Biogas production from lignocellulosic agricultural residues

Microbial approaches for enhanced efficiency

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Cover: Scanning Electron Microscope (SEM) photo showing pure cultured *Clostridium* sp. Bciso-3 degrading cellulose, isolated from an industrial-scale anaerobic digester.
(Photo: Tong Liu. Colorized by Johnny Isaksson)

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Abstract

Methane, produced through microbial anaerobic digestion of various organic materials, is seen as a promising sustainable bioenergy source with the potential to reduce the current dependence on fossil fuels. Among organic materials, lignocellulosic materials, especially agriculture residues, are highly interesting due to high abundance and potential for methane production. However, low nutrient content and highly recalcitrant structure often limit process efficiency. This thesis presents the results of in-depth studies conducted in order to obtain new information about lignocellulose-degrading bacteria in biogas processes and to identify ways to enable more efficient biogas production.

Different biogas processes were investigated in terms of their overall microbial community (bacteria and archaea) and potential lignocellulose degraders. The results showed that the biogas processes differed with regard to overall microbial community and chemical composition, but also composition of the cellulose-degrading bacterial community. These differences significantly influenced the degradation efficiency of both cellulose and wheat straw in batch digestion systems and also performance during start-up of semi-continuous stirred tank reactor (CSTR) processes. A positive correlation was found between lignocellulose degradation efficiency and relative abundance of *Clostridium cellulolyticum*. Ammonia level in the inoculum was identified as the most significant factor potentially affecting microbial community structure and methane production from lignocellulosic materials. Microbial and chemical composition of the original inoculum sources also influenced long-term degradation of lignocellulose in CSTR and appeared to influence residual methane potential. Different molecular methods for microbial community analysis were explored, with the aim of building an appropriate pipeline for in-depth studies of lignocellulose degraders in anaerobic reactors.

This thesis provides novel information about the microbial communities involved in degradation of lignocellulosic materials and possible connections to process parameters. This information could potentially enable biogas production to be steered towards a more efficient and controllable process for degradation and biogas production from agriculture residues and plant-based materials.

Keywords: anaerobic digestion, lignocellulose, glycoside hydrolase families 5 and 48, biomethane potential, continuous stirred-tank reactor, co-digestion, residual methane potential, next-generation amplicon sequencing, terminal restriction fragment length polymorphism (T-RFLP).

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Biogasproduktion från lantbrukets lignocellulosrika restprodukter. Mikrobiella tillvägagångssätt för ökad effektivitet

Sammanfattning

Metan, som produceras genom mikrobiell nedbrytning av olika organiska material under anaeroba förhållanden, ses som en lovande hållbar bioenergikälla med potential att minska det nuvarande beroendet av fossila bränslen. I detta sammanhang representerar jordbruksrester, som finns tillgängligt i stor mängd, en stor metanpotential. Tyvärr har denna typ av material ofta ett lågt näringsinnehåll och ett högt innehåll av lignocellulosa, som är svårt att bryta ner och därför begränsar processens effektivitet. Denna avhandling presenterar resultat från studier som genomförts för att ta fram ny information om bakterier som bryter ner lignocellulosa i biogasprocesser. Målet var att identifiera sätt att möjliggöra en effektivare biogasproduktion.

Olika biogasprocesser undersöktes med avseende på sammansättningen av det mikrobiella samhället (bakterier och arkeer) och bakterier med potentiell förmåga att bryta ner lignocellulosa. För den mikrobiella analysen användes olika molekylära metoder. Resultaten visade att de olika biogasprocesserna var olika i avseende både till den kemiska sammansättningen och det mikrobiella samhället, inklusive de cellulosanedbrytande bakterierna. Dessa skillnader påverkade signifikant nedbrytningseffektiviteten av cellulosa och vetehalm i satsvisa metanproduktionsprocesser. Under dessa försök identifierades en negativ korrelation mellan nedbrytningseffektiviteten och halten ammoniak, samt en positiv korrelation med mängden av en specifik cellulosanedbrytande bakterie, *Clostridium cellulolyticum*. Uppstart av semi-kontinuerligt omrörda biogasreaktorer (CSTR) visade också tydliga skillnader i processprestanda beroende på ympens ammoniakhalt och på sammansättningen av det mikrobiella samhället. En koppling mellan låg nedbrytningseffektivitet och resterande metanpotential identifierades också.

Kunskap som genererats i denna avhandling kan potentiellt möjliggöra styrning mot en mer effektiv och kontrollerbar process för nedbrytning och biogasproduktion från jordbruksrester och växtbaserade material.

Nyckelord: anaerob nedbrytning (rötning), lignocellulosa, glykosidhydrolas familj 5 och 48, biometanpotential, CSTR, samrötning, restgas produktion, pyrosekvensiering, T-RFLP.

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Dedication

To me, myself, my mom: 丹, my wife: 馨, and my cat: Wasabi

“虽然你不是最聪明的，但是你一直在慢慢的进步。当别人选择放弃时，你从来没有停下脚步。”

我的母亲：周丹

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Liu, T., Sun, L., Müller, B. and Schnürer, A.* (2016). The microbial community structure in industrial biogas plants influences the degradation rate of straw and cellulose in batch tests. *Biotechnology for Biofuels* 9, 1-20.

II Liu, T., Sun, L., Müller, B. and Schnürer, A.* (2017). Importance of inoculum source and initial community structure for biogas production from agricultural substrates. *Bioresource Technology* 245, 768-777.

III Liu, T., Sun, L., Nordberg, Å. and Schnürer, A.* (2018). Substrate-induced response in biogas process performance and microbial community relates back to inoculum source. *Microorganisms* 6, 80-99.

IV Ahlberg-Eliasson, K., Liu. T., Nadeau, E. and Schnürer, A.* (2018). Forage types and origin of manure in codigestion affect methane yield and microbial community structure. *Grass and Forage Science* 73, 740-757.

* Corresponding author.

In addition to Paper I-IV, I contributed to the following paper within the time frame of this thesis work:

Šafarič, L., Yekta, S.S.[*], Liu, T., Svensson, B.H., Schnürer, A., Bastviken D. and Björn, A. (2018). Dynamics of a perturbed microbial community during thermophilic anaerobic digestion of chemically defined soluble organic compounds. *Microorganisms* 6, 105-118.

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My contribution to the papers included in this thesis was as follows:

I. Participated in planning the study and analysing the results. Performed some molecular work, monitored the reactors and was engaged in writing the article.

II. Participated in planning the study and analysing the results. Performed all molecular work and was involved in reactor operation. Main writer of the article.

III. Participated in planning the study and analysing the results. Performed all molecular work and was involved in reactor operation. Main writer of the article.

IV. Participated in analysing the results. Performed some molecular work and was engaged in writing the article.

List of figures

Abbreviations

AD	Anaerobic digestion
BMP	Biomethane potential
CSTR	Continuously stirred tank reactor
GH	Glycoside hydrolase
HRT	Hydraulic retention time
LC-MS	Liquid chromatography-mass spectrometry
NGS	Next-generation sequencing
OLR	Organic loading rate
PCR	Polymerase chain reaction
Qiime	Quantitative insights into microbial ecology
qPCR	Quantitative polymerase chain reaction
RMP	Residual methane potential
SBS	Sequencing by synthesis
SMP	Specific methane production
T-RFLP	Terminal restriction fragment length polymorphism
T-RFs	Terminal restriction fragments
VFA	Volatile fatty acid
WWTP	Wastewater treatment plant

1 Introduction

The development of the petroleum industry has led to a rapid rise in the world economy in the past century. However, the underlying resource, fossil fuel, is recognised as a limited energy resource. Furthermore, emissions of greenhouse gases (*e.g.* fossil fuel-derived carbon dioxide (CO_2) emissions) have become a global concern, since about 88% of global energy consumption derives from fossil fuels (Achinas *et al.*, 2017; Agency, 2015). To meet the environmental challenges and overcome the dependence on fossil fuel, European Union (EU) member states have decided to increase the proportion of renewable energy to 20% of total consumption by 2020 (Karmellos *et al.*, 2016). Progress towards this target is measured every two years and was proposed on 30 November 2016 to reach at least 27% renewables in final energy consumption in the EU by 2030 (Scarlat *et al.*, 2018).

Biogas is seen as one of the most important renewable energy resources that can replace part of the fossil fuel-based energy used today, and it shows great potential and many advantages, including both climate and economic benefits (Meyer-Aurich *et al.*, 2016). A biogas process can be implemented in small or large scale, which is important when designing flexible and sustainable energy solutions in both industrialised and developing countries (Holm-Nielsen *et al.*, 2009). Materials that can be used for biogas production include various types of waste products, such as manure, straw, municipal wastewater, food waste *etc.*, and dedicated energy crops (Vasco-Correa *et al.*, 2018; Appels *et al.*, 2011). Among these substrates, lignocellulosic materials, such as agricultural residues, are of great interest due to their high abundance and potential for biogas production (Azman *et al.*, 2015). By controlled use of wastes in a biogas process rather than *e.g.* dumping household waste in landfill or storing farm manure in open tanks, it is possible not only reduce the number of waste deposits, but also to decrease emissions of carbon dioxide and other greenhouse gases (Borjesson & Mattiasson, 2008). The biogas produced, containing the energy carrier methane, can be used for production of heat,

electricity and vehicle fuel after upgrading (removal of carbon dioxide and trace gases) (Holm-Nielsen *et al.*, 2009). The residues left after biogas production are rich in mineral nutrients and can be used as a fertiliser during crop production to replace fossil energy-requiring mineral fertilisers, thus enabling recycling of nutrients between urban and rural areas (Vasco-Correa *et al.*, 2018; Möller & Müller, 2012; Weiland, 2010; Holm-Nielsen *et al.*, 2009).

Microorganisms are essential for degrading organic material to biogas, in a process that involves various anaerobic digestion pathways and requires the combined activity of several groups of microorganisms with differing metabolic capacities (Angelidaki *et al.*, 2011). To obtain a stable biogas process, all these conversion steps and microorganisms must work in a synchronised manner (Vanwonterghem *et al.*, 2014a). When plant-based materials (*e.g.* agricultural residues) are used for biogas production, the first step of the microbiological process, hydrolysis, becomes rate-limiting. It has been suggested that the crystalline structure of the lignocelluloses obstructs degradation in the initial step, and thus the hydrolysis of these insoluble compounds becomes slow (Mulat & Horn, 2018; Lynd *et al.*, 2002a; Noike *et al.*, 1985).

Some of the obstacles with degradation of these types of materials can be overcome by various pre-treatment methods, making the material more accessible to microbial and enzymatic attack (Martínez-Gutiérrez, 2018). An alternative strategy is to increase the efficiency of the active microbial community. Numerous studies have been devoted to examining anaerobic cellulose-degrading bacteria and their enzymatic capabilities, in efforts to clarify the degradation mechanisms and identify ways to enhance degradation rates. Most of these studies have been performed on samples from gut and soil ecosystems (Tsavkelova & Netrusov, 2012; Lynd *et al.*, 2002a) (Do *et al.*, 2018; Ransom-Jones *et al.*, 2012; Morrison *et al.*, 2009b; Miron *et al.*, 2001), while only a few have examined cellulose-degrading bacteria in biogas digesters (Jia *et al.*, 2018; Bozan *et al.*, 2017; Azman *et al.*, 2015; Sun *et al.*, 2013; Yan *et al.*, 2012). Consequently, insufficient information is available on cellulose-degrading communities in biogas processes and on possibilities to enhance the degradation rate by 'microbial steering', *i.e.* by supporting the growth of highly efficient cellulose-degrading bacteria or communities.

1.1 Hypothesis

The overall hypothesis tested in this thesis work was that: Increased knowledge of the microorganisms involved in hydrolysis of lignocellulosic materials can enable biogas production to be steered towards a more efficient and controllable process for degradation and biogas production from agriculture residues and plant-based materials.

1.2 Aim

The main aim of this thesis was to provide novel information about lignocellulose-degrading bacteria in biogas processes and thereby enable a more efficient biogas production from lignocellulosic materials.

Specific objectives of the work described in Papers I-IV were to:

1. Search for correlations between the degradation rate of cellulose and straw and the bacterial community structure, including potential cellulose-degrading bacteria (**I**).

2. Investigate the importance of the inoculum source for efficient biogas production from lignocellulosic materials in a continuously operated process and the dynamics of the microbial community shaped by the substrates and operating parameters used (**II**, **III**).

3. Examine the impact of adding an energy-rich co-substrate to anaerobic reactors operating with different lignocellulosic based substrates, regarding the reactor performance and microbial community (**IV**).

2 Biogas production

Biogas is the name given to a biologically produced specific gas mixture mainly composed of methane (52-85%), carbon dioxide (14-48%) and some small quantities of nitrogen, oxygen, hydrogen, hydrogen sulphide, ammonia and hydrocarbons (C2-C7) and some traces of organic compounds of sulphur, chlorine, fluorine, silicon *etc*. (Zamorska-Wojdyła *et al.*, 2012). Methane (CH_4) is an energy-rich and economically valuable energy resource. Methane can be produced through anaerobic digestion, a complex microbiological process requiring the combined activity of several groups of microorganisms with different metabolic capacities (Schnürer, 2016). At least four different groups of microorganisms (*i.e*. performing hydrolysis, acidogenesis, acetogenesis and methanogenesis) are involved (Schnürer, 2016) (Figure 1).

The substrate fed to a biogas process, such as manure, crop residues, food wastes or municipal sewage sludge, is mainly composed of polysaccharides (such as starch, cellulose, hemicellulose, pectin *etc*.), proteins and lipids. Most of these complex organic compounds are too large for a single organism to bring into the cell for its metabolism. Thus, in the first degradation step, the compounds are degraded (hydrolysed) to soluble sugars, peptides, amino acids and fatty acids, by the action of extracellular enzymes produced by microorganisms (Adekunle & Okolie, 2015). In the second step, the fermenting bacteria use these monomers as carbon and energy sources in their metabolism and, as a result, they produce alcohols, organic acids, carbon dioxide, hydrogen, hydrogen sulphide and ammonia (sometimes called intermediate products). These compounds can then be utilised by acetogens in the third step, producing mainly acetic acid, hydrogen and carbon dioxide. In the last step, methanogens (archaea) use mainly acetate, formate, methyl compounds, hydrogen and carbon dioxide as carbon and energy sources, forming carbon dioxide and methane (biogas) as the final products. According to the known methanogenic pathways, these methanogens can be categorised as hydrogenotrophic methanogens, acetoclastic methanogens and methylotrophic

methanogens (Kleinsteuber, 2018). The hydrogenotrophic methanogens perform a very important role, as they 'pull' many of the preceding oxidation reactions, *e.g*. oxidation of acids. These oxidation reactions are endergonic under standard conditions and can only proceed at a low partial pressure of hydrogen, *i.e*. in the presence of hydrogenotrophic methanogens. The hydrogen and carbon dioxide produced during the acidogenesis and acetogenesis steps can be converted to acetate through homoacetogenesis, which can also affect the partial pressure of hydrogen (Ye *et al.*, 2014; Collet *et al.*, 2005). The conversion of acetate to methane can proceed through two different pathways, depending on prevailing environmental conditions such as ammonia and volatile fatty acid (VFA) level and temperature: 1) the acetoclastic pathway, which involves acetoclastic methanogens cleaving acetate into methane and carbon dioxide; and 2) the syntrophic acetate oxidation (SAO) pathway, where acetate is first metabolised into hydrogen and carbon dioxide by syntrophic acetate-oxidising bacteria (SAOB) and is later used by hydrogenotrophic methanogens for methane production (Westerholm *et al.*, 2016; Schnürer *et al.*, 1999; Zinder & Koch, 1984).

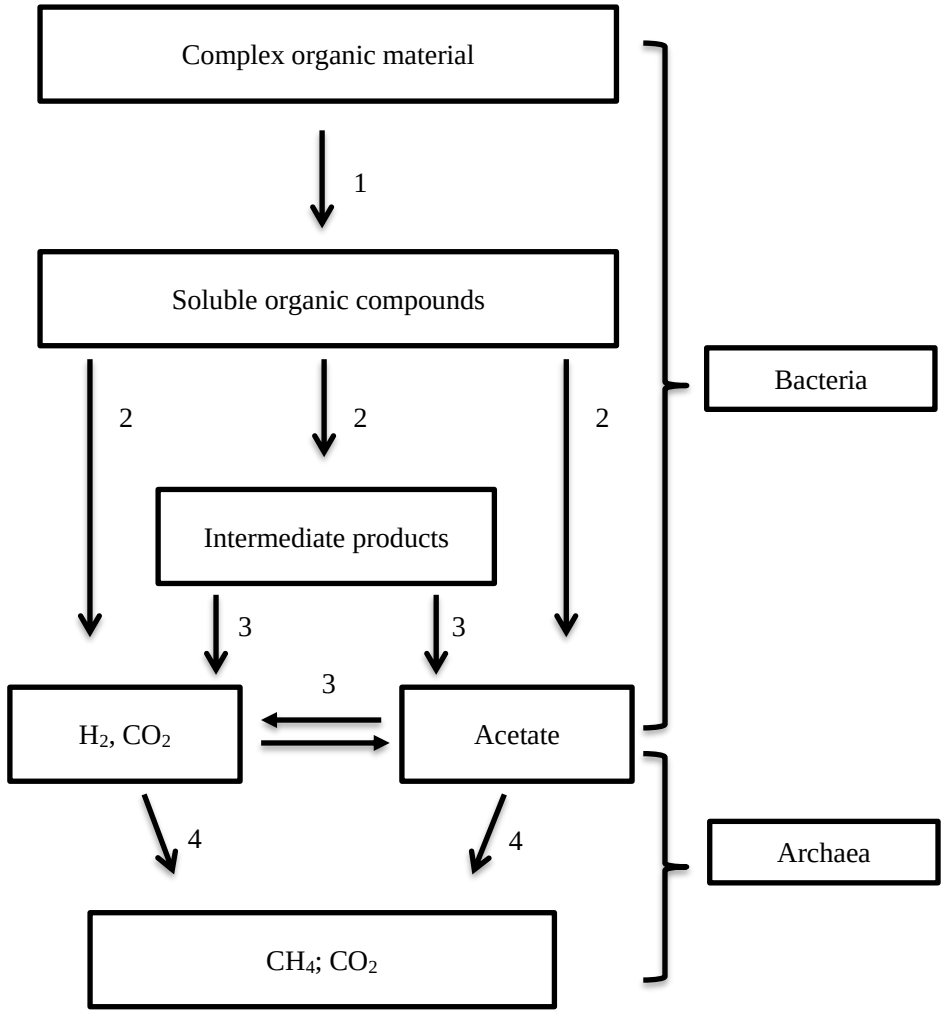

Figure 1. The anaerobic digestion process leading to biogas production. Organic materials are first hydrolysed to soluble organic compounds such as amino acids, fatty acids and sugars (1. Hydrolysis). Then, depending on different kinds of microorganisms, these soluble organic compounds are converted to intermediate products such as alcohols and fatty acids (2. Acidogenesis). In the next step, the intermediate products are utilised by acetogens to form hydrogen (H_2), carbon dioxide (CO_2) and acetate (3. Acetogenesis and syntrophy). Finally, methanogens consume mainly CO_2, H_2 and acetate to produce methane (CH_4) and CO_2 as the metabolic end-products (4. Methanogenesis). Diagram adapted from Pap *et al.* (2016) and Schnürer *et al.* (2016) (Pap & Maróti, 2016; Schnürer, 2016).

2.1 Current status of biogas production in the EU and Sweden

Biogas production has been continually increasing in the EU and its member states for some years. By 2015, there were more than 17,400 biogas plants installed in the EU, producing in total 18 billion m^3 methane (equal to ~654 PJ), which corresponded to 50% of global biogas production in 2015 (Scarlat *et al.*, 2018). The biogas production situation and applications vary between EU countries from several perspectives, including: 1) the production sources (*i.e.* landfill gas, wastewater treatment, anaerobic digestion and thermochemical processes); 2) the feedstock used (*i.e.* energy crops, agricultural residues, biowaste and municipal waste, industrial waste, sewage *etc.*); and 3) the downstream usage of the biogas (*i.e.* for electricity, heat and transportation) (Scarlat *et al.*, 2018). For example, in Germany biogas is mainly produced through the anaerobic digestion process using around half energy crops and half agricultural waste (calculation based on wet weight of material), while in Sweden it is mainly produced from sewage sludge at wastewater treatment facilities (Stambasky *et al.*, 2016). In contrast to other EU countries, the biogas produced in Sweden is mainly upgraded to vehicle fuel (65%), while less is used for generation of electricity and heat (Johan & Linus, 2018). Sweden is a world leader in the use of upgraded biogas in the transportation sector, where the amount used corresponds to 75% of all biogas used for vehicles in Europe (Scarlat *et al.*, 2018) (Figure 2).

Figure 2. Biogas buses refuelling at a biogas station (Photo: Anna Schnürer, Uppsala).

Moreover, the EU has adopted the target of increasing the share of renewables to at least 27% of final energy consumption by 2030 (final, 2014). This target could be achieved by contributions from further development of the biogas sector. The increase in biogas production in the EU after 2003 was achieved in the first instance by the development of anaerobic digestion processes (treating various organic materials, including energy crops), followed by landfill gas and biogas from wastewater treatment (Scarlat *et al.*, 2018). Organic wastes, especially agricultural wastes, have been highlighted as having great potential for future biogas production (Scarlat *et al.*, 2018; Meyer *et al.*, 2017). Within the EU target for 2030, Sweden has a more specific target of reaching 49% renewables in final energy consumption and reducing use of fossil fuels in the transport sector by 80% from 2010 to 2030 (https://2030-sekretariatet.se/english/). The theoretical biogas production potential in Sweden has been calculated to be about 54 PJ/year, which is nearly seven times the current annual production of biogas (around 7.6 PJ/year). The agricultural waste sector has again been suggested to represent a major part of this potential (Meyer *et al.*, 2017) (Figure 3).

Figure 3. Grass bedding mixed with cattle manure, an agricultural waste with high potential for biogas production.

3 Lignocellulosic materials as a substrate for biogas production

When lignocellulosic materials are used as a substrate for anaerobic digestion, the first step, hydrolysis, usually becomes rate-limiting for the whole process, due to the recalcitrant structure of the plant cell wall (Mulat & Horn, 2018; Lynd *et al.*, 2002a). Moreover, lignocellulosic materials are characterised by low nutrient content, giving low methane yield compared with other substrates, such as food and municipal wastes (Li *et al.*, 2013; Chynoweth *et al.*, 1993). To overcome this disadvantage of using lignocellulosic materials, many approaches have been suggested, involving both substrate optimisation (*e.g.* pre-treatment and co-digestion) and optimisation of process configuration (*e.g.* improved process design). These are discussed in more detail below.

3.1 Structure of lignocellulose

Lignocellulose is widely present in plants in the form of microfibrils in the cell wall, which makes plants strong (Li *et al.*, 2009). It is abundant in most kinds of plants, comprising *e.g.* around 100% in cotton flower parts (Bayané & Guiot, 2010) and around 40-50% in different agriculture residues (*e.g.* rice straw, rice husk, maize stalks *etc*.) (Gani & Naruse, 2007). In the linear structure of microfibrils, acetal bonds provide a strong binding force between each cellulose unit. Each linear cellulose strain interacts with the neighbouring strains forming a sheet structure, which is similar to the β-sheet structure in the DNA molecule. These cellulose strains are covered by hemicellulose, which has several branched glucose structures that are further reinforced by the mesh of lignin (Figure 4). Lignin is a complex aromatic structure that cannot be significantly degraded by microorganisms in the anaerobic environment (Prochazka *et al.*, 2012). The rigid structure of the plant cell wall,

lignocellulose, is almost unreachable by enzymes produced by microorganisms (Akin, 1988), thus restricting the degradation efficiency (Bayané & Guiot, 2010). Consequently, the hydrolysis rate is the main limitation in biogas production using lignocellulosic materials (Mulat & Horn, 2018; Noike *et al.*, 1985).

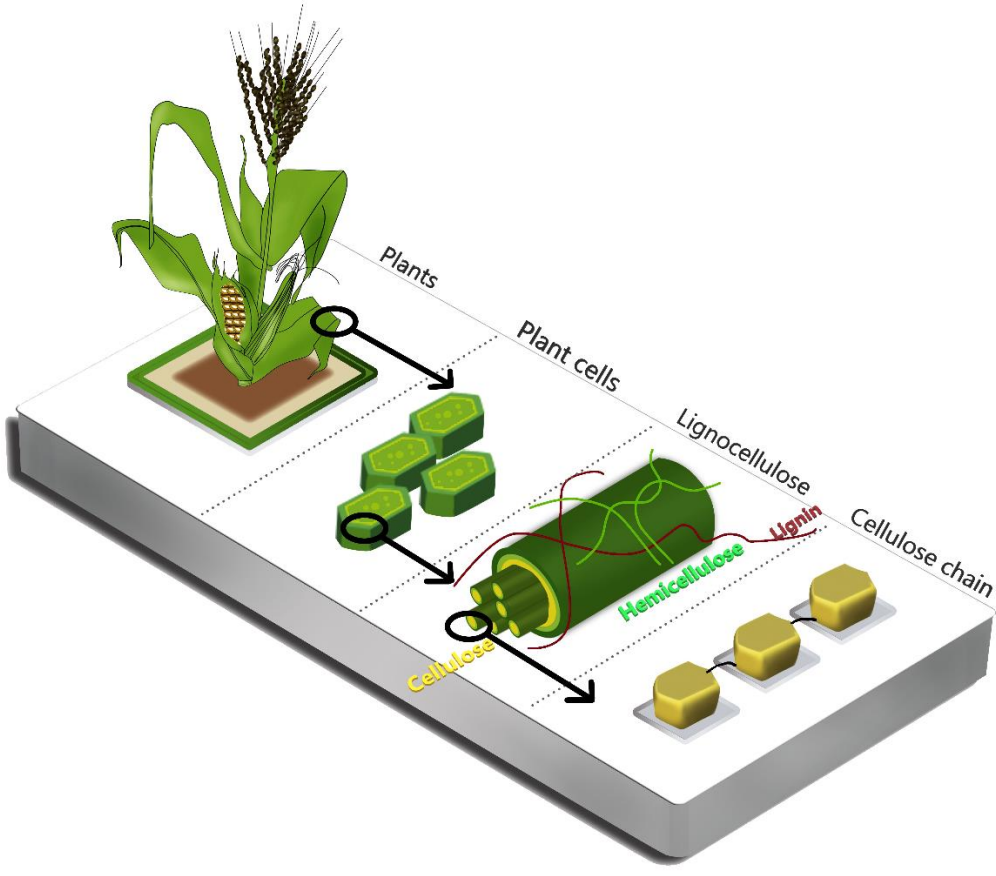

Figure 4. Microstructure of a typical plant cell wall, indicating the relationship between cellulose, hemicellulose and lignin (Modified from https://www.total.com).

3.2 Lignocellulosic substrate optimisation

As mentioned above, the biodegradability of lignocellulosic materials can be increased by a pre-treatment with the purpose of removing lignin, hydrolysing hemicellulose, decreasing cellulose crystallinity, increasing the porosity of materials and making the material more accessible to microbial and enzymatic attack (Monlau *et al.*, 2013). Different pre-treatment methods for lignocellulosic materials have been explored, for example mechanical, thermal, chemical and biological methods (Monlau *et al.*, 2013). However, most pre-treatment methods require expensive specialist equipment with substantial energy requirements. In addition, toxic products such as furfurals, 5-hydroxymethylfurfural (HMF), organic acids and phenols may be formed and cause inhibition of the microbial process (Sawatdeenarunat *et al.*, 2015).

Lignocellulose-rich materials typically also have a high carbon to nitrogen (C/N) ratio, low levels of micronutrients and, often, a low energy content (Li *et al.*, 2013). However, through co-digestion, the substrate mixture can be designed to optimise the composition of nutrients, balance the C/N ratio *etc*. and achieve higher methane yields (Ebner *et al.*, 2016; Macias-Corral *et al.*, 2008; Lehtomäki *et al.*, 2007; Sosnowski *et al.*, 2003). Many substrates have been tested for co-digestion in biogas production from lignocellulose-rich material. For example, lignocellulose-rich cattle manure has been evaluated in co-digestion with food waste (Awasthi *et al.*, 2018; Ebner *et al.*, 2016) and stillage (Westerholm *et al.*, 2012) and co-digestion has been shown to give enhanced methane yield compared with mono-digestion of the manure. Process stability and volumetric biogas yield from lignocellulose-rich materials with a low C/N ratio, such as corn stovers (Li *et al.*, 2014), switchgrass (Zheng *et al.*, 2015) and other agricultural residues, have been shown to improve when these materials are co-digested with nitrogen-rich animal manure (Neshat *et al.*, 2017; Zhang *et al.*, 2013).

When diluted agricultural residues (such as liquid manure) are used, co-digestion with lignocellulosic materials can also be applied to achieve a higher organic loading rate (OLR), with only minor effects on hydraulic retention time (HRT). This is particularly important, as relatively long hydraulic retention time is typically needed for degrading lignocellulose-rich materials (Neshat *et al.*, 2017; Mata-Alvarez *et al.*, 2014). Positive effects, such as increased methane yield, of combining lignocellulose-rich agricultural substrates with various high-energy co-substrates, including protein- and sugar-rich materials, have been demonstrated in several different studies (Ahlberg-Eliasson *et al.*, 2017; Neshat *et al.*, 2017; Mata-Alvarez *et al.*, 2014) and in this thesis (**III**, **IV**).

An issue to consider when selecting a co-substrate for lignocellulosic materials is that some co-materials can result in decreased degradation efficiency. For example, a negative effect of proteins on anaerobic digestion of carbohydrate-rich materials has been observed and has been attributed to high ammonia levels (Breure *et al.*, 1986). Similar results are presented in this thesis, with negative effects, specifically on cellulose degradation, observed following high levels of ammonia release during degradation of proteins (**I**, **II**). A decrease in the degree of degradation efficiency and specific methane production was also observed in this thesis work when digesting lignocellulose-rich material with milled feed wheat, resulting in elevated ammonia levels (**III**). The low degree of degradation efficiency is unfavourable, as it represents a loss of energy and could potentially lead to higher methane emissions during digestate storage (Liebetrau *et al.*, 2013); (**III**).

3.3 Anaerobic digestion process configurations for lignocellulose-rich material

Various configurations can be used for biogas production depending on the practical needs (*e.g.* different production purposes, characteristics of the feedstock *etc*.). Depending on the feeding frequency, biogas plants are generally categorised as batch, fed-batch or continuous processes (Schnürer, 2016).

In a batch process, all the materials are added at once and the four steps of the biogas production process proceed in one reactor at the same time. The advantages of the batch process are that it is cheap, easy to operate and allows nearly 100% degradation of the organic material in a substrate. However, the batch process usually requires a long time to digest the organic material and toxic compounds such as ammonia can accumulate, since the internal reactor contents are not exchanged during the process (Schnürer & Jarvis, 2018; Raposo *et al.*, 2012). Batch-type processes are typically used for small-scale production of biogas, mainly in Asia, but are also common in Germany, especially for dry materials (total solids content >15%), which are often rich in lignocellulosic material (Kothari *et al.*, 2014; Rajendran *et al.*, 2012).

Batch processes are also commonly used in laboratory biogas trials, for example during determination of the biomethane potential (BMP) of a certain substrate (Schnürer *et al.*, 2017) (**I**, **II**, **III**, **IV**). BMP value is usually high for substrates like food waste, vegetable oil, and cheese whey while substrates like agriculture and forest residuals, containing high levels of lignocellulose, have

lower biomethane potential (Labatut *et al.*, 2011). The final biomethane potential and the degradation efficiency (time to reach the final biomethane potential) of a substrate can be used to guide the set-up of the biogas reactor. The biomethane potential can also be used to evaluate the importance of different inocula for the degradation of various materials (Perrotta *et al.*, 2017; Elbeshbishy *et al.*, 2012) (**I**, **II**, **III**) (Figure 5). For lignocellulose-rich materials, a significant difference in degradation was seen in this thesis depending on the characteristics of inoculum, with different physicochemical and microbial components (**I**, **III**) (De Vrieze *et al.*, 2015b; Gu *et al.*, 2014).

Figure 5. Sealed serum bottles on a rotary shaker in a biomethane potential (BMP) test.

In a fed-batch process, materials are added successively over time, which allows for a more constant rate of gas production and a higher level of dilution of any toxic compounds accumulated compared with the batch process (Lim & Shin, 2013). However, the amount of gas produced rises quickly at the start of feeding and decreases gradually over time and the digester needs to be filled and emptied at intervals, which causes irregular gas production compared with a continuous process (Li *et al.*, 2011). Examples of using lignocellulosic material in a methanogenic fed-batch process are rare in the literature. However, one study found that using a fed-batch process gave a higher methane yield than a batch or semi-continuous process when degrading lignocellulosic material (grass and maize silage) with anaerobic sludge from pig slurry fermentation after supplementation of rumen anaerobic fungi (Prochazka *et al.*, 2012).

A continuous process is the most commonly used method in industrial biogas production (Schnürer *et al.*, 2017). The major advantages of a continuous process are that the substrate is added continuously or semi-continuously, in parallel with removal of the reactor contents, thus giving constant production of biogas. Continuously stirred tank reactors (CSTR) are often used for a continuous process (or a semi-continuous process) (Moestedt *et al.*, 2014; Usack *et al.*, 2012). Continuously stirred tank reactors can be applied at different scales from a few litres (laboratory-scale) to hundreds of cubic metres (commercial or full-scale) (Schnürer, 2016). Thus, a CSTR can be used as a laboratory or pilot test system before scaling up (Kaparaju *et al.*, 2009; Kaparaju *et al.*, 2008) (Figure 6). Previous studies have also shown similar process performance during laboratory-scale and full-scale operation (Westerholm *et al.*, 2018; Grim *et al.*, 2015; Moestedt *et al.*, 2014). There are many studies on the use of CSTR with lignocellulosic materials, focusing on various research questions, *e.g.* comparisons of methane yield using different lignocellulosic feedstocks (Martínez-Gutiérrez, 2018), evaluations of the importance of seeded inoculum (**II**, **III**), effects of co-digestion (Li *et al.*, 2014; Comino *et al.*, 2012; Nges *et al.*, 2012) (**IV**), impacts of pre-treatment (Carrere *et al.*, 2016), process operating parameters (*e.g.* hydraulic retention time, organic loading rate and temperature) (Shi *et al.*, 2017; Zhou *et al.*, 2017; Risberg *et al.*, 2013) or feeding strategy (Mauky *et al.*, 2015) (**III**) and determination of lignocellulolytic microbes (Yu *et al.*, 2018; Zhou *et al.*, 2017; Sun *et al.*, 2015; Qiao *et al.*, 2013; Lissens *et al.*, 2004) (**II**, **III**). Continuously stirred tank reactors have also been shown to give higher efficiency of lignocellulosic material degradation than other types of continuous reactor configurations, such as the leach bed-upflow anaerobic sludge blanket (USAB) (Fu & Hu, 2016; Nizami & Murphy, 2010).

Figure 6. Left: A series of continuously stirred tank reactors (CSTR). *Right*: SLU full-scale biogas plant at Lövsta (Photo: Anna Schnürer).

The anaerobic digestion process configuration can also be categorised by different process stages, *e.g*. single-stage, two-stage and multiple-stage processes (Achinas *et al.*, 2017). In a single-stage process, all materials are digested in a single reactor and the four degradation steps in the biogas production process take place at the same time and in the same chemical environment. To achieve better biogas performance, the biogas process can also be set up as a multiple-stage system (Ward *et al.*, 2008). In the example of a two-stage anaerobic digester, all four degradation steps proceed in both digesters, but the second digester is fed with the reactor contents from the first reactor (Parawira *et al.*, 2008). This type of design allows two digesters to work with different operating parameters (such as temperature, agitation speed *etc*.) and allows the first stage to focus on hydrolysis and acidogenesis. It has been used for complex substrates, such as lignocellulose-based materials (Akobi *et al.*, 2016; Ward *et al.*, 2008). Higher methane concentration and greater efficiency can be achieved with a two-stage process design compared with a single-stage design (Colussi *et al.*, 2013; Parawira *et al.*, 2008; Taherzadeh & Karimi, 2008).

However, an obvious drawback of multiple-stage anaerobic digestion is the high cost compared with the one-stage process. Thus, there are few commercial multiple-stage anaerobic digestion systems for processing lignocellulose-based materials in operation today (Achinas *et al.*, 2017). Multiple-stage anaerobic digestion processes (as opposed to multiple-*phase* anaerobic digestion systems) sometimes also include recirculation of reactor contents (*e.g*. from methanogenic phase to hydrolytic phase) (Azbar & Speece, 2001). This has been applied as an additional approach to optimise the degradation of lignocellulosic materials. For example, by recirculating the reactor contents in an anaerobic digestion process, it is possible to: 1) achieve a longer retention time and consequently the time available for degradation can be prolonged (Estevez *et al.*, 2014); 2) reach optimal conditions for hydrolytic bacteria (in terms of pH, water content and alkalinity), which are better maintained in the process (Dandikas *et al.*, 2018); and 3) preserve micronutrients (Aslanzadeh *et al.*, 2013). Thus, several studies using this concept for degradation of lignocellulosic material have found increased methane yield compared with a non-recirculating reactor.

3.4 Process regulating parameters

In addition to digester configuration, different operating parameters, such as organic loading rate (OLR), hydraulic retention time (HRT), temperature and stirring, need to be considered during set-up of an anaerobic digestion process (Schnürer, 2016). Important parameters are *e.g*. OLR and HRT, which are often interlinked so that a higher OLR usually leads to shorter HRT. The organic loading rate can be defined in kilograms or grams of volatile solids (VS) per day and cubic metre or litre of reactor volume. Overloading with organic materials may cause accumulation of volatile fatty acids (VFAs), as the methanogenic step cannot keep up with the acidogenic and acetogenic steps (Franke-Whittle *et al.*, 2014). However, due to the slow degradation rate, the risk of VFA accumulation due to overloading is relatively low when using lignocellulosic material compared with when using *e.g*. sugar-rich or lipid-rich materials (Schnürer & Jarvis, 2018; Cavaleiro *et al.*, 2009). A high load can also reduce the HRT, which, as mentioned above, can have a negative impact on the degradation efficiency. Hydraulic retention time is defined as the time that the substrate remains in a digester. In a CSTR, the HRT can be approximated as volume of liquid phase divided by effluent flow rate. The HRT varies in different biogas digesters and normally ranges from 10 to 30 days, but is sometimes longer (Mao *et al.*, 2015). The actual magnitude of the HRT applied is dependent on many different parameters, such as the characteristics of the input substrate and the operating temperature. Due to the intricate structure of lignocellulosic materials limiting the hydrolysis efficiency in an anaerobic digestion process, a comparatively long HRT (>30 days) is typically needed (Shi *et al.*, 2017).

Another important parameter for the anaerobic digestion process is the operating temperature, where an appropriate temperature can potentially improve the methane production performance (Schnürer *et al.*, 2017). For a digester operating with lignocellulosic materials, the operating temperature is usually set around 37 °C (Sawatdeenarunat *et al.*, 2015) (**I**, **II**, **III**, **IV**). However, studies have shown that digestion of lignocellulosic materials at different temperatures is possible (Risberg *et al.*, 2013; El-Mashad *et al.*, 2004). A higher operating temperature has been suggested to give a higher hydrolysis rate of lignocellulosic material and even a higher methane yield (Moset *et al.*, 2015; Labatut *et al.*, 2014; Veeken & Hamelers, 1999). However, some studies have found no significant difference in performance and methane yield using the same substrate (manure/straw) at different temperatures (Risberg *et al.*, 2013).

Feeding regime (*e.g*. different feeding intervals) is another parameter that could be used to achieve flexible and efficient biogas production in a continuous process (Mulat *et al.*, 2016). However, the effect of feeding regime on biogas production performance can vary depending on the substrate used and the feeding interval (Piao *et al.*, 2018; Ziels *et al.*, 2018; Ziels *et al.*, 2017; Mulat *et al.*, 2016; Mauky *et al.*, 2015; De Vrieze *et al.*, 2013). Very few studies have investigated the effect of feeding regime on the degradation of lignocellulosic materials. In this thesis, a less frequent feeding regime that involved adding milled feed wheat as a co-substrate load all at once, compared with in two portions two hours apart, in CSTRs operating with a grass-manure mixture was found to give slightly higher cellulose conversion activity (**III**).

One additional parameter recently suggested to be of importance for the efficiency of degradation of lignocellulosic material and the final methane yield is the nature of the inoculum, including both physicochemical and microbial characteristics (Perrotta *et al.*, 2017; De Vrieze *et al.*, 2015a; De Vrieze *et al.*, 2015b) (**II**, **III**). For example, in Paper **I** lower cellulose degradation efficiency was seen in batch processes seeded with inoculum taken from biogas plants fed with wheat-based stillage, slaughterhouse waste and grass, compared with inoculum from a process fed with mixed sludge. In Paper **II**, CSTRs operating with different inoculum sources showed a significant difference in degradation efficiency for a grass-manure mixture, especially in the initial phase of the process. In Paper **III**, it was concluded that the original inoculum can profoundly influence biogas production performance in the long term and affect microbial responses to process operation changes.

3.5 Process monitoring parameters

When an anaerobic digestion process is set up, parameters of the reactor contents, such as volatile fatty acid concentration, pH, alkalinity and ammonia level, and parameters of the gas phase, such as methane, carbon dioxide and hydrogen sulphide, are regularly monitored and can be used in combination to evaluate the biogas production performance (Schnürer *et al.*, 2017). These parameters can be further subdivided into process efficiency measures, such as specific methane production (SMP), volatile solids reduction *etc*., and process stability measures, such as volatile fatty acid concentration, ammonia level *etc*.

Specific methane production is defined as the normalised volume of methane produced per gram of volatile solids in the substrate. A decreasing value of SMP for a substrate with a certain biomethane potential (BMP) may indicate less efficient substrate degradation (**III**). However, for a biogas plant,

the volumetric yield is often continuously recorded, while SMP might be less frequently considered. For example, decreased degradation efficiency caused by a recalcitrant substrate such as lignocellulosic materials can be masked by increased volumetric yield due to increased load, as shown in this thesis (**III**). Furthermore, several studies have shown that low efficient substrate degradation can increase the risk of methane emissions during storage of the digestate (*i.e.* residual methane potential, RMP), which is unfavourable from both an economic and an environmental perspective (Ahlberg-Eliasson *et al.*, 2017; Ruile *et al.*, 2015) (**IV**). This risk can be measured as the volatile solids (VS) reduction in reactor contents (*i.e.* VS of substrate compared with VS of the reactor contents). Moreover, a combination of BMP/RMP analysis has recently been proposed for the anaerobic digestion process, to better evaluate the degradation efficiency of the substrate (Li *et al.*, 2017; Rico *et al.*, 2015).

Volatile fatty acids are intermediate products produced during anaerobic digestion of organic compounds and the VFA concentration is considered one of the most important indicators for judging the stability of an anaerobic digestion process (see Chapter 2 of this thesis) (Drosg, 2013). Accumulation of VFAs can be caused by *e.g.* temperature fluctuations and substrate overloading (Schnürer *et al.*, 2017; Ferguson *et al.*, 2016). It can lead to a pH drop, which inhibits the methanogens and results in a decrease in methane production (Schnürer *et al.*, 2017). When the rate of acidogenesis is higher than the rate of methanogenesis, acetate and propionate often accumulate more than other VFAs such as butyrate and valerate, as demonstrated in this thesis (**II**, **III**). A high propionate to acetate ratio can be used as an early indicator of a risk of process failure (Marchaim & Krause, 1993). Methods to remedy VFA accumulation in an anaerobic digestion process include reducing the organic loading rate, extending the hydraulic retention time and adding trace elements, aiming to rebalance the relative rate of the acidogenesis and methanogenesis steps (Choong *et al.*, 2016; Ferguson *et al.*, 2016; Moestedt *et al.*, 2013).

The level of alkalinity indicates the buffering capacity within the anaerobic digestion process. When acids such as VFAs accumulate, the alkalinity typically shows a decrease before a pH drop (Drosg, 2013). Thus, the VFA/alkalinity ratio can be measured to monitor the stability of a reactor, especially when there is a high risk of acidification (Schnürer *et al.*, 2017).

Ammonium is formed during the degradation of protein-rich materials. Free ammonia, in equilibrium with ammonium, is toxic to microbes and strongly inhibits the anaerobic digestion process (Westerholm *et al.*, 2016) (**I, II**). However, a gradual increase in ammonia level permits development of ammonia-tolerant communities (Müller *et al.*, 2016). Decreasing the temperature and the pH can push the equilibrium between ammonium (NH_4^+)

and ammonia (NH_3) towards the ammonium side, and is thus often used to mitigate ammonia inhibition (Schnürer, 2016).

Another important factor is pH, which is affected by process parameters such as temperature, alkalinity, VFA concentration and ammonium level. (Fitamo *et al.*, 2017; Shi *et al.*, 2017; Franke-Whittle *et al.*, 2014). Different microbes have different optimal growth pH ranges. For the acidogenic bacteria, a pH range down to 4.5-5.0 can be tolerated (Chandra *et al.*, 2012), while the optimal pH range for methanogens is around 6.7-8.0. Thus, most single-stage biogas plants operate at around neutral pH to maximise the methanogenesis step (Schnürer *et al.*, 2017).

When using lignocellulosic materials as the main substrate, ammonia/ammonium and VFAs are unlikely to accumulate due to the high C/N ratio, slow hydrolysis rate and relatively long hydraulic retention time and low organic loading rate applied in the anaerobic digestion process (Cavaleiro *et al.*, 2009; Ward *et al.*, 2008). However, process imbalances can still arise, as lignocellulosic materials are often combined with co-substrates, such as proteins, to balance the C/N ratio and improve the gas yield (Neshat *et al.*, 2017) (**III**).

In addition, recent studies have suggested microbial monitoring as a possible way to evaluate and manage the process (Carballa *et al.*, 2015; Lebuhn *et al.*, 2015). By following the community dynamics or analysing specific key groups, such as the methanogens, and correlating their abundance to specific process parameters, it may be possible to predict instability or steer the community in a desired direction. These correlations are discussed in more detail in Chapter 4.

4 Microbial communities

As mentioned in Chapter 2, biogas is produced by a complex network of microbes with differing and complementary metabolisms. Thus, to optimise and achieve better regulation of a biogas process, an in-depth understanding of the important microbial agents is needed (Kleinsteuber, 2018; Carballa *et al.*, 2015; Lebuhn *et al.*, 2015; Vanwonterghem *et al.*, 2014b). In a typical methanogenic CSTR, members of the phyla Firmicutes and Bacteroidetes are often found to dominate the bacterial community, while members of the phylum Euryarchaeota tend to dominate the archaeal community (Güllert *et al.*, 2016; Luo *et al.*, 2016; Pore *et al.*, 2016; Satpathy *et al.*, 2016; Watanabe *et al.*, 2016; Sun *et al.*, 2015; Lu *et al.*, 2014) (**I**, **II**, **III**, **IV**). However, some other bacterial phyla such as Proteobacteria, Chloroflexi and Fibrobacteres can also be abundant (Schnürer, 2016; Vanwonterghem *et al.*, 2014a) as confirmed here (**I**, **II**, **III**, **IV**). Moreover, within the fungal community, the phylum Neocallimastigomycota has been shown to dominate (Dollhofer *et al.*, 2015).

Many recent studies have found that microbial communities can be shaped by the operating parameters of the anaerobic digestion process and can thus affect the biogas production performance (Grohmann *et al.*, 2017; Pap & Maróti, 2016; Satpathy *et al.*, 2016; Sun *et al.*, 2016; Westerholm *et al.*, 2016; De Francisci *et al.*, 2015; Rui *et al.*, 2015; Sundberg *et al.*, 2013; Cardinali-Rezende *et al.*, 2012; Kampmann *et al.*, 2012). This was also demonstrated in Papers **I-IV** in this thesis (Figure 7).

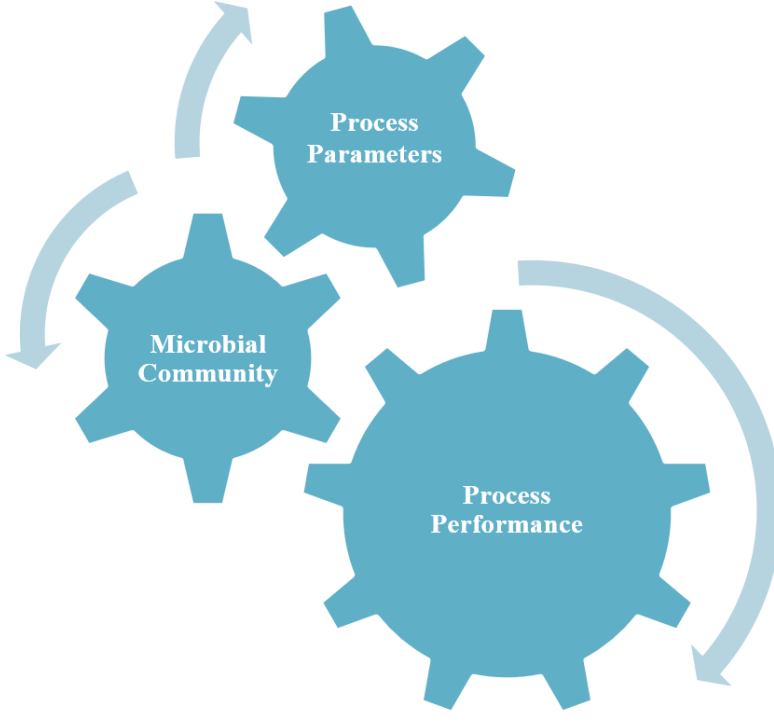

Figure 7. Operating parameters affecting microbial community and thus potentially biogas production performance.

4.1 Lignocellulose degraders in the anaerobic environment

In studies using different isolation and molecular microbiological methods, various anaerobic lignocellulose degraders have been found in diverse anaerobic environments, including soil, anaerobic digesters, aquatic environments such as sludge and sediment, animal gut environments such as the rumen, termites, dung beetles, *etc.* (Saini *et al.*, 2017; Azman *et al.*, 2015; Dollhofer *et al.*, 2015; Estes *et al.*, 2013; Ransom-Jones *et al.*, 2012; Morrison *et al.*, 2009a; Lynd *et al.*, 2002b; Leschine, 1995) (**I**, **II**, **III**, **IV**). These anaerobic lignocellulose degraders are widely distributed in genera within the bacteria and fungi domain, but have also been found recently in the archaea domain (Cragg *et al.*, 2015).

Many types of anaerobic bacteria have been demonstrated to have the ability to degrade or potentially utilise lignocellulose as a carbon source. These can be found in genera such as *Clostridium*, *Ruminococcus*, *Fibrobacter*, *Acetivibrio*, *Butyrivibrio*, *Halocella*, *Bacteroides*, *Spirochaeta*, *Thermotoga*, *Echinicola*, *Mahella*, *Marinilabilia*, *Prevotella*, *Flavobacterium* and *Streptomyces* (Azman *et al.*, 2015; Sun *et al.*, 2013; Tsavkelova & Netrusov, 2012) (**I**, **II**, **III**) (Figure 8).

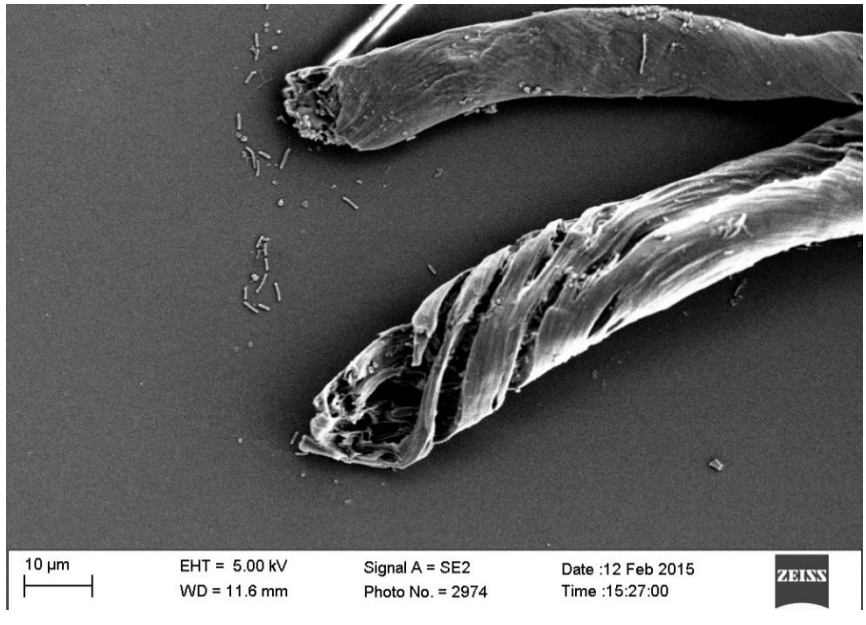

Figure 8. Scanning electron microscope (SEM) image of material isolated from an industrial-scale anaerobic digester, showing pure-cultured *Clostridium* sp. Bciso-3 degrading cellulose.

The relative abundance of these genera typically varies depending on the anaerobic environment. For example, the best-studied genus, *Clostridium*, has been found to be more abundant in landfilled sludge than genera such as *Fibrobacter* and *Ruminococcus*, but less abundant in the rumen (Ransom-Jones *et al.*, 2012; Burrell *et al.*, 2004). In anaerobic digestion processes operating with lignocellulosic materials as the main substrate, the relative abundance of different genera can also vary depending on factors such as the composition of the substrate, process configuration and operating parameters (Azman *et al.*, 2015). However, the phyla Bacteroidetes and Firmicutes often dominate, followed by phyla such as Proteobacteria and Actinobacteria (Güllert *et al.*, 2016; Azman *et al.*, 2015; Sun *et al.*, 2013). This was also the case in the anaerobic digestion processes studied in this thesis (**I**, **II**, **III**, **IV**). Recent studies using metatranscriptomics and metaproteomics approaches have revealed information on the active, lignocellulose degraders in the anaerobic digestion processes, rather than simply all microbes present. The results confirm the important roles of lignocellulose degradation by the genus *Clostridium* (Jia *et al.*, 2018; Güllert *et al.*, 2016; Lü *et al.*, 2014). New knowledge on members of the genus *Clostridium* has also been used to guide the design of bioaugmentation strategies for improving the lignocellulose degrading efficiency and methane yield in different anaerobic digestion processes (Tsapekos *et al.*, 2017; Poszytek *et al.*, 2016).

For fungi, the best-studied anaerobic cellulase producers are members of the family Neocallimastigaceae, including the genera *Neocallimastix*, *Orpinomyces* and *Piromyces* (Cheng *et al.*, 2018; Dollhofer *et al.*, 2015; Viikari *et al.*, 2009). These genera have been widely found in the gastrointestinal tract of ruminants and most non-ruminant herbivores (Dashtban *et al.*, 2009), but have lately been identified also as part of the community in anaerobic digesters (Dollhofer *et al.*, 2015). The first anaerobic lignocellulolytic fungus to be identified was *Neocallimastix frontalis*, isolated from sheep rumen fluid (Orpin, 1975; Braune, 1913). Later studies have demonstrated that members of the genera *Neocallimastix*, *Orpinomyces* and *Piromyces* are able to utilise different carbohydrates and produce hydrogen, carbon dioxide, acetate, formate, lactate and ethanol as metabolic end-products (Gruninger *et al.*, 2014; Dashtban *et al.*, 2009; Hodrová *et al.*, 1998; Joblin & Naylor, 1989). Notably, these fungi can also develop an invasive rhizoid system that penetrates plant cell walls, combined with secretion of various carbohydrate-hydrolysing enzymes, thus improving the accessibility of plant structures to bacterial action (Dollhofer *et al.*, 2015). Moreover, an ability of anaerobic fungi to degrade lignin has been reported in several studies (Dollhofer *et al.*, 2015; Gruninger *et al.*, 2014; Haitjema *et al.*, 2014; Kazda *et*

al., 2014). This suggests the potential to enhance degradation of lignocellulosic material for biogas production by enhancing the growth of these fungi.

A few species of hyperthermophilic archaea belonging to the genus *Pyrococcus*, such as *Pyrococcus furiosus*, *Pyrococcus horikoshii* and *Pyrococcus glycovorans*, have also been found to produce endoglucanases such as glycoside hydrolase (GH) families 5 and 12 (Kishishita *et al.*, 2015; Ando *et al.*, 2002; Barbier *et al.*, 1999). These archaea can live under extremely high temperatures (around 100 °C) and in high-salt environments, and could thus potentially be applied in a pre-treatment step before biogas production.

4.2 Enzymatic depolymerisation of cellulose and hemicelluloses

Lignocellulose is degraded by the collective action of multiple carbohydrate-active enzymes, including glycoside hydrolases, produced by microorganisms (Jia *et al.*, 2018; Young *et al.*, 2018; Cragg *et al.*, 2015; Malherbe & Cloete, 2002). The glycoside hydrolases are classified based on amino acid sequence similarities and grouped into different enzyme families, such as GH 5, 6, 7, 8, 9, 10, 11, 12, 26, 44, 45, 48, 51, 60, 61 and 74 (Henrissat, 1991). Notably, most cellulases secreted by the anaerobic cellulose-degrading bacteria belong to GH families 5, 9 and 48 (endo-β-1,4-glucanase) (Vanwonterghem *et al.*, 2016; Pereyra *et al.*, 2010) (Figure 9).

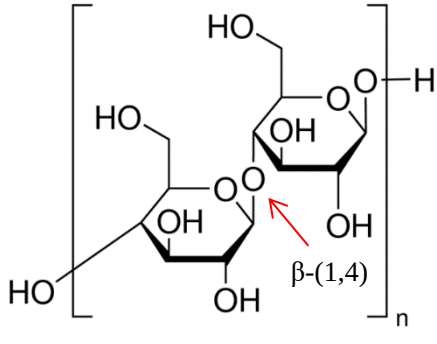

Figure 9. Structure of the cellulose chain.

Depending on the environment (aerobic/anaerobic), the strategy used by microbes for cellulose degradation is somewhat different (Tomme *et al.*, 1995). In the aerobic environment, fungi (such as the phyla Ascomycetes and Basidiomycetes) and bacteria (such as the genera *Cellulomonas*, *Cellvibrio* and

Cytophaga) typically use non-complexed cellulase systems, which secrete cellulase-hydrolysing enzymes (Malherbe & Cloete, 2002; Mullings & Parish, 1984). However, in the anaerobic environment, fungi (such as the family Neocallimastigaceae) and bacteria (such as the genus *Clostridium*) typically contain a relatively more complex cellulase system, including a membrane-bound enzyme complex (cellulosome) (Gruninger *et al.*, 2014; Pereyra *et al.*, 2010).

The cellulosome assists in the degradation process by synchronising different type of enzymes performing different reactions (Bayer *et al.*, 2004). A typical cellulosome contains a scaffolding protein chain (without enzymatic activity), which has many enzyme binding domains, named cohesions. There are also different types of cohesins, *e.g*. *Clostridium thermocellum* has two, type I and type II. A corresponding domain on glycoside hydrolases, called dockerin, can selectively bind to the type-I cohesins of the primary scaffolding protein CipA. The terminal X-dockerin dyad of CipA can then bind to three types of type-II cohesins of anchoring scaffoldings, named SdbA, Orf2p and OlpB. These three types of type-II cohesins bind to the cell surface with an S-layer homology module (Bayer *et al.*, 2008; Boisset *et al.*, 1999; Bayer *et al.*, 1998). Cellulose is bound by the carbohydrate binding module (CBM) on the scaffolding protein chain, which results in linkage of the cellulosic material and the cellulosome complex (Shoseyov *et al.*, 2006).

In addition, recent studies have regrouped glycoside hydrolase family GH 61 and carbohydrate binding module CBM33 into a new family due to their capacity for catalysing oxidative cleavage of polysaccharides. This new family, which is called lytic polysaccharide monooxygenases (LPMOs) (Horn *et al.*, 2012), has been found in fungi, bacteria and viruses (Chiu *et al.*, 2015; Kohler *et al.*, 2015; Vaaje-Kolstad *et al.*, 2010). These enzymes have been demonstrated to specifically break and loosen the polysaccharide chains, which creates new attack points for cellobiohydrolases (CBHs), thus increasing the accessibility of cellulose to microorganisms (Johansen, 2016; Hemsworth *et al.*, 2015). It is known that LPMOs require molecular oxygen (O_2) for their activity (Johansen, 2016). However, a recent study has shown that hydrogen peroxide (H_2O_2) can act as a co-substrate instead of molecular oxygen, which suggests that LPMOs can work under anaerobic conditions (Bissaro *et al.*, 2016). However, so far these enzymes have not been shown to be present in an anaerobic environment.

4.3 Lignocellulolytic communities and influence of digester parameters

In an anaerobic digestion process, lignocellulose degradation is usually not performed by a single fungus or bacterium, but by a complex microbial community (Jia *et al.*, 2018; Young *et al.*, 2018; Pereyra *et al.*, 2010). In this thesis and in other studies, the composition and structure of the lignocellulolytic community (as part of the overall microbial community) has been shown to be influenced by process parameters such as temperature, volatile fatty acid concentration and ammonia content (Jia *et al.*, 2018; Sun *et al.*, 2013) (**I**, **II**, **III**).

Temperature is one of the most important factors shaping microbial communities. At different temperatures, community structure, diversity and the activity of microorganisms all vary and the stability of the reactor is then highly dependent on the resilience of the microbial community (Westerholm *et al.*, 2018; Westerholm *et al.*, 2017; Luo *et al.*, 2016; De Vrieze *et al.*, 2015c; Westerholm *et al.*, 2015). Typically, higher operating temperature results in higher relative abundance of the phylum Firmicutes than of the phylum Bacteroidetes and lower microbial diversity compared with operation in mesophilic conditions (Westerholm *et al.*, 2017; Luo *et al.*, 2016; Westerholm *et al.*, 2015; Moestedt *et al.*, 2014). Studies specifically focusing on the response of the lignocellulolytic community to temperature changes in the anaerobic digestion process are rare. However, changing the operating temperature from 39 to 50 °C was shown to increase the ratio of Firmicutes to Bacteroidetes in a pilot-scale biogas reactor operating with rice straw (Yu *et al.*, 2018). In another study, higher temperature (55 °C compared with 37 °C) resulted in an increase in the relative abundance of an uncultured order MBA08 (class Clostridia) and a decrease in community diversity in a CSTR process operating with steam-exploded straw and manure (Sun *et al.*, 2015). Furthermore, temperatures below 4 °C and above 50 °C have been demonstrated to strongly decrease the degree of adhesion between bacteria and cellulose, thus potentially lowering the cellulose degradation efficiency (Miron *et al.*, 2001).

Volatile fatty acid content has been shown to inhibit microbial groups to different degrees (Ma *et al.*, 2015; Chen *et al.*, 2014; Franke-Whittle *et al.*, 2014). For the lignocellulolytic community, a negative correlation was seen in Paper **I** between the VFA content of the inoculum and the relative abundance of potential cellulose degraders, such as *Mahella australiensis* 50-1 BON and *Echinicola vietnamensis*. In Paper **III**, a decrease in the degrading efficiency of cellulose was also found to be associated with an increase in acetate and

propionate content. However, the correlation seen between VFA content and cellulose degradation could possibly be an indirect effect of ammonia inhibition, which often gives rise to accumulation of VFAs (**III**).

Ammonia level, combined with temperature, also significantly affects microbial community structure, both the overall structure (Hu *et al.*, 2017; De Vrieze *et al.*, 2015c) (**I**) and that of specific groups of microorganisms, such as the community of acetogenic bacteria (Moestedt *et al.*, 2016), syntrophic acetate-oxidising bacteria (SAOB) (Müller *et al.*, 2016) and the methanogens (Westerholm *et al.*, 2016). At present, there is little information available in the literature regarding the impact of ammonia on the degradation of lignocellulose and on lignocellulolytic bacteria. However, in this thesis work, ammonia level was shown to be negatively correlated with the relative abundance of specifically *C. cellulolyticum* (**I**) in a batch process and with the cellulose degradation efficiency in both a batch (**I**) and semi-continuous process (**II**).

5 Microbial community analysis techniques

Various methods can be employed to study microbial community structure. These are normally categorised into: 1) culture-dependent techniques, including *e.g*. clone library, isolation and characterisation; or 2) culture-independent techniques. The culture-independent techniques can be further categorised based on different study purposes into: i) identification (cloning library, denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP), Sanger sequencing, microarray, next-generation sequencing *etc*.); ii) community dynamics changes (DGGE, T-RFLP, single-stranded conformation polymorphism (SSCP), Sanger sequencing, next-generation sequencing *etc*.); iii) quantification (quantitative polymerase chain reaction (q-PCR), fluorescence *in situ* hybridisation (FISH) *etc*.) (**I**, **II**, **III**, **IV**) functionality (advanced FISH, stable isotope probing (SIP), metatranscriptomics, metaproteomics *etc*.) (Cabezas *et al.*, 2015). Alternatively, these techniques can be classified according to the level of gene products recovered from *e.g*. transcripts and proteins into: metagenomics, metatranscriptomics and metaproteomics) (Hassa *et al.*, 2018; Kameshwar & Qin, 2018; Kleinsteuber, 2018; Aguiar-Pulido *et al.*, 2016; Gawad *et al.*, 2016; Goswami *et al.*, 2016; Prince *et al.*, 2014; Fry, 2004).

5.1 Methods applied to study lignocellulolytic communities

Isolation and cultivation of pure culture is a very important way to study a lignocellulolytic microorganism. Many potential lignocellulolytic microorganisms, including aerobic and anaerobic fungi and bacteria, have been isolated from various environments such as termites, rumen, paper mill, manure, wood fermenter, anaerobic digestion process *etc.* (Pereyra *et al.*, 2010; König *et al.*, 2006; Schwarz, 2001). Anaerobic bacteria can be cultivated in an anaerobic medium, using the following preparation steps: boiling the medium (to reduce the amount of oxygen), adding reducing agents, adding a substrate such as cellulose, cellobiose, filter paper *etc.* (to enrich lignocellulolytic microorganisms), and exchange of gas phase in the bottle to nitrogen gas (N_2) or N_2/CO_2 (Westerholm *et al.*, 2010). Isolation often starts with enrichment of lignocellulolytic microorganisms, followed by *e.g.* use of the agar shake method to pick single colonies from a dilution series from the previously enriched culture (Sun, 2015) (Figure 10).

Figure 10. Left: Anaerobic serum bottles of lignocellulolytic microorganism-enriched culture. *Right:* Anaerobic glass tubes with agar for picking single colonies.

Besides the culture-based method, some molecular tools have been employed to study lignocellulolytic communities. As mentioned in section 3.2, lignocellulose is degraded by different glycoside hydrolases, which are grouped in different families based on amino acid similarities (Henrissat, 1991). Based on this information, Pereyra *et al*. (2010) designed a degenerated primer set to specifically target the major glycoside hydrolase genes (*cel5* and *cel48*) in anaerobic digestion processes (Pereyra *et al.*, 2010). These primers were further adapted to quantitative PCR (qPCR) and revealed dynamic changes in these genes in two different biogas reactors (Pereyra *et al.*, 2010). However, using qPCR can only show the overall changes in *cel5* and *cel48* genes in the reactor samples, and not the microbial community structures

containing these genes. Thus, the same primer sets were used in studies by Sun *et al.* (2013) and in work performed in this thesis (**I**, **II**, **III**), where the analysis was combined with T-RFLP and Sanger sequencing of clone libraries. These studies successfully revealed the population and structure of the potential lignocellulolytic degraders in anaerobic digestion processes set up with different inoculum sources and operated with agricultural substrates. The method of combining T-RFLP and sequencing of clone libraries has been widely used to study the microbial community structure in different ecosystems (Theuerl *et al.*, 2018; Ramos *et al.*, 2010; Dickie & FitzJohn, 2007; Wang *et al.*, 2004). However, there are some limitations to this method. For example: 1) the principle behind T-RFLP is that the length of terminal restriction fragments (T-RFs) should vary with various microorganisms and restriction enzyme(s) used (Liu *et al.*, 1997). However, one T-RF can represent several operational taxonomic units (OTUs) if they have the same number of bases at the first cutting site from the restriction enzyme; and 2) the community diversity is limited by the sequenced number of clones. These disadvantages can be somewhat mitigated by increasing the number of sequenced clones and using different enzymes in combination for the cutting. However, the method fails to provide as high resolution of the microbial community as next-generation sequencing.

Next-generation sequencing has been wildly applied for microbial community studies due to the advantages of including a high number of sequences per reaction, high max parallelisation and high throughput compared with Sanger sequencing (Ansorge, 2009; Morozova & Marra, 2008). Several recent studies, included Papers I-III in this thesis, have used different next-generation sequencing technologies (e.g. Roche454, illumina (Solexa), Ion Torrent and SOLiD) to scan the potential lignocellulolytic communities in e.g. dung beetles, termites, manure, anaerobic digestion processes fed with lignocellulosic materials etc. The aim of these studies has been either to identify previously undiscovered lignocellulolytic degraders or to investigate the correlation between the lignocellulolytic communities and the performance of an anaerobic digestion process (Ahlberg-Eliasson *et al.*, 2018; Chew *et al.*, 2018; Vanwonterghem *et al.*, 2016; Azman *et al.*, 2015; Estes *et al.*, 2013; Xia *et al.*, 2013) (**I**, **II**, **III**) .

In addition, next-generation sequencing has been applied in functional genomics studies relating to lignocellulolytic degraders. For example, Wei *et al.* (2015) and Wang *et al.* (2015) first sequenced DNA samples extracted from a mesophilic and thermophilic biogas digester, respectively, using the GSFLX sequencing system (Roche 454). They recovered several novel glycoside hydrolase genes from these metagenome datasets and heterologously expressed

these genes in *Escherichia coli* to study their biochemical characteristics (Wang *et al.*, 2015; Wei *et al.*, 2015). Vanwonterghem *et al*. (2016) used the Illumina HiSeq platform and a gene-centric metagenomic approach to compare the glycoside hydrolase profiles over time in different anaerobic digestion environments (Vanwonterghem *et al.*, 2016). These studies greatly expanded existing knowledge of possible application of the glycoside hydrolases and novel lignocellulolytic degraders, especially rare and uncultured species.

Moreover, when pure isolates are obtained, metagenome assembly and binning studies can be complemented by single-cell genomics with the help of next-generation sequencing (Yilmaz & Singh, 2012). Single-cell genomics can be used to assemble the genome of a bacterial species that is present at relatively low abundance in a metagenomics sample, or the genomes of completely unknown microorganisms (Gawad *et al.*, 2016). For example, complete genome sequencing of the cellulolytic anaerobic bacteria *Herbivorax saccincola* Type Strain GGR1 and *Herbinix luporum* SD1D is reported by Alexander *et al*. (2018) and Daniela *et al*. (2016), respectively. Their results revealed the presence of abundant carbohydrate-active enzymes (CAZymes) in these two bacteria (Pechtl *et al.*, 2018; Koeck *et al.*, 2016).

In recent studies, there has been an increasing trend for employing combined meta-omics methods, including metagenomics, metatranscriptomics and metaproteomics, to analyse lignocellulolytic communities (Kleinsteuber, 2018). For example, Güllert *et al*. (2016) compared microbial community structure by: i) 16S rRNA gene tag sequencing (using the Roche 454 platform) and ii) taxonomic origin of the cellulolytic glycoside hydrolase genes retrieved by the metagenomic data (using the Illumina HiSeq 2500 platform). The results indicate differences in cellulose degradation efficiency between biogas fermenter contents, elephant faeces and cow rumen fluid, possibly caused by differences in amount of transcribed cellulase (Güllert *et al.*, 2016).

Jia *et al*. (2018) reconstructed 107 population genomes from enrichment cultures and found only one sub-group to be highly transcribed in the metatranscriptomes. For the cellulose degraders, different genes were seen to be activated under different culture conditions. These findings deepen understanding of the relationship between a microbial population and the functional roles of active players in cellulosic biomass degradation (Jia *et al.*, 2018).

Furthermore, metaproteomics have been applied to study the metabolic activity of the lignocellulolytic communities by extracting total proteins, which are then digested with *e.g.* trypsin, followed by liquid chromatography-mass spectrometry (LC-MS) analysis (Heyer *et al.*, 2013). In a study combining metagenomics and metaproteomics, Hanreich *et al*. (2013) found that the

phylum Firmicutes seemed to play a major role for cellulose degradation, even though a fewer glycoside hydrolase genes were detected than in the phylum Bacteroidetes (Hanreich *et al.*, 2013). Moreover, a comparison of the taxonomic community structure recovered from a metaproteomic dataset and 16S rRNA gene tag pyrosequencing, together with fluorescent *in situ* hybridisation analyses, has revealed detailed lignocellulolytic functions in *Caldicellulosiruptor* spp. and the key role of *Clostridium thermocellum* for cellulose degradation (Lü *et al.*, 2014).

However, the use of metaproteomics to study lignocellulosic degradation groups in anaerobic digestion samples is still challenging in many ways (Heyer *et al.*, 2013). For example, the identification of proteins largely relies on the metagenomic database (Speda *et al.*, 2017b). The most used protein database, Swiss-Prot from the Universal Protein Resource (UniProt), contains around only 558 898 reviewed and annotated entries (last visited December 12, 2018), and most of these entries are not for bacteria and archaea. To overcome this problem, metaproteomic analysis can be performed based on a metagenome dataset recovered from the same samples (Hanreich *et al.*, 2013; Rademacher *et al.*, 2012). Another limitation is the sample complexity. To get high resolution in protein identification (*i.e.* identify as many proteins as possible), the extraction process needs to remove impurities such as humic organic matter, lipids, granules *etc.* (Keller & Hettich, 2009; Maron *et al.*, 2007; Hofman-Bang *et al.*, 2003). In addition, lignocellulosic bacteria are usually tightly attached to the fibres of biomass. Thus, the protein extraction method needs to be optimised in this regard to mitigate the loss of lignocellulosic bacteria. Several extraction methods have been tested in order to improve the protein yield from anaerobic digestate (Speda *et al.*, 2017a). The biases that can be introduced by using different databases and purification methods have been evaluated in an ongoing work (not included in this thesis). Preliminary results showed a significant improvement on the quality of identified proteins by using customised metagenomic database and purification method. However, obtaining specific microbial proteins from highly redundant and abundant environmental protein pools remains a great challenge (Heyer *et al.*, 2015).

6 Conclusions and future perspectives

Lignocellulosic materials, especially lignocellulosic residues, represent an important class of biomass that has not yet been fully utilised. Anaerobic digestion is believed to be one of the most feasible and economical tools for extracting the 'hidden' energy in lignocellulosic materials. Globally, several billion cubic metres of methane are produced yearly and demand is growing. The potential of using lignocellulosic materials to expand future production to meet this demand is impossible to ignore. However, the degradation efficiency of lignocellulosic materials in the anaerobic digestion process is still far from optimal. To increase use of lignocellulosic materials for methane production, a deeper understanding of the key agents in the degradation process, lignocellulosic microbes, is essential.

This thesis revealed the importance of the original inoculum for methane production using cellulose and wheat straw in a batch digestion system and also for the performance during start-up of semi-continuous stirred tank reactor (CSTR) processes. The microbial and chemical composition of the original inoculum sources was also revealed to influence the degradation of lignocellulose during long-term operation of CSTRs. Moreover, a positive correlation between the cellulose degradation rate of wheat straw and the level of *Clostridium cellulolyticum* was observed, indicating the possibility for steering the biogas production process to become more efficient by using a microbial approach. However, ammonia level appears to be one of the most important factors regulating the methane production performance of processes using lignocellulosic materials, possibly because it is a strong parameter shaping the microbial community structure and also the potential cellulose-degrading bacterial community. Lignocellulose-rich materials are often co-digested with energy-rich materials such as proteins in order to improve the C/N ratio. The data presented in this thesis suggest that degradation of proteins, giving high ammonia levels and high volatile fatty acid levels, results in

decreased lignocellulose efficiency. However, this decreased efficiency can be masked by increased volumetric yield due to increased load and higher energy content of the co-substrate. A low substrate degradation rate can potentially increase the risk of residual methane emissions during storage of the reactor digestate before use as a fertiliser.

The picture of the anaerobic lignocellulolytic microbial community is still far from complete. One important component of that community not covered in this thesis is the anaerobic fungi. Studies have shown that anaerobic fungi play an active role in lignocellulose degradation, even though their relative abundance in the overall microbial community is often low.

Furthermore, in this thesis only genomics-based analyses were performed and these are not sufficient to describe the anaerobic lignocellulolytic microbial community. Additional analyses relating to functions (*e.g.* proteomics and transcriptomics) are needed to fully identify the lignocellulose-degrading community and how to optimise it. Fortunately, with the rapid development in analytical methods and techniques and the corresponding growing databases, the cost of using transcriptomics- and proteomics-based approaches is becoming cheaper. When the complete guild is identified and a comprehensive and elaborate map of the lignocellulolytic microbial community becomes available, a customised inoculum adapted for each specific digestion task can be designed. This will help maximise methane production from the highly abundant lignocellulosic materials available world-wide.

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Popular science summary

The development of the oil industry has led to a rapid rise in the global economy in the last century. However, fossil fuel is a limited and non-sustainable energy resource. It is believed that, if no alternatives are developed in the future, energy constraints on the international community will become the main bottleneck in economic development. In addition, emissions of greenhouse gases (*e.g*. fossil fuel-derived carbon dioxide) have become a global concern, with about 88% of global energy consumption originating from fossil fuels. To overcome the environmental challenges and the dependence on fossil fuel, governments world-wide have formulated various policies to encourage the use of renewable energy.

Against this background, anaerobic digestion technology is highly interesting. In this process, various types of organic materials can be degraded under anaerobic conditions (without oxygen) into the end-product biogas, a renewable energy source. Anaerobic digestion is a multi-functional technology and it can be used simultaneously for waste management, production of renewable energy and production of an organic fertiliser. In addition, the biogas process can be implemented at small or large scale, which is important when designing flexible and sustainable energy solutions in both industrialised and developing countries. Materials that can be used for biogas production include various types of waste products, such as manure, straw, municipal wastewater, food waste *etc*., and dedicated energy crops. By controlled use of wastes in a biogas process, rather than dumping household waste in landfill or storing manure in open tanks, it is possible to reduce the volume of unwanted wastes and also decrease emissions of carbon dioxide, methane and other greenhouse gases. The biogas produced, containing the energy carrier methane, can be used for production of heat, electricity or vehicle fuel after upgrading (removal of carbon dioxide and trace gases). The residues left after biogas production are rich in plant nutrients and can be used as a fertiliser in crop

production, replacing fossil energy-requiring mineral fertilisers and enabling recycling of nutrients between urban and rural areas.

Microorganisms are essential for degrading organic materials to biogas, in a process that proceeds through various anaerobic digestion pathways and requires the combined activity of several groups of microorganisms. To obtain a stable biogas process, all these microorganisms must work in a synchronised manner.

Among the organic materials that can be used for biogas production, lignocellulosic materials, especially agriculture residues such as animal manure, straw, rice husks, corn stalks *etc*. are of great interest due to their high abundance world-wide. However, when lignocellulosic materials (including agriculture residues) are used for biogas production, the process efficiency is limited, due to the low nutrient content of these materials and the highly recalcitrant structure of their plant cell walls hindering microbial degradation. Thus, to achieve higher degradation efficiency of lignocellulosic materials for biogas production, a better understanding of the lignocellulose degraders (*i.e*. lignocellulolytic microorganisms) is needed.

This thesis examined possible lignocellulose degraders and studied the composition of their community. It also investigated possible links between changes in microbe community structure and environmental factors within the anaerobic reactor (*e.g.* process parameters, feedstock composition and feeding strategy). Different molecular methods (analysing DNA and proteins) for exploring the microbial communities were discussed, with the aim of building an appropriate pipeline for in-depth study of the lignocellulose degraders in biogas processes.

Populärvetenskaplig sammanfattning

Utvecklingen av oljeindustrin har lett till en snabb ökning av världsekonomin under det senaste århundradet. Men fossilt bränsle är en begränsad och icke-hållbar energiresurs och användningen leder dessutom till utsläpp av växthusgaser. Fossila bränslen står för ca 88% av den globala energiförbrukningen. För att övervinna miljöutmaningarna och beroendet av fossila bränslen har regeringar över hela världen formulerat olika strategier för att uppmuntra användningen av förnybar energi.

Mot denna bakgrund är anaerob (syrefri) rötning en mycket intressant teknik. I denna process kan olika typer av organiska material brytas ned av olika mikroorganismer till slutprodukten biogas, en förnybar energikälla. Anaerob nedbrytning är en multifunktionell teknik som kan användas för både behandling av avfall och produktion av förnybar energi och av organiskt gödselmedel. Dessutom kan systemet sättas upp både i liten eller stor skala, vilket är viktigt vid utformningen av flexibla och hållbara energilösningar i både industri- och utvecklingsländer. Material som kan användas för biogasproduktion inkluderar olika typer av avfall, såsom gödsel, halm, kommunalt avloppsvatten, matavfall m.m. och dedikerade energigrödor. Genom kontrollerad användning av avfall i en biogasprocess, snarare än deponering på soptipp eller lagring av gödsel i öppna tankar, är det möjligt att minska volymen av oönskat avfall och samtidigt minska även utsläppen av koldioxid, metan och andra växthusgaser. Den biogas som produceras kan sedan användas för produktion av värme, el eller fordonsbränsle efter uppgradering (avlägsnande av koldioxid och spårgas). Resterna som blir kvar efter biogasproduktionen är rik på växtnäringsämnen och kan användas som gödningsmedel i jordbruket och då ersätta fossila energikrävande mineralgödselmedel. Genom att använda rötresten som gödningsmedel möjliggörs också kretslopp av näringsämnen mellan städer och landsbygdsområden.

Olika mikroorganismer är avgörande för att anaerob nedbrytning av organiskt material till biogas ska fungera. Den mikrobiella processen fortskrider genom flera olika nedbrytningsvägar och kräver också aktivitet av flera olika grupper av mikroorganismer. För att erhålla en stabil biogasprocess måste alla dessa mikroorganismer också fungera på ett synkroniserat sätt. Bland de organiska material som kan användas för biogasproduktion är olika jordbruksrester, som djurgödsel, halm, blast etc. av stort intresse på grund av att dessa finns i stor mängd. Karaktäristiskt för detta material är att det ofta innehåller mycket lignocellulosa, vilket har ett lågt näringsinnehåll och en komplicerad struktur, något som hindrar mikrobiell nedbrytning. För att uppnå högre nedbrytningseffektivitet och biogasproduktion av denna typ av material behövs en bättre förståelse av de bakterier som bryter ner lignocellulosa i biogasprocesser.

Denna avhandling undersökte vilka bakterier som är närvarande i olika biogasprocesser och som potentiellt kan vara inblandade i nedbrytningen av lignocellulosa. Den utredde också möjliga kopplingar mellan sammansättningen på mikroorganismssamhället och driften av biogasprocessen. Frågor som belystes var till exempel; kommer sammansättningen av det cellulosanedbrytande bakteriesamhället påverkas av vilket material som bryts ner i biogasreaktorn? Är vissa bakterier viktigare än andra för att få en bra nedbrytning? För att studera mikroorganismerna i olika biogasprocesser användes olika s.k. molekylära metoder (analys av DNA). Det övergripande syftet med studierna var dels att hitta metoder att studera specifikt de bakterier som bryter ner lignocellulosa i biogasprocesser och dels att förstå vilka som är mest kritiska för att få en effektiv process samt vilka parametrar som påverkar dem.

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