	35.261	0.01	0.01	0.00	0.00	0.02	0.00	0.22	0.09
Phenols												
Phenol	Ph1	94	66+94	9.639	1.62	4.60	7.23	4.10	2.02	1.64	3.07	2.87
2-Methylphenol 77	Ph2	108	107+108	11.338	0.05	0.28	0.80	0.27	0.32	0.22	0.32	0.27
3/4-methylphenol	Ph3	108	107+108	11.845	0.69	4.47	2.50	1.56	1.65	2.09	3.66	5.44
dimethylphenol	Ph4	122	107+122	13.515	0.03	0.27	0.20	0.15	0.22	0.19	0.40	0.51
3/4-ethylphenol	Ph5	122	107+122	13.937	2.17	5.90	3.44	0.56	0.79	0.75	1.63	1.21
1,2 benzenediol catechol	Ph6	110	64+110	14.876	0.32	0.70	0.00	0.00	1.71	0.00	2.16	0.00
methyl-ethylphenol	Ph7	136	121+136	15.306	0.12	0.14	1.19	0.07	0.06	0.17	0.08	0.06
methyl-ethylphenol	Ph8	136	121+136	15.537	0.03	0.11	0.18	0.00	0.08	0.16	0.16	0.04
2-methoxy-1,4-benzenediol	Ph9	140	125+140	16.085	0.05	0.14	0.00	0.00	0.67	0.53	1.57	0.00
benzaldehyde, 4-hydroxy	Ph10	122	121+122	18.082	0.42	0.15	0.00	0.00	0.01	0.05	0.13	0.00
3,4-dimethoxyphenol	Ph11	154	139+154	18.143	0.00	0.04	0.00	0.08	0.14	0.34	0.52	0.56
1,3-benzenediol, 4-ethyl	Ph12	138	123+138	18.652	0.21	0.27	0.00	0.00	0.48	0.05	1.13	0.00
Phenol compound	Ph13	214	107 214	30.922	0.00	1.31	0.00	0.00	0.84	0.00	0.20	0.00
Phenol compound	Ph14	242	107+121	33.244	0.30	0.20	0.00	0.00	0.03	0.00	0.00	0.00
phenol compound	Ph15	240	131+225	34.561	1.20	0.24	0.00	0.00	0.00	0.00	0.00	0.00
diphenyl compound	Ph16	270	255+270	34.953	0.35	0.11	0.00	0.00	0.00	0.00	0.00	0.00
diphenyl compound	Ph17	270	255+270	35.215	0.23	0.05	0.00	0.00	0.00	0.00	0.00	0.00
dipphenyl compound 131 223 255 270	Ph18	270	255+270	35.678	0.11	0.01	0.00	0.00	0.00	0.00	0.00	0.00

List of quantified pyrolytic compounds with codes and contents per sample (continued).

Name	Grp	M[a]	M/z[b]	RT[c] (min)	FALmai1	FALmai2	FALman1	FALman2	HALmai1	HALmai2	HALman1	HALman2
dipphenyl compound 131 223 255 270	Ph19	270	255+270	35.900	0.42	0.07	0.00	0.00	0.00	0.00	0.00	0.00
Polysaccharides												
Acetic acid	Ps1	60	60	2.683	0.19	0.79	0.12	0.63	0.34	0.39	0.01	0.53
2-Methylfuran	Ps2	82	53+82	2.730	0.07	0.31	0.03	0.18	0.23	0.54	0.21	0.67
(2H)-Furan-3-one	Ps3	84	54+84	5.119	0.30	0.23	0.00	0.00	0.64	0.90	0.06	0.16
2-furaldehyde	Ps4	96	95+96	5.872	1.14	0.08	0.04	0.45	0.70	0.14	0.03	0.11
3-furaldehyde	Ps5	96	95+96	6.009	0.71	0.31	0.13	0.22	0.04	0.82	0.10	0.09
5H-furan-2-one	Ps6	84	55+84	7.253	0.00	0.20	0.00	0.00	0.34	0.00	0.02	0.00
2,3-dihydro-5-methylfuran-2-one	Ps7	98	55+98	7.887	0.03	0.19	0.02	0.00	0.40	0.51	0.04	0.00
5-methyl-2 furaldehyde	Ps8	110	109+110	8.859	0.05	0.11	0.00	0.00	0.58	0.56	0.08	0.04
Levoglucosenone	Ps9	126	68+98	12.268	0.08	0.12	0.00	0.00	0.66	0.38	0.00	0.00
benzofyran,2,3 dihydro-2-methyl	Ps10	134	119+134	16.876	0.03	0.46	0.00	0.00	0.03	0.00	0.06	0.07
Levoglucosan	Ps11	162	60+73	21.685	0.73	0.35	0.33	0.00	18.64	34.83	0.93	1.61
Terpenoids												
3,7,11,15,tetramethyl-2-hexadecen-1-ol (ttp)	T1		55+68	26.702	0.00	0.00	0.00	0.00	0.53	0.25	0.26	0.70
tritepenoid 3,7,11,15-tetramethyl-2-hexadecen-1-ol	T2	278	57+81	27.049	0.00	0.00	0.00	0.00	0.07	0.13	0.05	0.19
3,7,11,15-tetramethyl-2-hexaden-1-ol	T3	296	57+82	27.340	0.00	0.00	0.00	0.00	0.23	0.09	0.14	0.45
phytol (TTP)	T4	296	71+123	30.577	0.00	0.00	0.00	0.00	0.11	0.00	0.00	0.00

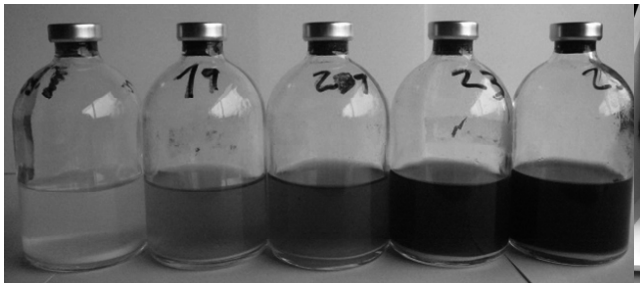
List of quantified pyrolytic compounds with codes and contents per sample (continued).

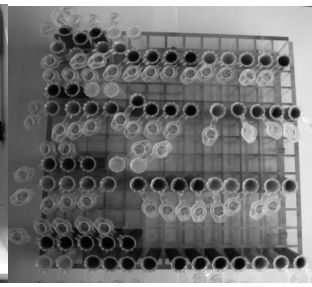
Name	Grp	M[a]	M/z[b]	RT[c] (min)	FALmai1	FALmai2	FALman1	FALman2	HALmai1	HALmai2	HALman1	HALman2
terpenoid	T5		69 81	38.878	0.02	0.13	0.14	0.14	0.36	0.40	0.32	0.40
Unknown												
Unknown	U1	110	54+82	10.293	0.05	0.40	0.01	0.25	0.04	0.07	0.09	0.00
69 82 95 110 167	U2		110+167	27.227	0	0.10	0	0	0	0	0	0
				Sum %	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00

(a) M=molecular mass (Daltons).
(b) M/z=masses used for quantification.
(c) RT=approximate retention time (min).

Chapter 5

Effect of Humic acid-like and Fulvic acid-like on the hydrolysis of cellulose and tributyrin





Abstract

Enzymatic hydrolysis of complex wastes is a critical step for efficient biogas production in anaerobic digesters. Inhibition of this hydrolytic step was studied by addition of humic acid-like (HAL) and fulvic acid-like (FAL), extracted from maize silage and fresh cow manure, to solutions of: cellulose Sigmacell Type 50 and cellulases from Aspergillus Niger; and tributyrin and lipases from Aspergillus Niger. The enzymatic experiments were performed under mesophilic conditions and a pH of about 7. Hydrolysis products, like glucose, glycerol and butyric acid were measured in order to determine the hydrolysis efficiency and its rate. HAL and FAL from maize and cow manure, in the ranges from 0.5 to 5 $g \cdot l^{-1}$, clearly inhibit the hydrolysis of cellulose. The hydrolysis of tributyrin is inhibited by HAL from maize and cow manure, in the ranges from 0.5 to 5 $g \cdot l^{-1}$, however not clearly inhibited by FAL from maize and cow manure. The latter might be due to differences in the HAL and FAL structural characteristics, and the mechanisms involved in the binding of the HAL and FAL to the enzymes.

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5.1 Introduction

The production of biogas by anaerobically digesting organic agricultural and industrial wastes has been applied on a farm scale since the 1980s, especially in northern European countries. However, due to an incomplete understanding of the inhibiting factors concerning anaerobic digestion, the design and operational conditions are still not optimized, which, with the present increasing demand for renewable energy sources, becomes a growing necessity.

Lignocellulosic biomass from agricultural wastes, like harvest residues and animal manure, is considered as an important source for the production of bio-energy, viz. bio-methane, bio-ethanol, and bio-hydrogen (Reith et al., 2003). Together with lipid-rich wastes from the food industry, which have high organic matter content, they are nowadays being co-digested in agricultural biogas plants (Gallert and Winter, 2005).

For the anaerobic digestion of these kind of complex organic wastes, the hydrolysis, which is the conversion of complex polymers, such as carbohydrates, lipids and proteins, into soluble monomers, is considered as the rate limiting step (Pavlostathis and Giraldo-Gomez, 1991). This conversion of biopolymers is catalysed by exoenzymes that are secreted from facultative and obligatory anaerobic acidogenic bacteria (Gallert and Winter, 2005). Consequently, the biogas production depends not only on the biodegradability of the organic waste, i.e., how much methane a certain matter can maximally produce, but also on the hydrolysis rate (El-Mashad, 2003).

Under anaerobic conditions, such as in animal's rumen, many different genera of acidogenic bacteria, such as Fibrobacter, can be found (Insam et al., 2010). Moreover, due to the lignocellulosic diet of the ruminants, the acidogenic bacteria present secrete hydrolytic enzymes capable of metabolizing cellulosic material (Park et al., 2007). Fibrobacter succinogenes is one of the most common rumen bacterium and can grow exclusively on crystalline cellulose (Yoshida et al., 2010). The enzymes secreted by the F. succinogenes hydrolyse cellulose into glucose, which is then converted into acetate and succinate (Nouaille et al., 2009).

HAL and FAL have been reported to have a scavenger effect on anaerobic enzymatic hydrolysis (Brons et al., 1985) similar to the reported inhibiting effect of lignin on cellulases (Bernardez et al., 1993; Palonen and Viikari, 2004), since they share the same complex structure of polyphenolic compounds.

Humic and fulvic acids are products of the biological decay of biota residues and behave like weak acid polyelectrolytes (Stevenson, 1994; Veeken, 1998). They consist of organic acids of low and high molecular weight, i.e. mono, oligo and polysaccharides, proteins, peptides, amino acids, lipids, waxes, polycyclic

hydrocarbons and lignin fragments (Saiz-Jimenez, 1992). They are mostly formed by carbon and oxygen, however they can vary in composition (Schulten and Schnitzer, 1993). Their oxygen containing functional groups are known to be responsible for their reactivity (Stevenson, 1994). They can be extracted from soils and sediments, and, from lignocellulosic biomass, such as animal manure and plant material, due to their physico-chemical characteristics; Humic acids (HA) are soluble above pH 3.5 and fulvic acids (FA) are soluble at all pH values (Chen and Wang, 2007). HA and FA are commonly reported in soil sciences. When extracted from anthropogenic origin matter, such as agricultural wastes, its nomenclature changes to HAL and FAL.

The objective of this study was to determine the inhibiting effect of the HAL and FAL extracted from maize silage and fresh cow manure on carbohydrates and lipids hydrolysis. In order to observe this inhibition, cellulose and tributyrin were used as model substrates.

5.2 Materials and methods

5.2.1 Experimental materials

Fresh cow manure and energy maize silage supplied by Wageningen University experimental farm (Nij Bosma Zathe, The Netherlands), were air-dried (40°C), and ground (≤ 2 mm) by a cutting mill (Retsch Muhle; type sm1). The dried and ground material was further used for HAL and FAL extraction, which was performed according to the method developed by the International Humic Substances Society (IHSS, 2009), with the addition of a few modifications related to the organic nature of the raw material (chaper 4). All extracted HAL and FAL were stored in a desiccator until use. Total COD of the extracted HAL and FAL were 1450 ± 9 $g \cdot kg^{-1}$ for HAL manure, 1950 ± 117 $g \cdot kg^{-1}$ for HAL maize, 1230 ± 11 $g \cdot kg^{-1}$ for FAL manure and 1310 ± 314 $g \cdot kg^{-1}$ for FAL maize. Total COD was titrimetric determined according to standard methods (APHA, 1998).

5.2.2 Enzymatic hydrolysis trials

For all experiments glass bottles were used. Each 100 ml bottle was filled with 0.5, 1.0, 2.5 or 5.0 $g \cdot l^{-1}$ extracted HAL or FAL in 60ml of potassium phosphate buffer (79 mM, pH 7). They were then closed with butyl rubber stoppers and aluminium clamps. HAL and FAL solutions were then sterilized in an autoclave

(Auto Koch, PBI International) at 120°C for 20 minutes and allowed to cool down. Sterilisation of HAL and FAL was necessary to remove microbial contamination of the trials. In order to determine the effect of sterilisation on the HAL structure, freeze dried HAL were sterilised by two methods: the already mentioned autoclave, and Gamma radiation with a dose of 25 $kGy \cdot min^{-1}$ (Isotron, the Netherlands). The structure of the sterilised HAL, in terms of COOH and OH groups, were then compared with the non sterilised HAL by FTIR. One mg of each freeze dried sterilised and non sterilised HAL samples were mixed with 100 mg KBr, making a pellet, and analysed on a FTIR (Tensor 27, Bruker) spectrophotometer.

Meanwhile, the bottles that were not going to have HAL or FAL addition (substrate control), were not sterilised in the autoclave, but left overnight in an 6 M HNO_3 bath, followed by extensive rinsing with Ultra Pure Water (Simplicity 185, Milipore). After drying, they were filled with the potassium phosphate buffer (79 mM, pH 7). An inorganic phosphate buffer was chosen instead of an organic sodium acetate buffer suggested in the Enzymatic Assay of Cellulase (EC 3.2.1.4) (Sigma-Aldrich), in order to avoid biological conversion of the acetate. Each bottle had 10 g $COD \cdot l^{-1}$ of cellulose (Sigmacell Type 50, 50 µm) or tributyrin (Glyceryl tributyrate, Sigma) and 1 $mg \cdot ml^{-1}$ of cellulases from Aspergillus Niger (Sigma) or lipases from Candida rugosa (Sigma), as shown in Table 5.1. The addition of substrate and enzymes was done next to a flame to achieve aseptic conditions. All bottles were then placed in an incubator (Model 420, Thermo Electron Corporation) at 35 ± 2˚C and 150 rpm. Initial conditions of each trial duplicate are shown in Table 5.1. Two types of controls were performed, the above mentioned substrate control, without HAL or FAL addition, to determine the cellulose or tributyrin hydrolysis at the set operational conditions (pH 7 and 35°C); and HM control, without cellulose or tributyrin addition, to determine the hydrolysis of cellulose or tributyrin incorporated in the HAL and FAL. Since the experiments were carried out in eight independent starting times, substrate hydrolysis controls were performed at each of the eight times. All trials were performed in duplicate.

Table 5.1. Experimental set-up for cellulose (C) hydrolysis by cellulases (c) and tributyrin (T) hydrolysis by lipases (L), with addition of HAL and FAL from fresh cow manure and silage maize.

Trials	HAL;FAL ($g \cdot l^{-1}$)	Initial pH	Trials	HAL;FAL ($g \cdot l^{-1}$)	Initial pH
C + c *	—	6.95 (0.00)	**T + L***	—	7.11 (0.00)
C + c + HALmanure	0.50 (0.01)	6.95 (0.00)	**T + L + HALmanure**	0.52 (0.03)	7.13 (0.02)
C + c + HALmanure	1.00 (0.01)	6.95 (0.00)	**T + L + HALmanure**	1.02 (0.03)	7.14 (0.00)
C + c + HALmanure	2.48 (0.02)	6.95 (0.00)	**T + L + HALmanure**	2.48 (0.01)	7.14 (0.00)
C + c + HALmanure	4.93 (0.02)	6.95 (0.00)	**T + L + HALmanure**	4.92 (0.00)	7.11 (0.00)
c + HALmanure	0.51 (0.00)	6.95 (0.00)	**L + HALmanure**	0.50 (0.00)	7.11 (0.01)
c + HALmanure	1.01 (0.01)	6.95 (0.00)	**L + HALmanure**	1.01 (0.00)	7.10 (0.00)
c + HALmanure	2.51 (0.00)	6.95 (0.00)	**L + HALmanure**	2.50 (0.01)	7.11 (0.01)
c + HALmanure	5.00 (0.00)	6.95 (0.00)	**L + HALmanure**	4.98 (0.02)	7.12 (0.00)
C + c *	—	6.97 (0.00)	**T + L ***	—	7.10 (0.00)
C + c + HALmaize	0.50 (0.00)	6.97 (0.00)	**T + L +HALmaize**	0.50 (0.00)	7.10 (0.00)
C + c + HALmaize	1.00 (0.00)	6.97 (0.00)	**T + L + HALmaize**	0.99 (0.00)	7.08 (0.00)
C + c + HALmaize	2.48 (0.01)	6.97 (0.00)	**T + L + HALmaize**	2.47 (0.00)	7.04 (0.00)
C + c + HALmaize	4.91 (0.00)	6.97 (0.00)	**T + L + HALmaize**	4.94 (0.03)	7.00 (0.00)
c + HALmaize	0.51 (0.00)	6.97 (0.00)	**L + HALmaize**	0.50 (0.00)	7.10 (0.00)
c + HALmaize	1.01 (0.00)	6.97 (0.00)	**L + HALmaize**	1.00 (0.00)	7.08 (0.00)
c + HALmaize	2.51 (0.01)	6.97 (0.00)	**L + HALmaize**	2.50 (0.00)	7.04 (0.00)
c + HALmaize	5.01 (0.01)	6.97 (0.00)	**L + HALmaize**	4.98 (0.02)	7.00 (0.00)

* Substrate control.

Data expressed as mean (standard deviation).

Table 5.1. Experimental set-up for cellulose (C) hydrolysis by cellulases (c) and tributyrin (T) hydrolysis by lipases (L), with addition of HAL and FAL from fresh cow manure and silage maize (continued).

Trials	HAL;FAL ($g \cdot l^{-1}$)	Initial pH	Trials	HAL;FAL ($g \cdot l^{-1}$)	Initial pH
C + c *	—	6.97 (0.00)	**T + L ***	—	7.10 (0.00)
C + c + FALmanure	0.52 (0.01)	6.97 (0.00)	**T + L + FALmanure**	0.49 (0.00)	7.10 (0.00)
C + c + FALmanure	0.99 (0.00)	6.97 (0.00)	**T + L + FALmanure**	0.99 (0.00)	7.10 (0.00)
C + c + FALmanure	2.46 (0.00)	6.97 (0.00)	**T + L + FALmanure**	2.46 (0.00)	7.10 (0.00)
C + c + FALmanure	4.93 (0.00)	6.97 (0.00)	**T + L + FALmanure**	4.91 (0.00)	7.10 (0.00)
c + FALmanure	0.533	6.97	**L + FALmanure**	0.50 (0.01)	7.06 (0.00)
c + FALmanure	1.01 (0.00)	6.97 (0.00)	**L + FALmanure**	1.00 (0.00)	7.13 (0.04)
c + FALmanure	2.49 (0.00)	6.97 (0.00)	**L + FALmanure**	2.50 (0.00)	7.10 (0.00)
c + FALmanure	5.01 (0.02)	6.97 (0.00)	**L + FALmanure**	4.97 (0.00)	7.14 (0.01)
C + c *	—	6.98 (0.00)	**T + L ***	—	7.10 (0.01)
C + c + FALmaize	0.50 (0.01)	6.98 (0.00)	**T + L + FALmaize**	0.50 (0.00)	7.09 (0.00)
C + c + FALmaize	0.99 (0.00)	6.98 (0.00)	**T + L + FALmaize**	0.99 (0.00)	7.12 (0.01)
C + c + FALmaize	2.49 (0.01)	6.98 (0.00)	**T + L +FALmaize**	2.47 (0.01)	7.11 (0.00)
C + c + FALmaize	4.94 (0.01)	6.98 (0.00)	**T + L + FALmaize**	4.91 (0.00)	7.14 (0.03)
c + FALmaize	0.51 (0.02)	6.98 (0.00)	**L + FALmaize**	0.51 (0.00)	7.11 (0.00)
c + FALmaize	1.01 (0.02)	6.98 (0.00)	**L + FALmaize**	1.00 (0.00)	7.11 (0.00)
c + FALmaize	2.50 (0.00)	6.98 (0.00)	**L + FALmaize**	2.50 (0.01)	7.11 (0.01)
c + FALmaize	4.99 (0.01)	6.98 (0.00)	**L + FALmaize**	4.98 (0.00)	7.11 (0.01)

* Substrate control.

Data expressed as mean (standard deviation).

5.2.3 Microbially catalysed hydrolysis trials

5.2.3.1 Cultivation and enrichment of acidifiers

Fibrobacter succinogenes was isolated from rumen fluid and anaerobically grown in a basal medium described by Stams et al. (Stams et al., 1993). To enrich this basal medium and enable growth, 100 $mg \cdot l^{-1}$ of yeast, 0.5 $mg \cdot l^{-1}$ of vitamin K, 1 mM of cystein, 2.5 $mg \cdot l^{-1}$ of hematin and 81 mg of cellulose (Sigmacell Type 50, 50 µm) were also added. The isolation and growth of the *Fibrobacter succinogenes* is here shortly described. Five ml of rumen fluid were added to 200 ml of the enriched basal medium. After 2 days of incubation at 30°C, 2 ml of this batch culture were added to 50 ml of the enriched basal medium. After 5 days of incubation at 37°C, serial dilutions up to 10^9 were performed by adding the previously grown culture into 50 ml of the enriched basal medium. The diluted cultures were grown for 7 days at 37°C. After microscope inspection to ensure sufficient bacterial population growth, 0.5 ml of the higher dilution batch was added, together with 50 ml of the enriched basal medium, to the 100 ml batch bottles used for the microbially catalysed enzymatic hydrolysis trials.

5.2.3.2 Batch trials

Each 100 ml batch bottle was then filled with 0.05, 0.5 and 5 $g \cdot l^{-1}$ of HAL extracted from silage maize, except for the substrate control (no HAL). In each batch bottle 81 mg of cellulose (Sigmacell Type 50, 50 µm) (1.92 g $COD \cdot l^{-1}$) was added, except for the HAL control (no substrate). As explained in the enzymatic hydrolysis trials (5.2.2), substrate and HAL controls were performed. All trials were performed in duplicate.

5.2.4 Chemical analysis

Samples were taken at short time intervals so to follow the hydrolysis step. They were directly analyzed by diluting them with 12% H_2SO_4 and centrifuged (Microlite, Thermo IEC) at 10000 rpm for 10 minutes. The supernatant of the centrifuged samples was used for glucose analysis for the trials where cellulose was added, and for glycerol and butyric acid for the trials where tributyrin was added. Glucose was determined by High Performance Liquid Chromatography (HPLC) equipped with an OA-1000 organic acids column (30 cm ID 6.5 mm)

(Alltech, USA) (70°C), a Refractive Index (RI-71) detector and a Gynkotek M480 high precision pump. The mobile phase was 1.25 mmol H_2SO_4 at flow rate of 0.4 ml·min^{-1}. Glycerol and butyric acid were determined by HPLC equipped with a Hi-Plex H column (300 x 6.5 mm) (Varian part nr. 1F70-6830) (70°C), a Hi-Plex H Guard precolumn (Varian part nr. PL1670-0830), a Refractive Index (RI-71) detector and a Gynkotek M480 high precision pump. The mobile phase was 5 mM H_2SO_4 at flow rate of 0.6 ml·min^{-1}.

VFA was also measured to determine if any contamination by acidifiers was taking place during the trials. Samples for VFA analysis were diluted with 3% formic acid and centrifuged (Microlite, Thermo IEC) at 10.000 rpm for 10 minutes. The supernatant was analysed in a Hewlett Packard 5890A gas chromatogram equipped with a 2m × 6 mm × 2 mm glass column packed with Supelco port, 100-120 mesh, coated with 10% Fluorad FC 431. The flow rate of the carrier gas, i.e. nitrogen saturated with formic acid, was 40 ml·min^{-1}, and the column pressure was 3 bar. The temperatures of the column, the injector port, and the flame ionization detector were 130, 200, and 280°C, respectively. pH was measured with a Multi 340i meter and SenTix P14 electrode (WTW, Germany).

Succinate, acetate and propionate, from the microbially catalysed hydrolysis trials, were analysed in a HPLC, and hydrogen and biogas were analysed in a GC as described by Stams et al. (1993).

5.2.5 Calculations

To determine the hydrolysis efficiency (%), as shown in equation 5.1, the hydrolysis products of each substrate were measured during each trial. Glucose was the product of cellulose hydrolysis, while glycerol and butyric acid were the products of tributyrin hydrolysis:

$$Hydrolysis\,(\%) = \frac{\sum COD_{hydrolysis\ products,t=x}}{COD_{total,t=0}} \times 100 \qquad (5.1)$$

Where the $COD_{hydrolysis\ products,t=x}$ was the amount of glucose or glycerol and butyric acid produced during respectively, the cellulose or tributyrin hydrolysis, at t=x, expressed as mg COD·l^{-1}. The $COD_{total,\ t=0}$ was the amount of cellulose or tributyrin added at the beginning of the trial, t=0, also expressed as mg COD·l^{-1}.

The first order hydrolysis constant (k_h) of the cellulose COD was calculated according to Angelidaki and Sanders (Angelidaki and Sanders, 2004) and is here presented as equation 5.2.

$$\frac{COD_{total,t=0} \cdot f_h - \sum COD_{hydrolysis\ products,t=x}}{COD_{total,t=0} \cdot f_h} = e^{-k_h t} \quad (5.2)$$

Where f_h was the maximum biodegradable fraction of substrate (cellulose or tributyrin) measured during the substrate control trials.

5.3 Results and discussion

5.3.1 Enzymatic hydrolysis trials

FTIR spectra of the non-sterilised and sterilised HAL (results not shown) were similar in terms of hydroxyl and carboxyl functional groups, which are known to be the main cause for HAL and FAL reactivity (Stevenson, 1994), indicating that sterilisation does not effect the binding capacity of HAL.

5.3.1.1 Cellulose hydrolysis

Hydrolysis of cellulose without HAL and FAL addition (substrate control) reached a maximum of 6%. Incomplete hydrolysis of Sigmacell type 50 cellulose has been reported (Martins et al., 2008; O'Sullivan et al., 2008), however the reasons for it are not fully understood. Many researches have indentified that the reported delay and incomplete hydrolysis are related to substrate accessibility, crystallinity and degree of polymerization (Zhang and Lynd, 2004). This might also be the case, but the main reasons for such extremely low hydrolysis during our trials, are pH and probably glucose accumulation. The trials were performed at a pH of approximately 7, whereas the optimum pH of cellulases from Aspergillus Niger is 5, therefore decreasing their enzymatic activity. The pH of the trials was set to approximately 7 so to simulate farm-scale anaerobic digester's conditions, which tend to be even slightly alkaline, to enable methanogenic conditions. Previous experiments, not shown here, confirmed that higher hydrolysis of cellulose was reached when the pH was decreased down to 5. The other reason might have been glucose accumulation, which has been reported to inhibit hydrolysis,

therefore repressing cellulase activity (Gallert and Winter, 2005). It has also been reported that the degree of inhibition might vary according to the enzymes used (Holtzapple et al., 1990), however, no study on hydrolysis inhibition due to glucose using the same cellulases and conditions used here has been published, so concentrations could not be compared.

Cellulose was hydrolysed into glucose mostly within the first 24 hours, as shown in Figure 5.1. The error bars indicate the standard deviation between the eight substrate control trials. The average hydrolysis rate found for all eight substrate control trails was higher in the beginning of the run followed by a lower rate after the 24 hours. Results show a five times higher k_h for the first 24 h compared to the whole trial, as shown in Figure 5.1. This might have been due to a decrease in accessibility of the β-glucosidic bonds of cellulose by the cellulases, once the accessible cellulose chain-ends of the 3D structure cellulose particles have been enzymatically attacked (Zhang and Lynd, 2004). It might also be due to other modifications that occur during the saccharification process, as adsorption of inactive cellulase on cellulose's surface, blocking further hydrolysis, and/or innate structural characteristics of the cellulose, as the degree of polymerization and crystallinity. However, for a correct evaluation of these characteristics, better quantitative methods have to be developed (Mansfield et al., 1999).

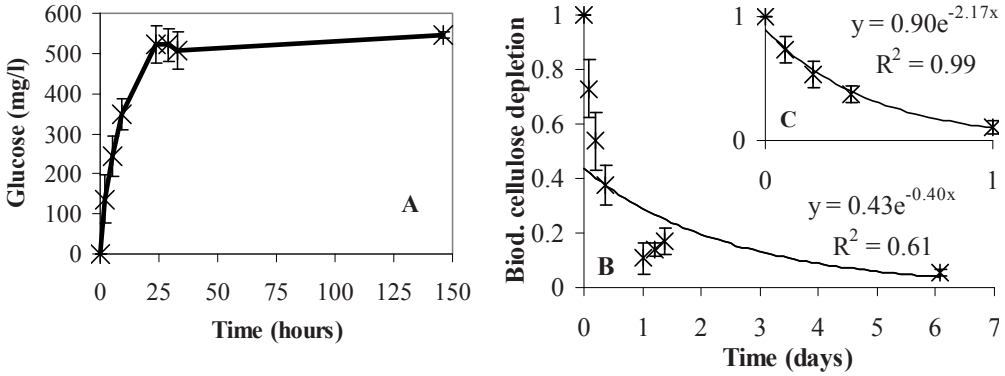

Figure 5.1. Glucose produced during enzymatic hydrolysis of cellulose without HAL and FAL addition (substrate control) (**A**) and biodegradable cellulose depletion during the whole trial (**B**) and during the first 24 h (**C**).

First-order kinetics described the hydrolysis of cellulose in the first 24 h, Figure 5.1 (C), with almost a perfect fit, however this fit was less good for the whole trial, Figure 5.1 (B), showing a R^2 of 0.61. First-order kinetics is commonly

used for anaerobic hydrolysis of organic matter due to the generally good data correlation, even though other kinetics, such as second-order, surface-limiting and Contois, have been reported to better fit a number of substrate, namely at high organic loading rates (Myint and Nirmalakhandan, 2006; Vavilin et al., 2008).

Although cellulose hydrolysis was as low as 6% for the substrate controls, for the trials where HAL and FAL from manure and maize was added, cellulose hydrolysis was almost nonexistent, as shown in Figure 5.2, clearly indicating inhibition of cellulose hydrolysis due to HAL and FAL.

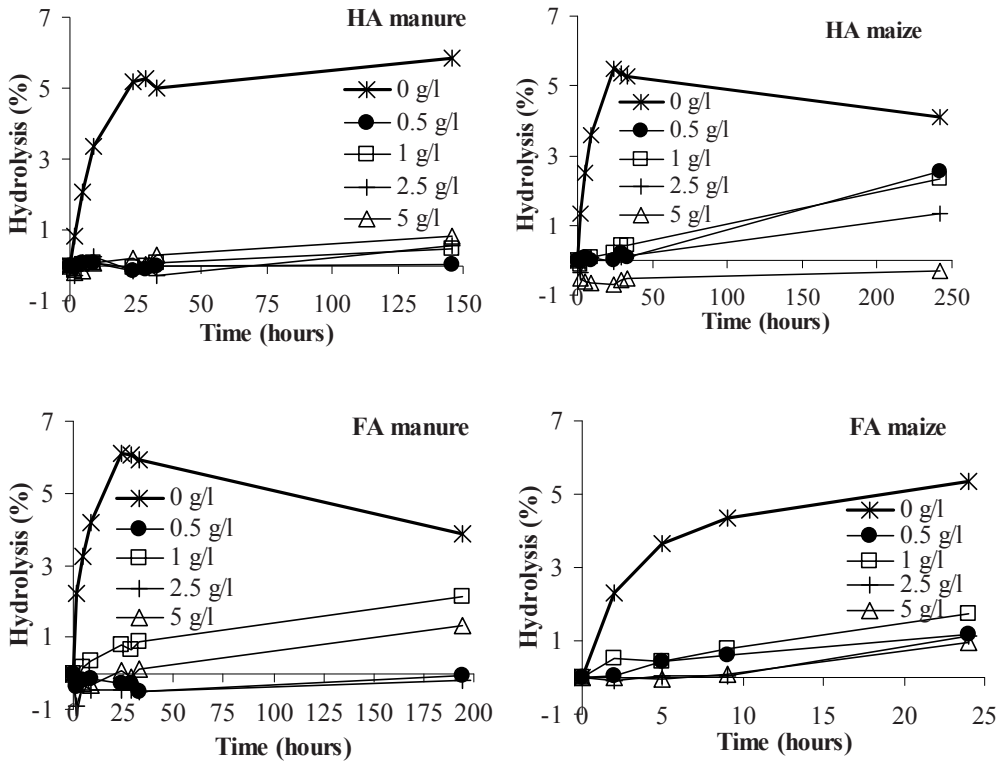

Figure 5.2. Hydrolysis of cellulose without HAL and FAL (×) and with HAL manure, HAL maize, FAL manure and FAL maize at concentrations of 0.5 (●), 1 (□), 2.5 (+) and 5 $g \cdot l^{-1}$ (Δ).

Except for the HAL manure trials, the other trials show a slight increase of hydrolysis at the end of the trials, indicating that the hydrolysis of cellulose might have just been delayed. However, when extrapolating the increasing hydrolysis

lines it is still clear that the hydrolysis rate of cellulose was reduced by HAL and FAL.

We believe that the carboxyl and phenolic hydroxyl functional groups of the HAL and FAL bind to the cellulases, preventing them from hydrolysing the cellulose, as has been reported to happen with lignin compounds and cellulases in cellulose hydrolysis (Pan, 2008), and with soil HAL and proteases on amino-acids hydrolysis (Jorobekova et al., 2005).

The clear inhibiting effect of HAL and FAL on cellulose hydrolysis is supported by the k_h reported for each trial and presented in Table 5.2.

The average substrate controls k_h, as presented in Table 5.2, were 1.67 ± 1.03 d^{-1} for the whole trial and 2.56 ± 1.27 d^{-1} for the first 24 h. The higher value for the former hydrolysis rate constants shows that this step, as mentioned before, occurs almost completely within the first 24 h of exposure. The large standard deviation of each constant indicates that, even though the maximum amount of hydrolysed cellulose is identical in all eight trials, there are differences in its rates, which might be related to the cellulose structural characteristics mentioned above. To make sure that these differences were not just related to operational conditions, pH development and formation of other products were studied. pH data were correlated with glucose production, resulting in no pH influence on cellulose hydrolysis (data not shown). From the collected VFA data, from acetic acid (C2) to caproic acid (C6), only C2 was found in the substrate control trials, representing a maximum of 2% of the total cellulose conversion. Since the C2 concentration throughout the whole trial was identical, the hypothesis of acidifiers contamination was discarded. Moreover, since the amount of C2 found in a cellulase and potassium phosphate buffer solution was only 10% of the maximum C2 found in the substrate controls, it was suggested that the remaining C2 must be some residual amounts present on the used Sigmacell cellulose, however no references were found in this respect.

The k_h data from all the trials with HAL and FAL addition did not provide a negative correlation between the amount of HAL and FAL added and the hydrolysis rate constant, because, even at 0.5 g HAL or FAL·l^{-1}, the inhibiting effect was already overwhelming. The very low amount of cellulose hydrolysed during these trials, especially during the first 24 h, resulted in cellulose depletion curves with poor R^2, as shown in Table 5.2. As mentioned before, since at the end of these trials there seemed to be, in some cases, an increase in cellulose conversion to glucose, the k_h was slightly higher with better R^2, however, far from the results achieved by the substrate controls.

Table 5.2. pH and kh for cellulose hydrolysis trials. Data expressed as mean (standard deviation).

Trials	[HAL;FAL] (g·l^{-1})	pH	Whole trial		First 24 h	
			kh	R2	kh	R2
HAL manure	0	6.91 (0.01)	0.425	0.697	1.798	0.996
	0.5	6.92 (0.01)	0.007	0.792	0.019	0.263
	1	6.90 (0.01)	0.010	0.896	0.001	0.001
	2.5	6.80 (0.01)	0.019	0.903	0.010	0.014
	5	6.70 (0.01)	0.053	0.954	0.048	0.615
HAL maize	0	6.95 (0.00)	1.500	0.922	2.134	0.999
	0.5	6.94 (0.01)	0.053	0.985	0.001	0.001
	1	6.92 (0.01)	0.048	0.994	0.048	0.752
	2.5	6.87 (0.01)	0.026	0.960	0.019	0.086
	5	6.80 (0.01)	0.002	0.064	0.070	0.416
FAL manure	0	6.95 (0.00)	2.916	0.851	4.447	0.980
	0.5	6.97 (0.01)	0.002	0.103	0.022	0.167
	1	6.92 (0.02)	0.050	0.913	0.156	0.913
	2.5	6.93 (0.02)	0.005	0.088	0.017	0.019
	5	6.90 (0.06)	0.038	0.788	0.084	0.204
FAL maize	0	6.96 (0.00)	1.841	0.915	1.841	0.915
	0.5	6.96 (0.01)	0.211	0.961	0.211	0.961
	1	6.94 (0.01)	0.307	0.962	0.307	0.962
	2.5	6.90 (0.02)	0.214	0.907	0.214	0.907
	5	6.80 (0.03)	0.182	0.903	0.182	0.903

The VFA data of the trial with HAL (manure and maize) addition had only a maximum of 0.2% of the total cellulose conversion, and only on the trials with 5 g HAL or FAL·l^{-1}, but the trials with FAL (manure and maize) addition had the same 2% of the total cellulose conversion as the substrate controls. However, this time instead of being all in the C2 form, it was mostly b-valeric acid (b-C5) and n-caproic acid (n-C6), which might indicate release of these fatty acids during the braking down of the cellulose entrapped in the FAL.

5.3.1.2 Tributyrin hydrolysis

Hydrolysis of tributyrin without HAL and FAL addition (substrate control) reached an efficiency of 100% for all eight trials. The observed high hydrolysis efficiencies were reached due to the chosen low concentrations of tributyrin that were added to each trial (0.012 g·ml^{-1}), therefore preventing a too fast decrease in pH which,

in its turn, practically enabled its manual rise with KOH, in order to keep it close to neutrality. According to Wu and Tsai (2004) tributyrin concentrations higher than 0.033 $g \cdot ml^{-1}$ result in acidification of the solution due to the butyric acid production, decreasing the pH, therefore denaturing the lipases and reaching lower hydrolysis efficiencies.

Unlike in the cellulose trials, the lipases used in the tributyrin trials, had an optimum pH of 7.7, which was favourable to the operational conditions chosen for these trials.

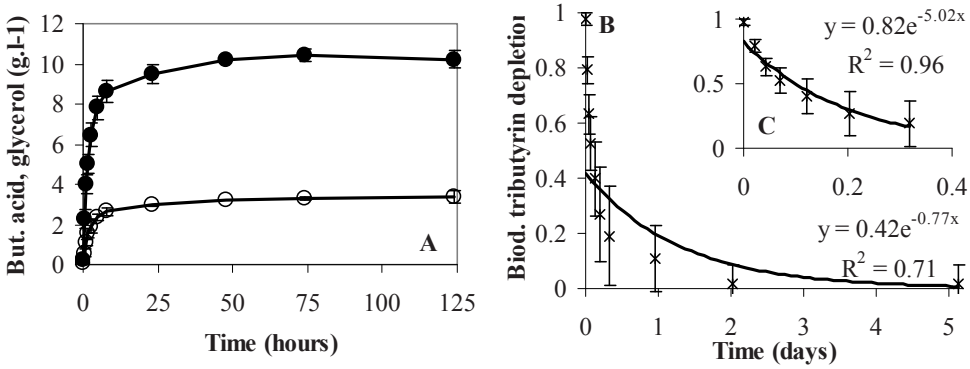

Figure 5.3. Butyric acid (and glycerol produced during enzymatic hydrolysis of tributyrin (**A**), with no HAL and FAL addition (substrate control) and biodegradable tributyrin depletion during the whole trial (**B**) and during the first 8 h (**C**).

Tributyrin was hydrolysed into butyric acid and glycerol mostly within the first 8 hours, as shown in Figure 5.3. The error bars indicate the standard deviation between the eight substrate control trials. Hydrolysis of tributyrin was always faster than of cellulose, as found in literature (Jurado et al., 2008; O'Sullivan et al., 2008). Similar to the results from the cellulose trials, the average hydrolysis rate found for all eight tributyrin substrate control trials was faster in the beginning of the run followed by a slower rate after approximately 8 hours, which resulted in a k_h seven times higher for the first 8 h than for the whole trial, as shown in Figure 5.3. This indicates that, also in lipids hydrolysis the availability and surface area of the substrate might play a role on the rate of the process. Jurado et al. (2008) reported that tributyrin hydrolysis proceeds in two partially overlapping stages. The first and fastest stage, is the hydrolysis of an outer ester group of the surface molecules of tributyrin, which leads to the formation glycerol dibutyrin. As the concentration of surface tributyrin molecules decreases, the hydrolysis of the remaining ester group of the dibutyrin molecules takes place. This second stage is much slower because it needs an appropriate arrangement of

the dibutyrin molecules surface (Jurado et al., 2008). Lu et al. (2007) not only reported the existence of the two same hydrolytic stages, but also compared the overall first-order kinetics with a combined first and second stage first-order kinetics, when hydrolysing lipids and proteins from fish residues with ammonia addition, concluding that the latter equation described the process more adequately. However, their suggested equation did not fit our experimental data, most likely due to the different source of lipids.

Table 5.3. pH and kh for tributyrin hydrolysis trials. Data expressed as mean (standard deviation).

Trials	[HAL and FAL] ($g \cdot l^{-1}$)	pH	Whole trial		First 8 h	
			kh	R2	kh	R2
HAL manure	0	6.90 (0.03)	1.474	0.843	5.448	0.962
	0.5	6.93 (0.07)	1.104	0.963	2.263	0.858
	1	6.91 (0.12)	2.755	0.937	3.278	0.961
	2.5	6.82 (0.14)	1.044	0.944	2.114	0.868
	5	6.94 (0.03)	0.833	0.942	2.074	0.901
HAL maize	0	6.83 (0.08)	5.047	0.761	4.286	0.967
	0.5	7.33 (0.19)	0.180	0.911	0.770	0.889
	1	7.24 (0.29)	0.175	0.785	1.133	0.823
	2.5	7.33 (0.24)	0.125	0.956	0.353	0.772
	5	7.11 (0.10)	0.168	0.919	0.624	0.796
FAL manure	0	7.03 (0.02)	0.456	0.750	4.442	0.917
	0.5	6.84 (0.02)	0.446	0.608	5.647	0.936
	1	6.87 (0.08)	5.146	0.762	5.458	0.998
	2.5	6.95 (0.04)	3.828	0.877	4.200	0.987
	5	6.93 (0.07)	5.266	0.618	2.974	0.994
FAL maize	0	6.94 (0.03)	2.374	0.747	6.326	0.959
	0.5	6.82 (0.01)	2.258	0.831	4.990	0.922
	1	6.79 (0.01)	3.326	0.775	8.510	0.970
	2.5	6.89 (0.04)	3.250	0.750	8.746	0.980
	5	6.83 (0.03)	3.934	0.879	8.069	0.965

Once again the hydrolysis was calculated as the overall process and the fastest stage, that generally gave better R^2, indicating a better fit of the chosen first-order kinetics, as shown in Table 5.3.

With regard to the average substrate controls, k_h values were 2.34 ± 1.97 d^{-1} for the whole trial and 5.13 ± 0.95 d^{-1} for the first 8 h. The significant difference between the two hydrolysis rate constants shows that this step, as mentioned

before, occurs almost completely within the first 8 h. The higher standard deviation observed for the k_h of the whole trial in relation to the k_h of the first 8 h, indicates that the slower degradable tributyrin might become randomly accessible to the lipases at different times throughout the remaining trial. This difference would have likely been minimized when higher lipase concentrations would have been applied, since the point where there is saturation of the interface due to enzyme-adsorption could have been reached and thus maximum hydrolysis rates achieved (Jurado et al., 2008).

Tributyrin hydrolysis and its rate were inhibited by HAL from manure and maize, especially during the first 8 h, as is shown in Figure 5.4 and Table 5.3. However, this was not verified for the trials where FAL were added. This difference in observed sensitivity is not fully understood, since the mechanisms involved in the binding are not known. The few researches on the enzyme-HA complex formation indicate possible electrostatic and hydrophobic interactions and other types of intermolecular interactions as binding mechanisms. However insufficient knowledge of HA and FA structure prevents further conclusions (Jahnel and Frimmel, 1994; Jorobekova et al., 2005). The binding of hydrolytic enzymes to lignin, which mechanism might be identical to HA and FA, has been ascribed to the functional groups of lignin, especially phenolic hydroxyl groups, however the lignin structural characteristics still raise doubts on other binding mechanisms (Palonen and Viikari, 2004; Pan, 2008). The HAL and FAL from manure and maize used in this study were characterized by up to 30% of polysaccharides, while the aliphatics, which include lipids, were low for the HAL (up to 6%) and nonexistent for the FAL (chapter 4). It could be that the lipases first bind to the lipids present in the HAL and then form a lipase-HAL complex, therefore leaving the added tributyrin free in the solution and not available to the enzymes, which is not observed in the FAL trials because they do not have lipids in their structure. When better techniques for identifying the binding mechanisms of HAL and FAL with enzymes are developed, future results may correlate the inhibiting effect of HAL and FAL with their chemical and physical properties. Such insights may then give rise to mitigating the inhibitory effects of humics and fulvics on hydrolysis.

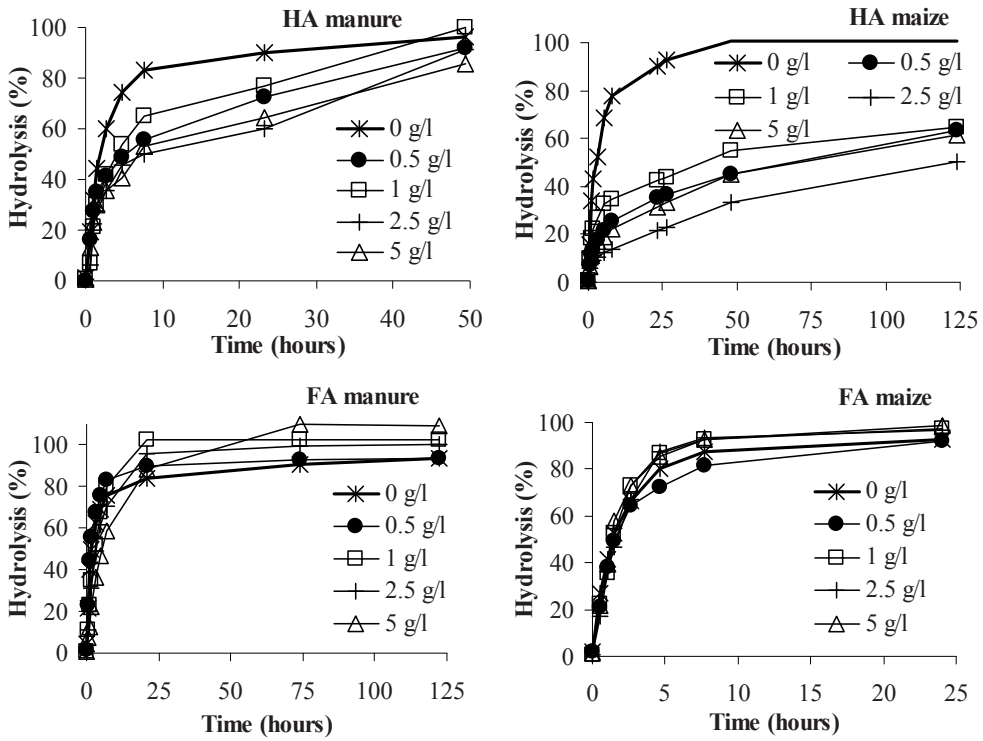

Figure 5.4. Hydrolysis of tributyrin without HAL and FAL (×) and with HAL manure, HAL maize, FAL manure and FAL maize at concentrations of 0.5 (●), 1 (□), 2.5 (+) and 5 $g \cdot l^{-1}$ (Δ).

The inhibiting effect of HAL, and especially from maize, on the hydrolysis of tributyrin was clear. However a correlation between the hydrolysis efficiency and rate, and the concentration of added HAL from maize was not achieved, since the lowest and highest concentrations of HAL showed similar k_h values (Table 5.3). Moreover, similar to the cellulose trials, hydrolysis efficiencies indicate that low HAL concentrations already had a large inhibiting effect on tributyrin hydrolysis.

Compared to the cellulose trials, in the tributyrin trials, there was a bigger variation of pH throughout each trial due to the aforementioned formation of butyric acid. Consequently, the pH was carefully monitored during the trials. Even though the pH was manually corrected to neutrality at each sampling point, there was still a decreasing tendency, especially at the beginning of the trials, when hydrolysis was faster. However, there was no reciprocal correlation with the pH and the hydrolysis rate, indicating that the pH variations were too modest to influence the reactivity of the HAL and FAL by deprotonation of its reactive

functional groups. Therefore, validating the inhibitory effect of HAL on tributyrin hydrolysis.

5.3.2 Microbially catalysed hydrolysis trials

Cellulose was mostly converted into succinate and acetate even though small amounts of propionate were also recorded for the experiments with HAL maize addition. The small amounts of propionate formed most likely originated from the conversion of extra substrate present in the HAL, as when subtracting the propionate of the HAL controls, a maximum of 1 mM of propionate was left.
Cellulose hydrolysis and acidification were clearly inhibited by HAL maize addition, as shown particularly in the left graph of Figure 5.5.

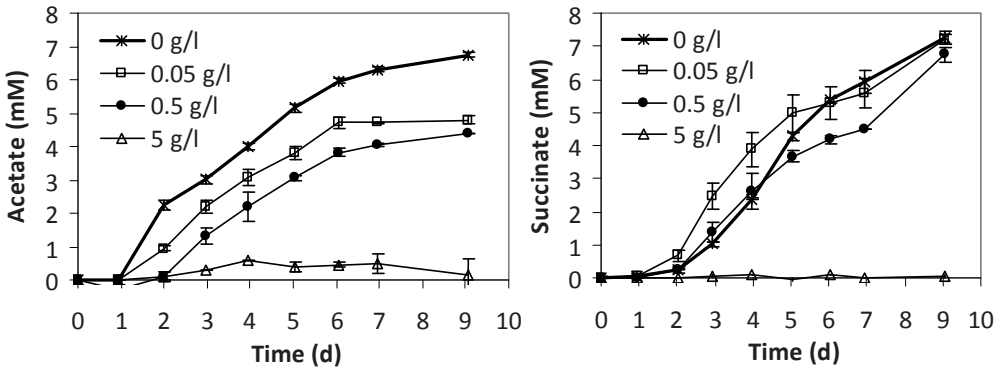

Figure 5.5. Acetate (left) and succinate (right) formation during anaerobic degradation of cellulose with and without addition of HAL extracted from maize.

The lower succinate concentration (right graph of Figure 5.5) for the trial without HAL maize addition in relation to the two trials with 0.05 and 0.5 $g \cdot l^{-1}$ HAL maize addition, between days 1 and 5, is not fully understood. It might be due to: 1) HAL maize accelerated the reduction of fumarate to succinate or 2) something provoked the delay in succinate formation for the trial without HAL maize addition 3) low concentrations of HAL maize accelerated the reduction of fumarate to succinate, yet high concentrations of HAL maize had an inhibiting effect.

HAL have been reported to increase reduction rates by serving as redox mediators for the reduction of compounds (Field and Cervantes, 2005; Hernandez and Newman, 2001; Lovley et al., 1998). This could have been the case for the HAL

addition trials, where the HAL transferred electrons to reduce fumarate to succinate, therefore, indicating that an increase in HAL would accelerate the reduction reaction and enhance succinate formation. However, this was not the case, since the fastest succinate formation was not at the highest HAL concentration, but at HAL concentrations of 0.05 > 0.5 > 0 and finally 5 $g \cdot l^{-1}$, that actually showed no succinate formation during the whole trial (Figure 5.5, right graph). Therefore, it might have been that at higher HAL maize concentration the inhibiting effect overruled the increased reduction rates verified at the lower HAL maize concentrations.

The other option is that for some unclear reason, for the trial where no HAL were added (0 $g \cdot l^{-1}$) there was a delay in the formation of succinate, as shown in Figure 5.6. This resulted in an S-shaped curved, that is actually not commonly found in enriched microorganisms batch trials, since the cultures have been enriched with cellulose, and therefore should immediately be able to convert it, as shown by Nouaille at al. (2009).

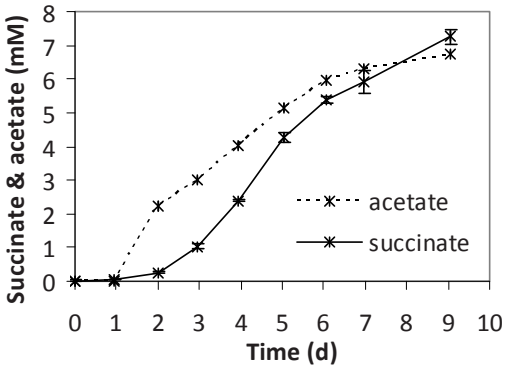

Figure 5.6. Acetate and succinate formation during anaerobic degradation of cellulose (no HAL maize).

Overall it can be concluded that hydrolysis was inhibited by HAL addition, as shown in Figure 5.7.

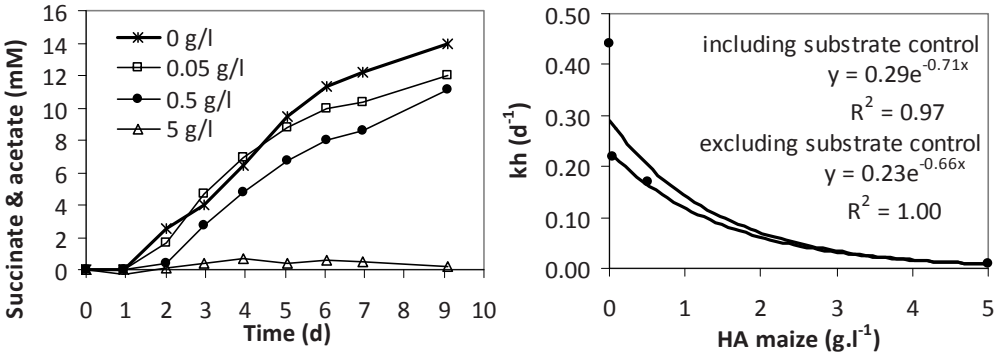

Figure 5.7. Succinate and acetate formation during anaerobic degradation of cellulose with and without addition of HAL extracted from maize (left). Cellulose first order hydrolysis rate constant (k_h) with and without HAL maize plotted against the HAL maize concentrations applied (right).

On the right graph of Figure 5.7 it can be seen that the increase in HAL maize concentration resulted in a decrease in k_h values. Even though it seems that the HAL maize effect on the hydrolysis and acidogenesis can be exponentially described, there were too few data points to conclude this, especially because there were no k_h values between 0.5 and 5 $g \cdot l^{-1}$.

5.4 General discussion on cellulose hydrolysis inhibition

It is clear that HM inhibits the hydrolysis of cellulose, however, it is not completely clear how this happens. When comparing the enzymatic hydrolysis trials with the microbially catalysed hydrolysis trials for cellulose hydrolysis at HAL maize concentrations of 0.5 and 5 $g \cdot l^{-1}$, it is clear that for the 5 $g \cdot l^{-1}$ no hydrolysis takes place in either trials, but for the 0.5 $g \cdot l^{-1}$ there is slow hydrolysis in the microbially catalysed hydrolysis trials. The reasons for such outcome are hypothesised below and illustrated in Figure 5.8. In the enzymatic hydrolysis trials cellulose hydrolysis did not take place probably because all enzymes added were bond to the functional groups of the HAL, as shown on **A** of Figure 5.8. However, it could also be that the HAL changed the active sites of the enzymes - known as non-competitive inhibition - therefore preventing cellulose hydrolysis.

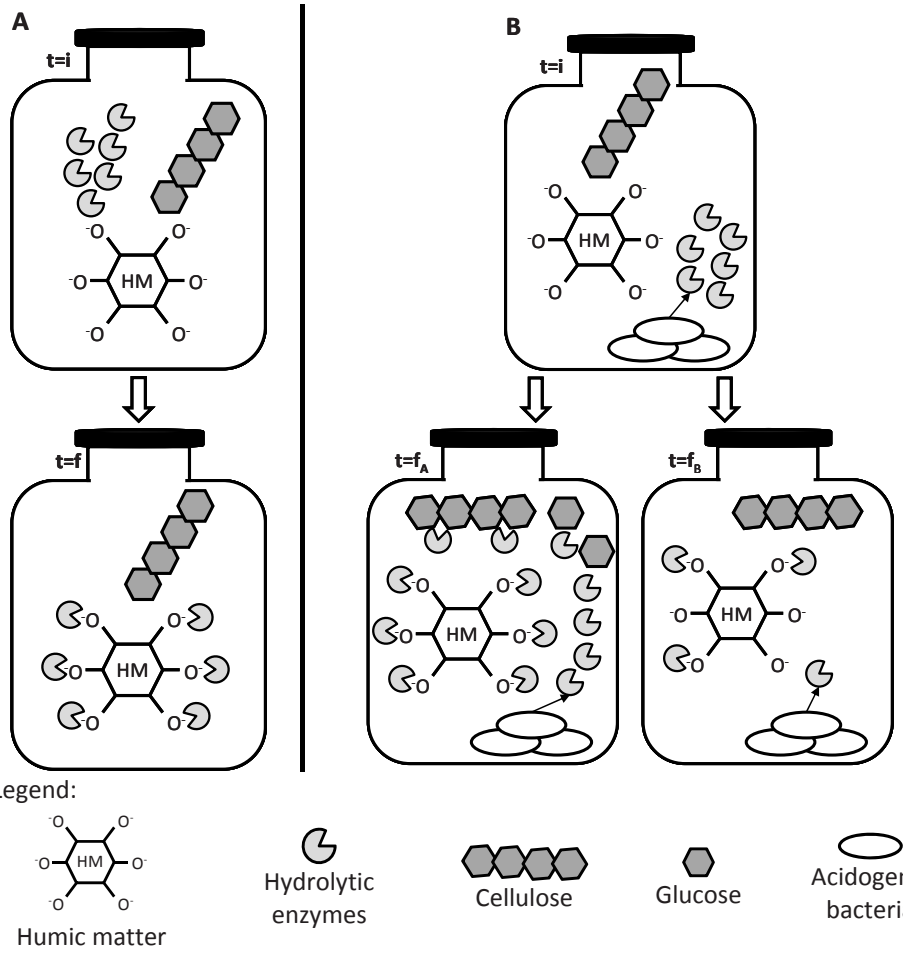

Figure 5.8. Schematic representation of possible cellulose hydrolysis inhibition in the presence of HM. For enzymatic hydrolysis experiments (**A**) hydrolytic enzymes are added in the beginning together with substrate and HM (**t=i**). When enzymes concentrations are limited, enzymes bind to HM instead of substrate (**t=f**), or HM changes the active-site of the enzyme still preventing substrate hydrolysis. For anaerobic hydrolysis experiments (**B**) acidogenic bacteria added in the beginning together with substrate and HM (**t=i**). When enzymes concentrations are not limited (**$t=f_A$**), enzymes bind to: 1) HM until all functional groups are taken and 2) to the substrate. When enzymes concentrations are limited due to inhibition of enzymes secretion by the acidogenic bacteria (**$t=f_B$**), the available enzymes bind to HM leaving the substrate un-hydrolysed.

In the microbially catalysed hydrolysis trials with HAL, cellulose hydrolysis was slower compared to the trials without HAL, indicating that there was

inhibition of cellulose hydrolysis. However, cellulose was still hydrolysed, indicating that probably the acidogenic bacteria kept on secreting enzymes to hydrolyse the cellulose, as reported by Sanders (Sanders, 2001). Therefore even though part of the enzymes were bond to the HAL, the excess of enzymes were able to hydrolyse the cellulose, as shown on **B**, $\mathbf{t=f_A}$ of Figure 5.8. Whether this competitive inhibition takes place from the beginning of the trial or only when the binding sites of the HAL functional groups are taken is not completely clear and more research on this phenomena is needed. On the other hand, it might be that together with the competitive inhibition, the secretion of the enzymes by the acidogenic bacteria is also inhibited, as shown on **B**, $\mathbf{t=f_B}$ of Figure 5.8. This later justification would explain why at the highest HAL concentration, 5 $g \cdot l^{-1}$, no hydrolysis took place. Hence, addition of hydrolytic enzymes would probably mitigate part of the inhibiting effect of HAL, because hydrolytic enzymes would still be present and, when in excess, able to hydrolyse the substrate.

5.5 Conclusions

HAL and FA from maize and cow manure, in the ranges from 0.5 to 5 $g \cdot l^{-1}$, clearly inhibit the enzymatic hydrolysis of cellulose. The enzymatic hydrolysis of tributyrin was inhibited by HAL from maize and cow manure in the ranges from 0.5 to 5 $g \cdot l^{-1}$, however it was not clearly inhibited by FA from maize and cow manure. The latter might be due to differences in the HAL and FA structural characteristics, and/or to the mechanisms involved in the binding of the HAL and FA to the enzymes. Possibly HAL and FA are able to form complexes with the lipid, having the lipid still available for enzymatic hydrolysis.

HAL from maize, in the ranges from 0.05 to 5 $g \cdot l^{-1}$, clearly inhibit anaerobic cellulose hydrolysis. Therefore, HAL and FA present in lignocellulosic biomass, such as animal manure and plant matter, are expected to inhibit hydrolysis, and therefore anaerobic co-digestion of complex waste.

Acknowledgments

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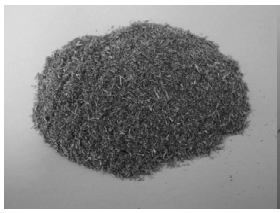
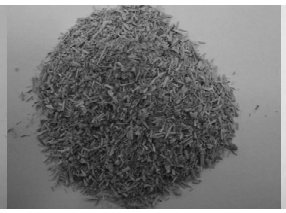
Chapter 6

Effects of thermo-chemical pre-treatment on anaerobic biodegradability and hydrolysis of lignocellulosic biomass


Abstract

The effects of different thermo-chemical pre-treatment methods were determined on the biodegradability and hydrolysis rate of lignocellulosic biomass. Three plant species, hay, straw and bracken were thermo-chemically pre-treated with calcium hydroxide, ammonium carbonate and maleic acid. After pre-treatment, the plant material was anaerobically digested in batch bottles under mesophilic conditions for 40 days. From the pre-treatment and subsequent anaerobic digestion experiments it was concluded that when the lignin content of the plant material is high, thermo-chemical pre-treatments have a positive effect on the biodegradability of the substrate. Calcium hydroxide pre-treatment improves the biodegradability of lignocellulosic biomass, especially for high lignin content substrates, like bracken. Maleic acid generates the highest percentage of dissolved COD during pre-treatment. Ammonium pre-treatment only showed a clear effect on biodegradability for straw.

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6.1 Introduction

Lignocellulosic biomass consists of lignin, cellulose and hemicellulose fractions, which quantitatively and qualitatively vary according to the plant material. Unlike cellulose and hemicellulose, lignin is a cross linked network hydrophobic polymer (Hatfield and Fukushima, 2005) that is fairly resistant to anaerobic degradation. Therefore, when cellulose is encrusted in lignin the cellulolytic enzymes will have hardly access to the cellulose, retarding or even preventing hydrolysis (Gallert and Winter, 2005).

In the anaerobic digestion of complex substrates, like lignocellulosic biomass, hydrolysis is considered as the rate limiting step (Pavlostathis and Giraldo-Gomez, 1991). Consequently, the biogas production depends for the most part on the biodegradability and hydrolysis rate (El-Mashad, 2003).

Pre-treatment of lignocellulosic biomass could accelerate the hydrolysis process and improve the final biogas production. Furthermore, when the hydrolysis process would be accelerated the hydraulic retention time of the digestion could be decreased which would be very attractive for anaerobic farm digesters.

Chemical pre-treatment of lignocellulosic biomass for renewable energy generation in the form of biogas is long considered to be economically unattractive due to the high price of chemicals in comparison to the low energy costs (Pavlostathis and Gossett, 1985). However, the current increase in energy prices and concomitantly increasing demand for renewable energy sources leads to a renewed interest in chemical pre-treatment.

Mosier et al. (2005) evaluated the effects of various pre-treatment methods on the chemical composition and structure of lignocellulosic biomass. They concluded that ammonium recycling percolation (ARP) and calcium hydroxide pre-treatments have a major effect on the removal and structural alteration of lignin. Diluted sulphuric acid pre-treatment, which has been extensively applied, also changes the lignin structure. However, the disadvantage of this pre-treatment method is that during the subsequent anaerobic digestion hydrogen sulphide is formed, lowering the quality of the produced biogas. Maleic acid pre-treatment has been hardly studied so far. Nevertheless, it has been reported to have a similar impact on cellulose degradation as diluted sulphuric acid (Mosier, 2001) and has showed promising results, enhancing hydrolysis of wheat straw for bio-ethanol production, however its major drawback is it's high cost (Schenk, 2005).

The objective of this study was to determine the effects of different pre-treatment methods on different lignocellulosic plant materials with respect to the biodegradability and hydrolysis rate of the pre-treated biomass. Hay, straw and

bracken, with different lignin contents were pre-treated with three chemical solutions, i.e. calcium hydroxide, ammonium carbonate and maleic acid. These chemicals were selected for the following reasons: calcium hydroxide pre-treatment can be performed at low temperatures (Kim and Holtzapple, 2005), the ammonium from the ammonium carbonate pre-treatment may possibly be reused (Kim and Lee, 2005), and maleic acid leaves no chemical residue. Moreover, both calcium hydroxide and ammonium (ARP) have been reported to achieve high sugar yields at low temperatures (Wyman et al., 2005). Hay, straw and bracken were selected based on their relatively high lignin content. Moreover, they are a suitable substrate for anaerobic farm co-digestion due to their availability and high organic matter content. Agricultural residues, like hay and straw, are already common (co)-substrates for anaerobic farm digesters (Møller et al., 2004). Bracken is not yet used as a (co)-substrate for farm digesters, however it can be considered since it is a common waste product from forestry activities (Hooper and Li, 1996; Salter, 2007). The applied pre-treatment methods performed in this research are following the procedures of Maas et al. (2008), Schenk (2005), and Hendriks (2006). Anaerobic digestion of the three untreated and pre-treated plant species was then followed in order to determine the maximum biodegradability and hydrolysis rate constants (k_h).

6.2 Material and methods

6.2.1 Experimental set-up

Three plant species, hay, straw and bracken, were grinded to particles of approximately 1 cm (Retsch Muhle; type sm1) and air-dried at 40°C. The air dried hay, straw and bracken had chemical oxygen demand (COD) / volatile solids (VS) ratios of 1.34, 1.25 and 1.36 g COD·g VS^{-1}, respectively. They were further homogenized to particles smaller than 1 mm by a cutting mill (Krups KM75) and freeze dried (Lyovat GT2) to be analysed on lignin content according to the van Soest (1991) method. One gram COD of each, ground and dried, plant specie was then pre-treated with calcium hydroxide, ammonium carbonate, and maleic acid, respectively, according to Maas et al. (2008), Schenk (2005) and Hendriks (2006), following the below described general procedure. Each plant species was added, together with the pre-treatment chemical, into stainless steel air tight reactor tubes of 110 ml. The content of each reactor was mixed and after 15 minutes the reactor tubes were closed and placed in a Haake B Fision oil bath filled with silicon oil. The temperature inside the reactor was measured with an Isopad TD2000

sensor. The reactor tube was air tight, resulting in a simultaneous temperature and pressure increase. Experimental conditions are shown in Table 6.1. After the defined pre-treatment time the reactor tubes were placed in ice water for 5 minutes to stop the reaction and the content was stored in plastic cups at 4°C for a few days until needed for the batch tests. All pre-treatments were performed in duplicate.

Table 6.1. Pre-treatment applied on hay, straw and bracken.

Chemical	Liquid in reactor (ml)	Concentration		Temperature (°C)	Pre-treatment time (h)	pH	
		(%)	($g \cdot l^{-1}$)			Before	After
Calcium hydroxide	110	10	-	85	16	11.8	10.2
Ammonium	100	-	4	120	2	8.3	8.3
Maleic acid	90	-	5.8	150	0.5	2	2.2

After pre-treatment, the plant material was anaerobically digested in batch bottles at 35±5˚C. Twelve batch experiments were carried out for 40 days. Each experiment was performed in duplicate including two controls with only inoculum sludge in order to correct for the COD that dissolves from the sludge into the medium (COD_{diss}), as well as the volatile fatty acids (VFA) and CH_4 produced by the control. In addition, two sludge plus maleic acid controls were used for the experiments where maleic acid was applied as pre-treatment. All test vials were inoculated with 4.2 g $VS \cdot l^{-1}$ of sludge from a maize digester (Corntec, Meppen) and 4.2 g $VS \cdot l^{-1}$ of granular sludge from an alcohol production anaerobic waste water treatment plant (Nedalco, Bergen op Zoom). The pH of all batch experiments was set to neutral at the beginning of the test. Trace elements, macro nutrients (Field, 1989) and 20 mM phosphate buffer (50 $ml \cdot l^{-1}$) were also added to each bottle. All experiments were performed in 0.5 l Scott bottles with two sampling ports, one for liquid sample to determine the intermediate digestion products and another for gas sample to determine the gas composition. All bottles had a total working volume of approximately 0.2 l and were fitted with an Oxitop® head for gas pressure measurement, together with the gas composition, to assess the cumulative methane production.

6.2.2 Chemical analysis

Macro COD, total solids (TS), volatile solids (VS), NH_4^+-N, PO_4-P and pH were determined according to standard methods (APHA, 1998). Samples for dissolved COD (COD_{diss}) and VFA were centrifuged at 10,000 rpm for 10 minutes in a Hermle Z300 centrifuge. After centrifuging, only the supernatant was used to analyse the COD_{diss} and VFA. COD_{diss} was analysed according to standard methods (APHA, 1998), and VFA and biogas composition according to El-Mashad (2003).

6.2.3 Calculations

Methane production was calculated from the pressure increase according to the ideal gas law and the methane present in the biogas. Maximum biodegradability, in percentage, meaning the maximum percentage of substrate COD that is converted to methane, was calculated according to El-Mashad (2004). Maximum biodegradability, in litres of methane produced, at standard temperature and pressure (STP), per gram of VS was calculated based on the COD/VS ratio of each batch experiment. Hydrolysis, acidogenesis and methanogenesis percentages, for the COD balances, were calculated according to El-Mashad (2004). The first order hydrolysis constant (k_h) of the biodegradable suspended COD remaining after pre-treatment was calculated according to Angelidaki and Sanders (2004) and is here presented as equation 6.1.

$$\frac{COD_{total,t=0} - (COD_{diss,t=x} + COD_{CH_4,t=x}) - COD_{ss,inert}}{COD_{total,t=0} - COD_{diss,inf} - COD_{ss,inert}} = e^{-k_h t} \quad (6.1)$$

Biodegradable suspended COD at t=x was calculated as the total COD at t=0 ($COD_{total,t=0}$) minus the combination of the dissolved COD at t=x ($COD_{diss,t=x}$) and the methane at t=x in COD ($COD_{CH4,t=x}$), and the inert suspended COD ($COD_{ss,inert}$). The biodegradable suspended COD after pre-treatment, therefore at t=0 ($COD_{ss,biod,\ t=0}$) was calculated as the $COD_{total,t=0}$ minus the dissolved COD at t=0 ($COD_{diss,t=0}$) caused by pre-treatment, and the $COD_{ss,inert}$ that is the suspended COD after t=∞ batch digestion.

The batch derived data were used to calculate the COD conversion to CH_4 in a continuously stirred tank reactor (CSTR) for different hydraulic retention time (HRT) according to equation 6.2.

$$CODcon. = \frac{COD_{ss,biod,inf} - \frac{COD_{ss,biod,inf}}{(1 + k_h \cdot HRT)} + (COD_{diss,inf} - COD_{diss,inert})}{COD_{total,inf}} \cdot 100 \qquad (6.2)$$

Inert dissolved COD ($COD_{diss,inert}$) is the dissolved COD after t = ∞ batch digestion. Biodegradable suspended COD influent ($COD_{ss,biod,inf}$) is the biodegradable suspended COD after pre-treatment, referred to as $COD_{diss,t=0}$ in equation (6.1). For equation (6.2), hydrolysis was assumed to be rate limiting from 10 days HRT onwards, assuming equal effluent VFA at increasing HRT (Van Velsen, 1981).

6.3 Results and discussion

Bracken had a lignin concentration of 184.5 ± 2.9 g·kg VS^{-1} which was 3.2 times higher than straw (57.1 ± 7.2 g·kg VS^{-1}) and 7.4 times higher than hay (24.9 ± 2.5 g·kg VS^{-1}). In accordance with Chandler et al. (1980), we found a reciprocal correlation between the lignin content and the biodegradability when relating the lignin content with the biodegradability of the substrate (Table 6.2). The results obtained after anaerobic digestion of untreated and pre-treated hay, straw and bracken are presented in Table 6.2.

Table 6.2. Maximum biodegradability, as percentage and l (STP) $CH_4 \cdot g\ VS^{-1}$, first order hydrolysis rate constant (k_h) with R^2, and pH during the test of hay, straw and bracken, both untreated and pre-treated, performed at 35°C. NH_4^+-N and PO_4-P at end of test run without discarding controls. Data expressed as mean ± standard deviation.

	Maximum Biodegradability		kh	R^2	pH	NH_4^+-N	PO_4-P
	(%)	($lCH_4 \cdot g\ VS^{-1}$)	(d^{-1})			($mg \cdot l^{-1}$)	($mg \cdot l^{-1}$)
Hay	66.9 ± 3.5	0.32 ± 0.02	0.088	0.95	7.0 ± 0.3	393 ± 18	190 ± 3
Hay + $Ca(OH)_2$	60.4 ± 2.5	0.28 ± 0.01	0.115	0.90	6.9 ± 0.2	430 ± 30	176 ± 21
Hay + maleic acid	63.6 ± 0.0	0.23 ± 0.00	0.197	0.16	7.3 ± 0.2	508.5 ± 0	288 ± 0
Hay + ammonium	64.7 ± 6.3	0.30 ± 0.03	0.409	0.91	7.5 ± 0.2	1697 ± 45	274 ± 6
Straw	57.6 ± 1.1	0.25 ± 0.00	0.081	0.95	7.0 ± 0.3	361 ± 6	179 ± 2
Straw + $Ca(OH)_2$	62.7 ± 0.0	0.26 ± 0.02	0.107	0.95	7.0 ± 0.3	447 ± 0	179 ± 0
Straw + maleic acid	42.5 ± 4.3	0.15 ± 0.02	0.240	0.81	7.3 ± 0.1	468 ± 12	275 ± 12
Straw + ammonium	72.2 ± 5.7	0.32 ± 0.03	0.073	0.98	7.6 ± 0.3	1595 ± 22	266 ± 96
Bracken	13.9 ± 0.2	0.07 ± 0.00	0.391	0.95	7.1 ± 0.2	356 ± 4	166 ± 0
Bracken + $Ca(OH)_2$	36.5 ± 2.6	0.17 ± 0.01	0.068	0.94	7.1 ± 0.2	420 ± 2	171 ± 6
Bracken + maleic acid	31.3 ± 0.6	0.11 ± 0.00	-	-	7.3 ± 0.2	449 ± 9	258 ± 7
Bracken + ammonium	19.2 ± 6.1	0.09 ± 0.03	0.068	0.98	7.7 ± 0.2	1507 ± 130	246 ± 18

All pre-treatments resulted in an increase in ammonium and phosphate concentration, which can be attributed to hydrolysis (Table 6.2), except for experiments with calcium hydroxide, indicating precipitation of phosphate with calcium.

The k_h values presented in Table 6.2 only indicate the hydrolysis rate of the residual suspended biodegradable material after thermo-chemical pre-treatment, since it is not possible to determine the rate of hydrolysis during the pre-treatment process. In the experiment with bracken pre-treated with maleic acid almost no hydrolyses of the residual matter was observed and, therefore, no hydrolysis rate constant could be calculated. In fact, during pre-treatment, hydrolysis of the initial complex substrates is already taking place, as illustrated by the results of the experiments with maleic acid, where the resulting k_h only represents the degradation of a small, approximately 10%, of the remaining biodegradable fraction.

As shown in Figure 6.1, the maleic acid generated the highest percentage of dissolved COD. For the ammonium the pre-treatment resulted in an increase of circa 10% in dissolved COD for hay and straw and almost 15% for bracken. For the calcium hydroxide the pre-treatment resulted in a less than 10% increase in dissolved COD for hay and almost 15% for straw and bracken.

Figure 6.2 illustrates the effect of the three chemical pre-treatment methods on the biodegradability of the used plant material. During the anaerobic digestion tests, the pre-treated bracken showed a significant improved biodegradability as compared to the untreated sample. Calcium hydroxide was the best pre-treatment method, increasing the biodegradability by 2.6 fold compared to the untreated bracken.

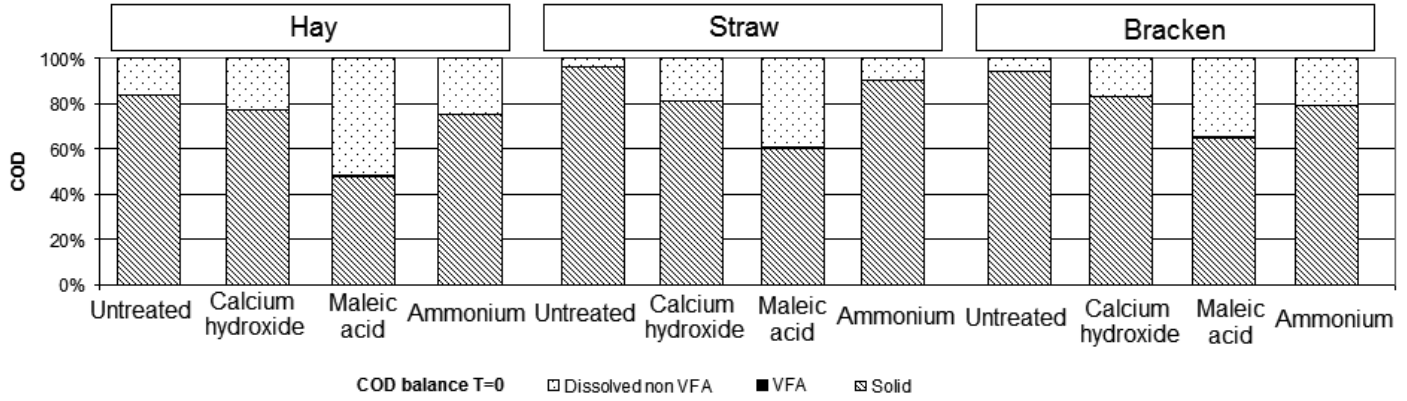


Figure 6.1. COD balance of untreated and pre-treated hay, straw and bracken at beginning of anaerobic batch tests.

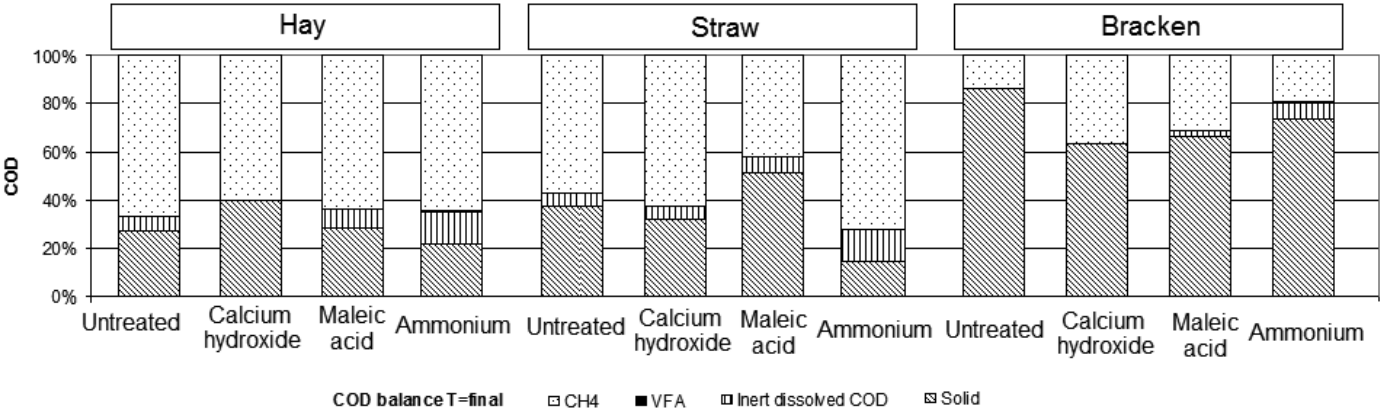


Figure 6.2. COD balance of untreated and pre-treated hay, straw and bracken at end of anaerobic batch tests.

On the other hand, the results of the experiments with pre-treated hay showed no improvement of the biodegradability in comparison with the untreated sample. Hay is shown to be an easy biodegradable plant material by itself. For the experiments with pre-treated straw, the results were not uniform for the three chemical pre-treatment methods. Calcium hydroxide and ammonium pre-treatment showed an increment in the biodegradability of 5 and 15% respectively, while pre-treatment with maleic acid generated no improvement of the biodegradability in comparison to the untreated sample, however its reason was not clear during this study. The ammonium pre-treatment resulted in a higher inert dissolved COD fraction than for the other pre-treatments, as can be seen in Figure 6.2.

Figure 6.3 shows the predicted effects of chemical pre-treatment on CH_4 recovery when digesting hay, straw and bracken in a CSTR at different HRTs, calculated using equation 6.2 and the batch test results. Pre-treatment of hay did not affect the biodegradability but only accomplished solubilisation of polymers during pre-treatment and/or increase in hydrolysis rate of the remaining polymers. Therefore only at lower HRTs, increased CH_4 recovery is evident. Ammonium and maleic acid pre-treatments showed the strongest effect. Except for maleic acid, pre-treatment of straw resulted in increased biodegradability, solubilisation and/or hydrolysis rate, and thus positively affecting the CH_4 recovery at both short and long HRTs. For bracken, calcium hydroxide and maleic acid pre-treatments mainly affected solubilisation, showing similar effects on long and short HRTs, whereas, ammonium pre-treatment only showed a slight effect.

Mladenovska et al. (2006) found an up to 16% increase in the methane yield when pre-treating the solid fraction of manure with an approximate 3 g NH_4^+-N·l^{-1}, for 40 minutes, at 120°C. Furthermore, Kurakake et al. (2001) reported an increasing ammonium requirement for pre-treating biomass with increasing lignin content. Additionally, (Dien et al., 2006) showed that the pre-treatment severity should increase with an increasing lignin content of the substrate. Apparently, optimal pre-treatment conditions depend on the exact content and structure of the lignocellulosic biomass (Wyman et al., 2005).

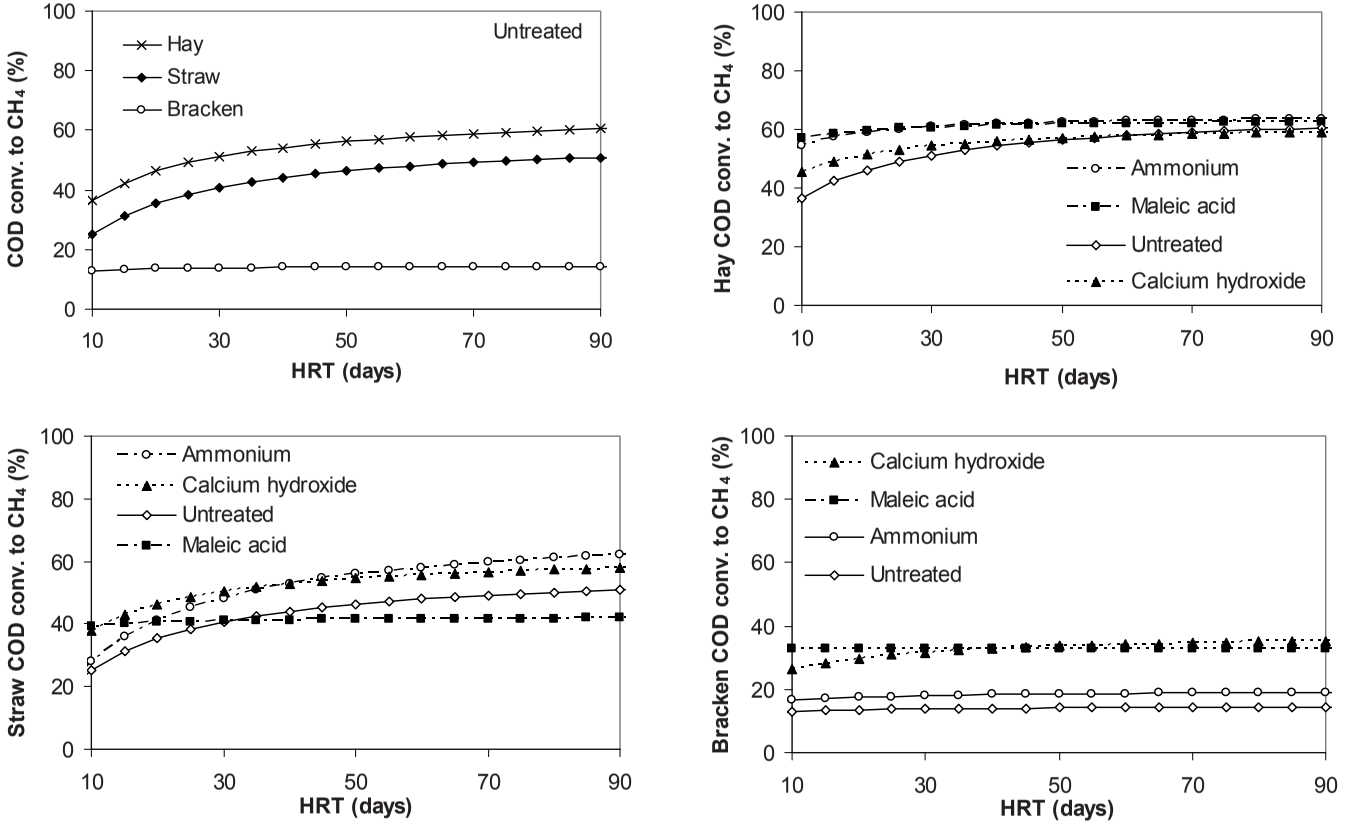


Figure 6.3. Calculated COD conversion to CH_4 during anaerobic digestion in a CSTR at 35°C of hay, straw and bracken untreated and pre-treated with ammonium, maleic acid and calcium hydroxide.

6.4 Conclusions

The biodegradability of lignocellulosic material increases with decreasing lignin content. When the lignin content is high, chemical pre-treatments have a clear positive effect on the biodegradability, but when it is low, this effect is lower or even absent.

Calcium hydroxide pre-treatment improves biodegradability, especially for high lignin content substrates. Maleic acid generates the highest percentage of dissolved COD during pre-treatment. Ammonium pre-treatment did not show a clear effect on biodegradability or hydrolysis rate.

When mathematically predicting the effects of pre-treatment on the CH_4 recovery in a CSTR at varying HRTs, results showed that at short HRTs ammonium and maleic acid have the strongest effects for low lignin content substrates, like hay. For high lignin content substrates, like bracken, calcium hydroxide and maleic acid mainly affected solubilisation showing similar effects on long and short HRTs.

For high lignin content substrates, $Ca(OH)_2$ is an attractive thermo-chemical pre-treatment since $Ca(OH)_2$ additions improve the biodegradability, whereas the pre-treatment can be performed at lower temperatures compared to the other pre-treatments. Moreover, $Ca(OH)_2$ is an inexpensive chemical.

Acknowledgments

The authors would like to acknowledge Katja Grolle for her technical support

Chapter 7

General Discussion and Summary

7.1 Introduction

Hydrolysis, the first step in the anaerobic digestion process, is considered as the rate limiting step in the anaerobic conversion of complex wastes, such as agricultural biowaste (Pavlostathis and Giraldo-Gomez, 1991), but has been an overlooked step in many anaerobic digestion studies (Sanders, 2001; Vavilin et al., 2008).

Hydrolysis enhancement is therefore one of the best approaches for optimizing current and future biogas plants using biomass as a primary energy source.

7.2 Anaerobic hydrolysis optimum conditions

Optimum conditions for achieving the highest hydrolysis rates, depend on the type of hydrolytic enzymes present and the kind of substrate(s) used for anaerobic digestion, as explained in **chapter 2**. Substrates high in lignocellulosic biomass, such as agriculture biowaste (e.g. manure and plant matter), have complex macro-molecular fibrous structure and are, therefore, more difficult to hydrolyse than lipid-rich waste streams. **Table 2.1.,** listing various hydrolysis rates' constants (K_h) for several substrates, shows large differences in dependence to the presence of fibrous material in the substrate. The reason for the slower hydrolysis of the lignocellulosic biomass is the low bio-availability of the cellulose and hemicellulose, which are anaerobically biodegradable, to the hydrolytic enzymes. Lignin encrusts cellulose, making it inaccessible to the hydrolytic enzymes (Gallert and Winter, 2005).

The optimum pH reported for hydrolytic enzymes depends on the type of enzymes present, and is moderate acidic for cellulases and slightly alkaline for proteases and lipases. Usually for anaerobic hydrolysis a neutral pH is favoured. The temperature effects on hydrolysis can be described by the Arrhenius equation. Temperatures between 20 and 40°C are usually favoured, and depend on the type of acidogenic bacteria present. Besides optimum environmental conditions, i.e. pH and temperature, and the accessibility and solubility of the substrate (**chapter 2)**, it has also been demonstrated that reduction in particle size and increase in substrates' surface will accelerate hydrolysis (e.g. Sanders, 2001). Therefore, physical pre-treatment methods, such as milling, are commonly applied to plant matter (Weiland, 2006). Finally, to accelerate anaerobic hydrolysis, disclosure of its inhibiting compounds and ways of mitigating them need to be attained.

7.3 Hydrolysis inhibition

7.3.1 Ammonia

Ammonia nitrogen is one of the most common inhibitors in the anaerobic digestion of complex wastes containing high concentrations of ammonia, such as animal manure, black water, and waste oil from gastronomy and olive oil production (Angelidaki and Ahring, 1994; Sanders, 2001; Zeeman, 1991). The inhibiting effect of high ammonia concentrations on methanogenesis has been well established. In contrast, the knowledge on the effect of ammonia on the hydrolysis is rather limited. In chapter 3, batch digestion of tributyrin and cellulose at varying ammonia nitrogen concentrations to 4.8 g NH_4^+-N·l^{-1} were performed, using adapted biomass. From these experiments, and previous ones (data not shown in this thesis) it was concluded that total ammonia nitrogen in the range of 2.4 to 7.8 g NH_4^+-N·l^{-1} (or 283 to 957 mg NH_3-N·l^{-1}) does not inhibit the hydrolysis of tributyrin or cellulose (**Figures 3.4 and 3.6**). This conclusion was further confirmed by mathematical analysis of the estimated variation of the first-order hydrolysis constant as a function of the total ammonia concentration.

Also in **chapter 3**, a lag phase for cellulose hydrolysis was observed. This lag phase, which lasted for about 4 days, was identical for all trials, independently of the varying ammonia concentrations, indicating that lag phase duration was not influenced by ammonia. In addition, in the enzymatic hydrolysis of cellulose trials (**chapter 5**), where no manure was added as inoculum, no lag phase was observed, indicating that the lag phase was apparently due to a compound present in the animal manure. In addition, similar cellulose hydrolysis lag phases were found in other studies where animal manure or leachate was used (O'Sullivan et al., 2008; Song et al., 2005), which justifies our assumption. Therefore, we concluded that the inhibition was not due to ammonia, but to some other compound present in animal manure as suggested by Van Velsen (1981) and Zeeman (1991).

7.3.2 HAL and FAL

The above mentioned inhibition was indeed verified in **chapter 5** where cellulose and tributyrin hydrolysis were inhibited by addition of humic acid-like (HAL) and fulvic acid-like (FAL), extracted from cow manure and silage maize.

HAL and FAL can be found in all organic matter, not only in soil, where they are extensively studied, but also in natural waters, sewage, and agricultural

biowaste. However, its concentrations vary greatly according to the source (Stevenson, 1994). In soil, humic matter (HM) varies greatly according to the climate, vegetation, type of soil, topography and age of the soil (Stevenson, 1994). In natural wasters, HM concentrations can vary from 20 $\mu g \cdot l^{-1}$ in groundwater to 30 $mg \cdot l^{-1}$ in surface water (Brum and Oliveira, 2007). Dissolved Organic Matter (DOM) from landfill leachates has been reported to consist 30 to 70% of HM (Tzoupanos et al., 2008). But also agricultural biowaste, such as animal manure and plant residues have HM. Fresh cow manure has about 7 g $HAL \cdot kg^{-1}$ of fresh matter (0.7 % w/w) and about 1.3 g $FAL \cdot kg^{-1}$ of fresh matter (0.1 % w/w), whereas silage maize has about 18 g $HAL \cdot kg^{-1}$ of fresh matter (1.5 % w/w) and about 3.5 g $FAL \cdot kg^{-1}$ of fresh matter (0.4 % w/w) (**chapter 4**). However, the maize yields may vary with harvest time and maize growth location.

The HAL and FAL extracted from cow manure and silage maize were used as model biowaste HM, and were chemically characterized in **chapter 4** by Elemental Analysis, ^{13}C CPMAS NMR, and Pyrolysis GC/MS and direct titrations. The detailed characterization of these HAL and FAL in terms of C, H, O, N, lignin, polysaccharides, acidity, functional groups and so on, is given in this chapter, facilitating the prediction of their reactivity during anaerobic hydrolysis in future work.

As mentioned before, in chapter 5, enzymatic hydrolysis experiments were performed where hydrolytic enzymes were added to cellulose or tributyrin. In addition, four concentrations of HM, 0.5, 1, 2.5 or 5 $g \cdot l^{-1}$, were also added. From these experiments it was clear that HAL and FAL from maize and cow manure, in the ranges from 0.5 to 5 $g \cdot l^{-1}$, clearly inhibit the enzymatic hydrolysis of cellulose (**Figure 5.2**). Also, the enzymatic hydrolysis of tributyrin was inhibited by HAL from maize and cow manure, in the ranges from 0.5 to 5 $g \cdot l^{-1}$. However no clear hydrolysis inhibition could be demonstrated by FAL from maize and cow manure (**Figure 5.4**). The latter might be due to differences in the HAL and FAL structural characteristics, and/or the mechanisms involved in the binding of the HAL and FAL to the enzymes. Possibly, HAL and FAL are able to form complexes with the lipid, having the lipid still available for enzymatic hydrolysis.

In order to determine if the same inhibiting effect was found during anaerobic digestion, HAL from maize were added to enriched cultures of *Fibrobacter succinogenes*, digesting the same type of crystalline cellulose, at mesophilic conditions. The results again clearly indicate that HAL from maize, in the ranges from 0.05 to 5 $g \cdot l^{-1}$, inhibit anaerobic cellulose hydrolysis. Therefore, it is here concluded that, HAL and FAL present in lignocellulosic biomass, such as animal manure and plant matter, do negatively effect the anaerobic hydrolysis rate, and therefore anaerobic (co)-digestion of complex waste.

7.4 Mitigating hydrolysis inhibition and optimizing anaerobic digestion

The main challenge for optimization of biogas plants is to manage the complexity of the anaerobic digestion process in terms of the required optimal conditions for the anaerobic bacteria and archaea, their syntrophic relations, and the interaction between the chemical compounds present. This is especially important when inhibiting compounds, such as HAL and FAL, are present because inhibition mitigation can interfere with anaerobic digestion optimal conditions.

In order to mitigate HAL and FAL inhibition on anaerobic hydrolysis, HM should be removed from the (co)-digestion substrates or inactivated, by binding them to an inorganic compound. Removal of HAL from surface water has been successfully achieved by several authors using techniques such as: coagulation by aluminium sulphate or iron chloride or calcium hydroxide; adsorption with natural absorbents, such as powdered activated carbon (PAC) and with cationic polymers, such as polyethylenimines (PEI's); and by precipitate flotation using cationic surfactants, such as cetyl trimethyl ammonium bromide (CTAB) and dodecylamine (DDA) (Brum and Oliveira, 2007). Combining coagulants and adding flocculants, such as calcium hydroxide with iron, have been shown to improve HAL removal efficiencies and to improve setting. However, this process has the disadvantage of increasing metal concentration in the liquid phase (Renou et al., 2008). Coagulation-flocculation techniques have been reported for removal of HAL from landfill leachates, both as pre- and post-treatment (Tzoupanos et al., 2008). Renou et al. (Renou et al., 2008), in their review paper on landfill leachate treatment options, indicated that flotation can remove almost 60% of the HAL and biofloculation more than 85%. The most efficient processes for HAL and FAL removal reported are, however, reverse osmosis (Renou et al., 2008) and ion exchange resins, such as MIEX®, for which a 90% HAL and FAL removal from ground water was reported (Fearing et al., 2004). However in all these studies the solids content was low, which is not the case for agricultural biowaste, making it more difficult to use such techniques. In high solids content waste streams, the HAL and FAL become less accessible, therefore interfering with the efficiency of the above mentioned HM removal technique. To reduce the solids concentration, a solid-liquid separation process needs to be applied. This includes separation by settling tanks or by centrifuges; mechanical removal of solids by forced filtration with screw presses; and drainage through fabric belts or screens (Hjorth et al., 2010). Since most of these processes are high in energy demand, they are economically less attractive for small-scale biogas plants. However, for large-scale plants they can be economically feasible as the removed HM have commercial value as a soil conditioner. Therefore, in order to accelerate the overall treatment

process and to enable HM removal, more complex systems should be implemented, as shown in Figure 7.1.

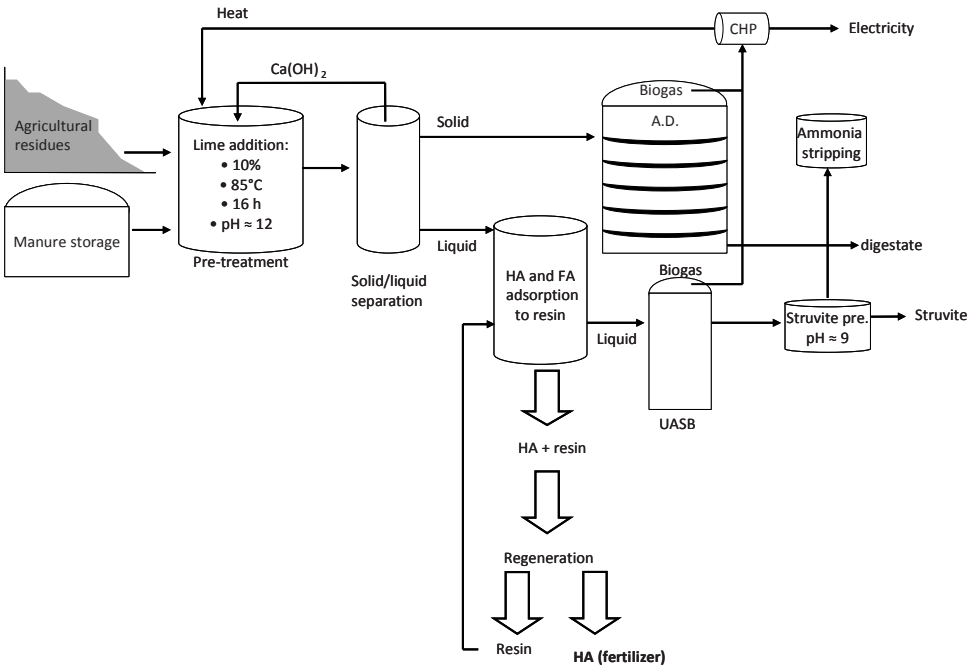


Figure 7.1. Optional set-up for large-scale biowaste treatment.

In this large-scale case, the precipitation of the HM with an ion exchange resin will enable HM removal. Moreover, anaerobically digesting the solids separated from the liquid will enable application of different operational conditions and recuperation of nutrients in inorganic fertilizer form, such as magnesium-ammonium-phosphates or struvite. Finally, pre-treating the lignocellulosic biomass with chemicals, such as calcium hydroxide under high temperature, will increase solubility and biodegradability, and reduce the time of treatment.

Pre-treatment methods, which vary according to the type of substrate and operational conditions, have been recently described in the reviews of Hendriks and Zeeman (2009), Mosier et al. (2005) and Taherzadeh and Karimi (2008) with regard to lignocellulosic biomass, Hjorth et al. (2010) regarding animal slurry, and Renou et al. (2008) with respect to landfill leachate. In this thesis, in **Chapter 6**, a pre-treatment method - maleic acid at 150°C -, which is not included in the mentioned reviews, was tested, in comparison with ammonium and calcium

hydroxide thermo-chemical pre-treatments. The results indicated that maleic acid is a very good pre-treatment, having the highest solubilisation of COD from the three tested methods. However, it is also the most expensive chemical, as recently analysed by Kootstra et al. (2009) who concluded that it costed 65 €·Mg^{-1} of dry feedstock. Another chemical investigated in **chapter 6**, was calcium hydroxide (lime). This chemical pre-treatment was not as effective as maleic acid in terms of COD solubility, but showed the highest improvement in terms of biodegradability of lignocellulosic biomass with high lignin content, such as bracken. Lime has the advantages of being inexpensive, safe to handle, also efficient at ambient temperatures, and recoverable from liquid as calcium carbonate (Mosier et al., 2005). Lime pre-treatment removes the lignin, acetyl and uronic substitutions on hemicellulose, therefore increasing accessibility of hydrolytic enzymes to cellulose and hemicellulose (Chang and Holtzapple, 2000). Under elevated temperatures, usually 85 to 150°C, the pre-treatment time is significantly reduced. This, however, does not influence the efficiency of the lime treatment, since identical digestibility (above 70%) has been reported for sugarcane bagasse at ambient temperatures for 192h, and at 120°C for 1h (Mosier et al., 2005). Therefore, lime addition at ambient temperatures is a good option for small-scale biogas plants, since farmers can just spray diluted lime (about 10%) onto the agricultural residues and store it for a few days, as shown in Figure 7.2.

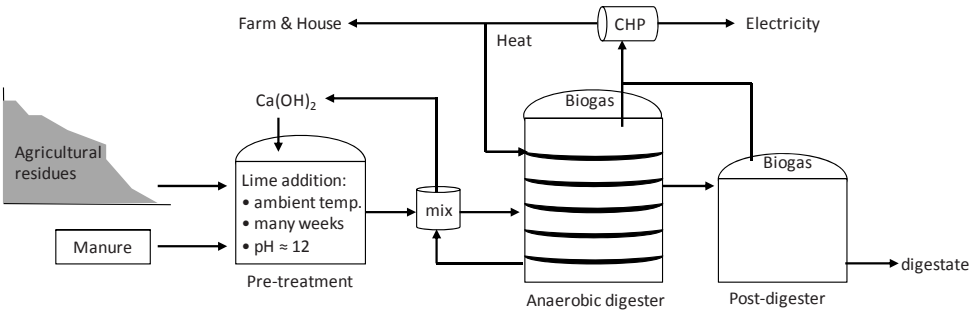


Figure 7.2. Suggested set-up for small-scale biowaste treatment.

Moreover, it is expected that, together with the breaking up of the lignocellulosic biomass, part of the HM will bind to the calcium, as at high pH the proton-binding sites are sufficiently dissociated (Tipping, 2002). Animal manure also contains high orthophosphate concentrations, which varies according to the type of animal, diet and storage of the manure (Hjorth et al., 2010). When adding calcium to the liquid fraction of manure, the orthophosphate might compete with the HM for calcium binding. However, this binding is strongly dependent on the

pH, concentrations of calcium, carbonate, orthophosphate and HM, and the binding sites. According to Borggaard et al. (2005), the amount of HAL adsorbed to iron and aluminium, which are good binding compounds, was higher than for phosphate, however the difference was not significant. This small difference is suggested to be due to the fact that HAL and phosphate bind differently to carboxylic groups, depending whether they occur isolated as single groups or as neighbouring COOH groups in aliphatic or aromatic structures (Borggaard et al., 2005). It should also be taken into consideration that animal manure contains ammonium and magnesium, and at pH > 9 struvite might be formed (Renou et al., 2008). In order to show the behaviour of calcium with HM and phosphate during pre-treatment and anaerobic digestion, model simulations with OLI Stream analyser (OLI Systems, NJ, USA) were performed and are here presented. The input data included NH_4^+-N, P, K, Cl, S, Ca and Mg concentrations from a mixture of dairy manure and food waste (El-Mashad and Zhang, 2007). Bicarbonate was adjusted so the stream had a neutral charge and the temperature was set to 35°C. Citric acid was used to simulate HM. Its three pka's are 3.13, 4.76 and 6.4. Calcium hydroxide was continuously added during simulation, as it would be the case in the pre-treatment suggested in Figure 7.2. The results indicate that at pH above 9.5 most of the calcium precipitates with carbonate forming calcite (Figure 7.3). The citric acid and phosphate present precipitated almost completely with calcium, leaving only a very small amount in the aqueous form, which is also bond to calcium or to magnesium. At a pH of 7.5, which is commonly found in anaerobic co-digesters, the binding of citric acid and phosphate is identical, however for the citric acid, more than half of it will be in the aqueous form ($Ca[C_6H_5O_7]^{-1}$). Struvite precipitation was not observed at the increasing pH, most likely because all the phosphate had a preference to precipitate with calcium forming hydroxyapatite ($Ca_5(OH)(PO_4)_3$) instead of struvite.

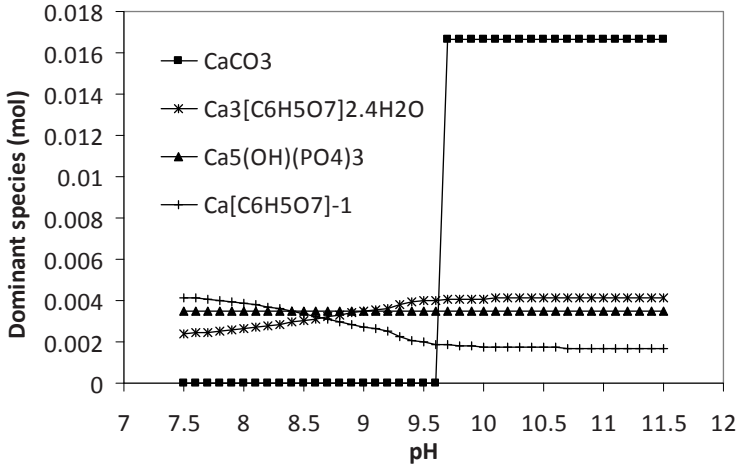


Figure 7.3. Predicted calcium bond compounds at varying pH.

These simulations, even though not performed with HM, give the indication that the HAL and phosphate, when excess of calcium is added as in the proposed pre-treatment, will bind to calcium and remain bonded throughout the biogas plant, therefore only leaving the treatment system in the form of digestate. However, these conclusions should be experimentally confirmed, before implementation.

In any case, having precipitation of HM with inorganics, such as Ca, Mg, K, P and N will only enrich the digestate, and therefore the fertilizer, as HM are known to increase soil quality and plant growth (Demirbas et al., 2006). The major concern is the ratio that these compounds will be present in the fertilizer in relation to the nutrients demand of the soil. Calcium is also needed for plant growth, however since an excess is being added for the pre-treatment it would be advantageous to reuse part of this calcium. When mixing the pre-treatment effluent with the liquid from the anaerobic digester, the pH will decrease and set to about 7.5. If needed acid addition can be applied to adjust the pH. At a pH of about 7.5, a third of the calcium will be present in the aqueous form, which can then be recycled to the pre-treatment process, as suggested in Figure 7.2.

From the above considerations it becomes clear that many possible interactions can be expected when pre-treating biowaste with calcium hydroxide. In choosing the most proper pre-treatment method some pre-conditions should be met, such as:

- Enhancement of agricultural residues digestibility, by removing lignin and making hemicellulose and cellulose available for hydrolytic enzymes. Therefore, reducing the HRT and increasing biogas yield.
- Enabling reuse of the pre-treatment chemical or upgrade the added chemical to a valuable fertilizer.
- Removal or inactivation of the HM binding sites before anaerobic digestion, thereby mitigating hydrolysis inhibition.
- Usage of the removed or bond HAL to enrich the inorganic fertilizer.

Hydrolysis enhancement can be achieved by increased accessibility of the biodegradable substrate and mitigation of inhibiting compounds, such as HAL and FAL. Mitigating the inhibition can be achieved either by removing the inhibiting compound, or by making it inactive, or by reducing the inhibitory effect with external agents.

7.5 Optimizing anaerobic hydrolysis with enzyme addition

Enzyme addition (shortly discussed at the end of **chapter 2**) has recently gained the attention of biogas plants' operators, because it has been shown that hydrolytic enzymes can accelerate, and even sometimes increase biogas production (Romano et al., 2009; Yunqin et al., 2010). Even though in some cases enzyme addition accelerates the hydrolysis, the mechanisms behind the observed phenomenon are not fully understood. Therefore, further research is needed to determine how the operational conditions, substrate characteristics, enzyme activity, and enzyme concentrations can influence the impact of enzyme addition on hydrolysis (Romano et al., 2009). When the microbial biomass is well adapted to the conditions in the reactor, and no inhibition is present, the acidogenic bacteria should be able to produce enough enzymes to hydrolyse the substrate (Sanders, 2001). Therefore, hydrolysis enhancement by enzyme addition can only be expected when the acidogenic bacteria are not able to produce enough hydrolytic enzymes and/or the enzymes are inhibited, which is the case for the experiments performed in **chapter 5**.

7.6 Future research

Results described in this thesis clearly shows that hydrolysis is inhibited by HAL and FAL. Therefore, research on the inhibition mechanisms, such as competitive or non-competitive, and inhibiting concentrations should be determined. In addition, experiments need to be conducted to elucidate the possible relation between the substrate characteristics and the degree of hydrolysis inhibition. Moreover, it is of interest to assess how HM compounds, known for their complex aromatic structure and reactive nature, such as lignin and quinones, impact anaerobic hydrolysis. Basic understanding of the mentioned factors is in fact a prerequisite for successfully mitigating possible inhibition effects.

Future research must include the search for non-expensive, easily available and safe to use mitigating agents, allowing not only for large-scale but also small-scale decentralised applications.

Another very important aspect that should be further investigated is the addition of hydrolytic enzymes to anaerobic digesters. It is important to understand the mechanisms and consequences of enzyme addition and, in relation to the substrate characteristics, determine the optimum type and enzyme concentrations for hydrolysis enhancement and/or mitigating its inhibition.

Summary

The increasing demand of renewable energy sources and reuse of wastes, challenges our society for better technological solutions for energy production. Co-digestion of agricultural biowaste, such as animal manure and plant residues, offers an interesting contribution to the renewable energy strategies. The numbers of full scale biogas plants is steadily increasing worldwide (Álvarez et al., 2010; EIA, 2009; Weiland, 2006). Biogas plants convert complex substrates, such as biowaste, into biogas that e.g. can be transformed to electrical energy and heat, which can be used in the farm and delivered to the main electricity grid. Due to its decentralised nature, the implementation of small-scale biogas plants can supply renewable energy to people without the need for large-scale infrastructural networks such as electricity grids, thereby solving part of the populations' energy demands (**chapter 1)**. In **chapter 2**, a review on the anaerobic hydrolysis step is presented. Hydrolysis – the first step of anaerobic digestion - is considered as the rate limiting step in the anaerobic digestion of complex waste, such as agricultural biowaste (Pavlostathis and Giraldo-Gomez, 1991). However, the hydrolytic process is still poorly described (Vavilin et al., 2008), which results in non-optimized digester volumes. Therefore, for properly designing anaerobic digesters, not only the biodegradability, or bio-methane potential (BMP), of complex waste needs to be assessed, but also the hydrolysis rate, which determines the maximum biogas production rate (El-Mashad, 2003). Hydrolysis enhancement can only be achieved when its biochemistry is unravelled and quantified (**chapter 2**). Moreover, it is essential to determine what is exactly impacting the hydrolysis step. The inhibition phenomena are thoroughly researched and **chapters 3 and 5** describe the effects of ammonia and humic matter on the hydrolysis of cellulose, a polysaccharide, and tributyrin, a lipid. The results from **chapter 3** indicate that ammonia nitrogen in the range of 2.4 to 7.8 g NH_4^+-N·l^{-1} (283 to 908 mg NH_3-N·l^{-1}) does not inhibit the hydrolysis of tributyrin. This was also verified for the hydrolysis of cellulose where additions of ammonia nitrogen in the range of 2.4 to 7.8 g NH_4^+-N·l^{-1} (333 to 957 mg NH_3-N·l^{-1}) were applied. In this chapter it was also possible to conclude that the lag phase of about 4 days observed during cellulose hydrolysis is commonly found for experiments were manure and leachate, which is more or less similar to manure in composition, are added (O'Sullivan et al., 2008; Song et al., 2005). This lag phase seems therefore to indicate, as suggested by Van Velsen (1981) and Zeeman (1991), that there are compounds present in the soluble fraction of animal manure that could cause hydrolysis inhibition. Brons et al. (1985) reported that HAL inhibit the hydrolysis of soluble protein. Therefore the amount and

characteristics of HAL and FAL extracted from fresh cow manure and silage maize were investigated in **chapter 4**. It was concluded that fresh cow manure has about 7 g HAL·kg^{-1} of fresh matter (0.7 % w/w) and about 1.3 g FAL·kg^{-1} of fresh matter (0.1 % w/w), while silage maize has about 18 g HAL·kg^{-1} of fresh matter (1.5 % w/w) and about 3.5 g FAL·kg^{-1} of fresh matter (0.4 % w/w). In order to understand the composition of the HAL and FAL, a detailed chemical characterization was performed with state of the art techniques, such as elemental analysis, ^{13}C CPMAS NMR and Pyrolysis GC/MS. Elucidation of the carboxylic and phenolic functional groups of the HAL and FAL, by direct titrations, was subsequently investigated in order to, in future works, predict their reactivity during anaerobic hydrolysis, since they are reported to be the reactive part of the HAL and FAL. In **chapter 5**, the effect of the HAL and FAL extracted from fresh cow manure and silage maize on the hydrolysis was investigated. It was concluded that HAL and FAL from maize and cow manure, in the ranges from 0.5 to 5 g·l^{-1}, clearly inhibit the hydrolysis of cellulose. The hydrolysis of tributyrin was inhibited by HAL from maize and cow manure, in the ranges from 0.5 to 5 g·l^{-1}. However no clear inhibition by FAL from maize and cow manure could be determined. The latter might be due to differences in the HAL and FAL structural characteristics, and/or the mechanisms involved in the binding of the HAL and FAL to the enzymes. It possibly might be that the HAL and FAL are able to form complexes with the lipid, therefore making it still available for enzymatic hydrolysis.

Besides the investigation of inhibiting compounds on anaerobic hydrolysis, pre-treatment of plant matter was also researched. Plant matter is high in lignocellulosic biomass. Lignocellulosic biomass consists of lignin, which is resistant to anaerobic degradation, cellulose and hemicellulose, and these fractions vary quantitatively, qualitatively and structurally according to the plant matter (Hatfield and Fukushima, 2005). Pre-treatment of plant material is particularly important in order to increase biogas production during co-digestion of manure with agriculture residues. Therefore, in **chapter 6**, three plant species, hay, straw and bracken, with varying lignin content, were thermo-chemically pre-treated with calcium hydroxide, ammonium carbonate and maleic acid and then anaerobically digested for 40 days. From the pre-treatment and subsequent anaerobic digestion experiments it was concluded that when the lignin content of the plant material is high, thermo-chemical pre-treatments have a positive effect on the biodegradability of the substrate. Calcium hydroxide pre-treatment improves the biodegradability of lignocellulosic biomass, especially for high lignin content substrates, like bracken. Maleic acid generates the highest percentage of dissolved COD during pre-treatment. Ammonium pre-treatment only showed a clear effect on biodegradability for straw.

In **chapter 7** the results of all chapters are discussed and solutions for further research are presented. A complete overview of this thesis outcome and pre- and post-treatment options are given in Figure 7.4.

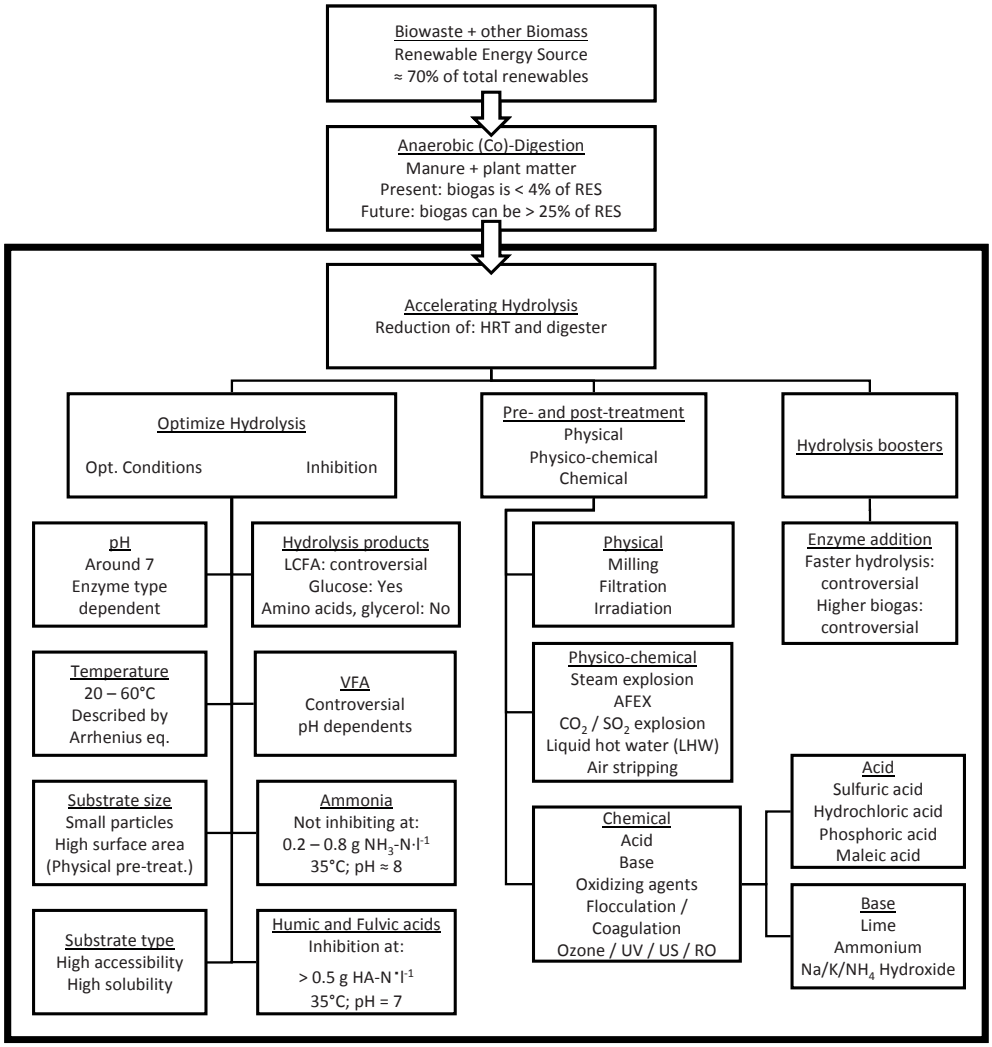


Figure 7.4. Overview of outcome of thesis.

Samenvatting

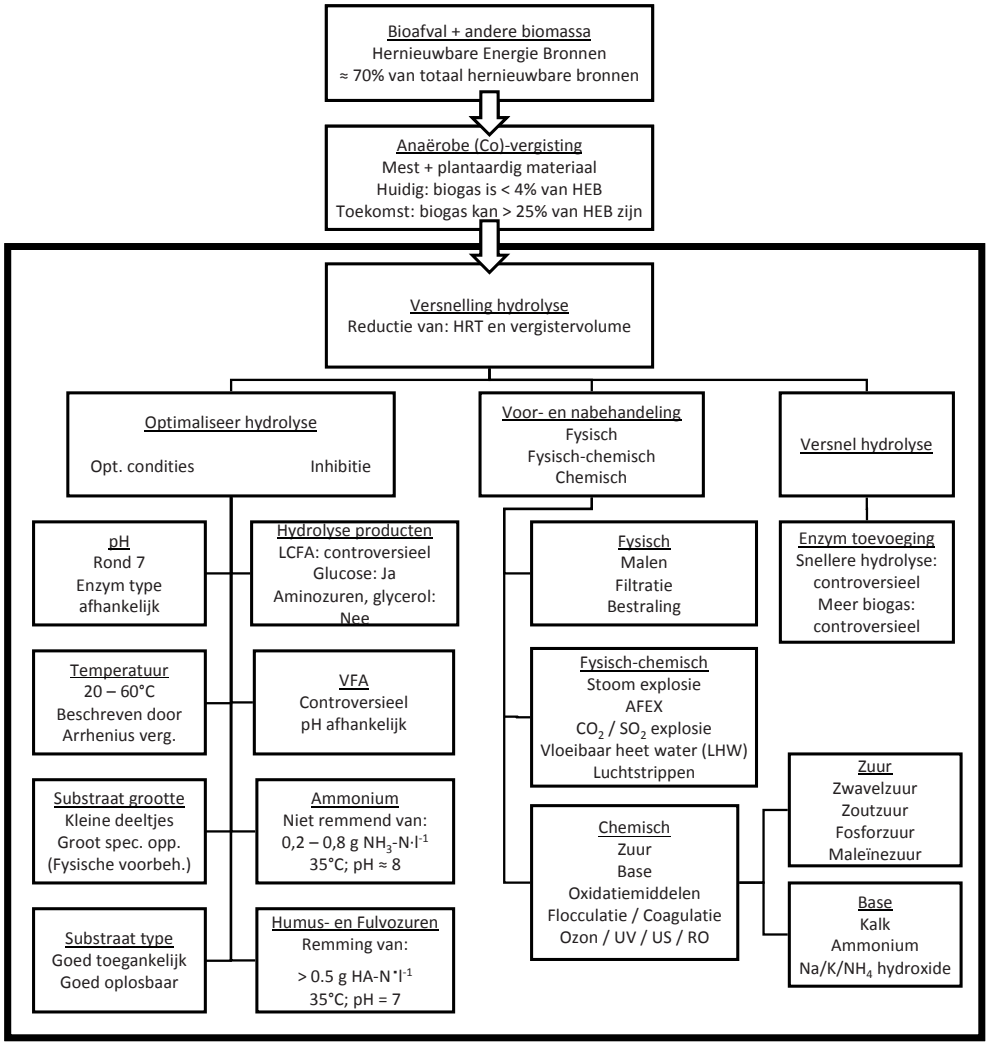
De toenemende vraag naar hernieuwbare energiebronnen en hergebruik van afvalstoffen, daagt onze samenleving uit voor betere technologische oplossingen voor de productie van energie. Co-vergisting van agrarisch bioafval, zoals dierlijke mest en plantaardige resten, biedt een interessante bijdrage in de transitie naar hernieuwbare energie. Het aantal biogasinstallaties groeit gestaag in de wereld (Álvarez et al., 2010; EIA, 2009; Weiland, 2006). Biogasinstallaties zetten complexe substraten, zoals bioafval, om in biogas. Het biogas kan bijvoorbeeld worden omgezet in elektrische energie en warmte, waarna vervolgens de warmte kan worden gebruikt op de boerderij en de elektriciteit kan worden afgegeven aan het elektriciteitsnet. Vanwege de decentrale aard kunnen kleinschalige biogasinstallaties hernieuwbare energie leveren aan mensen, zonder de noodzaak voor grootschalige infrastructurele netwerken, zoals elektriciteitsnetten, en waarmee tevens invulling wordt gegeven aan de energievraag van een deel van de bevolking (hoofdstuk 1). In hoofdstuk 2 wordt een overzicht van de anaërobe hydrolyse gepresenteerd. De hydrolyse – de eerste stap van anaërobe gisting – wordt beschouwd als de snelheidsbepalende stap in de anaërobe afbraak van complexe afvalstoffen, zoals agrarisch biologisch afval (Pavlostathis en Giraldo-Gomez, 1991). Echter, de hydrolyse is nog steeds onvoldoende beschreven (Vavilin et al., 2008), wat resulteert in niet-geoptimaliseerde vergistervolumes. Daarom dient voor het gedegen ontwerpen van anaërobe vergisters, niet alleen de biologische afbreekbaarheid, of bio-methaan potentieel (BMP), van complexe afvalstoffen te worden beoordeeld, maar ook de hydrolysesnelheid, die de maximale productiesnelheid van biogas bepaalt (El-Mashad, 2003). Verbetering van de hydrolyse kan alleen worden bereikt wanneer haar biochemie is ontrafeld en gekwantificeerd (hoofdstuk 2). Bovendien is het van essentieel belang om te bepalen wat precies invloed heeft op de hydrolyse. De inhibitie van de hydrolyse wordt grondig onderzocht en de hoofdstukken 3 en 5 beschrijven de effecten van ammoniak en humus op de hydrolyse van cellulose, een polysaccharide, en tributyrin, een lipide. Uit de resultaten van hoofdstuk 3 blijkt dat ammonium-stikstof in de range van 2,4 tot 7,8 g NH_4^+-N·l^{-1} (283 tot 908 mg NH_3-N·l^{-1}) de hydrolyse van tributyrin niet remt. Dit werd bevestigd voor de hydrolyse van cellulose, waar toevoegingen van ammonium-stikstof in de range van 2,4 tot 7,8 g NH_4^+-N·l^{-1} (333 tot 957 mg NH_3-N·l^{-1}) werden toegepast. In dit hoofdstuk wordt tevens geconcludeerd dat een opstartfase voor de hydrolyse van cellulose normaliter ongeveer 4 dagen in beslag neemt, indien mest en percolaat, dat min of meer in samenstelling vergelijkbaar is met mest, worden toegevoegd (O'Sullivan et al., 2008; Song et al., 2005). Deze opstartfase lijkt dan ook aan te

geven, zoals voorgesteld door Van Velsen (1981) en Zeeman (1991), dat er in de opgeloste fractie van dierlijke mest verbindingen aanwezig zijn die de hydrolyse-inhibitie zouden kunnen veroorzaken. Brons et al. (1985) rapporteerde dat humuszuur-achtigen (HAL) de hydrolyse van oplosbaar eiwit remt. Daarom werd de hoeveelheid en de kenmerken van HAL en FAL (fulvozuur-achtigen) uit verse koeienmest en snijmaïs onderzocht in hoofdstuk 4. Geconcludeerd werd dat verse koeienmest ongeveer 7 g HAL·kg^{-1} vers materiaal (0,7% w/w) en ongeveer 1,3 g FAL·kg^{-1} vers materiaal (0,1% w/w) bevat, terwijl snijmaïs ongeveer 18 g HAL·kg^{-1} vers materiaal (1,5% w/w) en 3,5 g FAL·kg^{-1} vers materiaal (0,4% w/w) bevat. Om de samenstelling van HAL en FAL te begrijpen is een gedetailleerde chemische karakterisering uitgevoerd met state of the art technieken, zoals elementaire analyse, ^{13}C NMR CPMAS en pyrolyse GC/MS. Vervolgens zijn door middel van rechtstreekse titraties de functionele carbonzuur- en fenolgroepen van HAL en FAL opgehelderd, om in toekomstig onderzoek hun reactiviteit tijdens anaërobe hydrolyse te voorspellen. Dit is van belang, aangezien zij gerapporteerd worden als het reactieve deel van HAL en FAL. In hoofdstuk 5 is het effect van HAL en FAL uit verse koeienmest en snijmaïs op de hydrolyse onderzocht. Geconcludeerd werd dat HAL en FAL uit maïs en koeienmest, in de range van 0,5 tot 5 g·l^{-1}, duidelijk de hydrolyse van cellulose remmen. De hydrolyse van tributyrin werd geremd door HAL uit maïs en koeienmest, in de range van 0,5 tot 5 g·l^{-1}. Er is echter geen duidelijke inhibitie door FAL uit maïs en koeienmest vastgesteld. Dit laatste kan te wijten zijn aan verschillen in structurele kenmerken van HAL en FAL en/of de mechanismen die betrokken zijn bij de binding van HAL en FAL aan de enzymen. Het zou eventueel kunnen dat HAL en FAL in staat zijn om complexen te vormen met een lipide, waardoor het dus nog steeds beschikbaar is voor enzymatische hydrolyse.

Naast het onderzoek van remmende stoffen op anaërobe hydrolyse, is tevens voorbehandeling van plantaardig materiaal onderzocht. Plantaardige materialen bevat veel lignocellulose biomassa. Lignocellulose biomassa bestaat uit lignine, dat resistent is tegen anaërobe afbraak, cellulose en hemicellulose. Deze fracties verschillen kwantitatief, kwalitatief en structureel, afhankelijk van het type plantaardig materiaal (Hatfield en Fukushima, 2005). Voorbehandeling van plantaardig materiaal is vooral belangrijk met het oog op toename van de productie van biogas tijdens de co-vergisting van mest met agrarische residuen. Daarom wordt in hoofdstuk 6, drie plantensoorten, hooi, stro en varens, met wisselende hoeveelheid lignine, thermo-chemisch voorbehandeld met calciumhydroxide, ammoniumcarbonaat en maleïnezuur en vervolgens gedurende 40 dagen anaëroob vergist. Vanuit de experimenten met de voorbehandeling en de daaropvolgende anaërobe vergisting werd geconcludeerd dat wanneer het

plantaardige materiaal veel lignine bevat, de thermo-chemische voorbehandelingen een positief effect hebben op de biologische afbreekbaarheid van het substraat. Voorbehandeling met calciumhydroxide verbetert de biologische afbreekbaarheid van lignocellulose biomassa, vooral bij substraten met hoge ligninegehalten, zoals varens. Maleïnezuur genereert het hoogste percentage van opgeloste COD tijdens de voorbehandeling. Voorbehandeling met ammonium toonde alleen een duidelijk effect op de biologische afbreekbaarheid voor stro.

In hoofdstuk 7 worden de resultaten van alle hoofdstukken besproken en worden oplossingen voor verder onderzoek gepresenteerd. Een compleet overzicht het resultaat van dit proefschrift en de voor- en nabehandelingopties zijn weergegeven in figuur 7.4.



Figuur 7.4. Overzicht van de resultaten van het proefschrift.

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About the author

Tânia Vasconcelos Fernandes was born on the 25th of August of 1976 in Lisbon, Portugal. In 2003 she obtained her diploma in Environmental Engineering at the Universidade Lusófona de Humanidades e Technologia, Lisbon. Her final internship, in 2003, was on the feasibility of anaerobic digestion of concentrated black water and kitchen refuse within the DESAR (Decentralized Sanitation and Reuse) concept at the sub-department of Environmental Technology at Wageningen University, The Netherlands. At the end of 2004 she started her PhD at this same department. Improving biowaste and wastewater treatment with sustainable solutions is one of her main goals.

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C E R T I F I C A T E

The Netherlands Research School for the
Socio-Economic and Natural Sciences of the Environment
(SENSE), declares that

Tânia Vasconcelos Fernandes

born on 25 August 1976 in Lisbon, Portugal

has successfully fulfilled all requirements of the
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Wageningen, 10 September 2010

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