Biogas Production from Lignocellulosic Materials

Microbial Community Structure

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Cover: Scanning electron micrographs of strain Dc1, cells and spores associated with the cellulose fibres.
(Photo: T. Liu)

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Abstract

Lignocellulosic materials such as agricultural plant residues are widely available in large amounts and can be used for production of biogas without the risk of competition for arable land. However, the intricate structure of lignocellulose, a major component of the plant cell wall, limits microbial degradation and consequently results in low degradation rate and low biogas yield. The aim of this thesis was to investigate microbial communities engaged in the degradation of lignocellulose and search for correlations with process operating parameters and degradation efficiency. Such information could be used in a long-term perspective for management of biogas processes towards more efficient degradation.

The bacterial and archaeal communities as well as functional gene communities of glycoside hydrolase family 5 (*cel5*) and 48 (*cel48*), representing potential cellulose-degrading bacteria, were investigated in laboratory-scale and industrial-scale digesters; and their incidence was related to process parameters. The laboratory-scale digesters were operated with manure, alone or in co-digestion with wheat straw (mechanically chopped or treated with steam explosion), at different temperatures. The results demonstrated that all digesters had similar overall process performance, *e.g*. degree of degradation and biogas yield, irrespective of straw addition or changes in temperature. However, the microbial communities, including potential cellulose-degrading bacteria, changed in response to the changes in operation, *e.g*. addition of straw, pre-treatment and operating temperature. In a survey of degradation efficiency of cellulose and wheat straw in 10 industrial-scale biogas plants in Sweden, free ammonia level was identified as a potential factor affecting degradation efficiency as well as the species richness and taxonomic composition of bacterial communities, including the *cel5* community.

This thesis presents novel information about microbial communities in biogas processes degrading lignocellulosic materials and their response to operating parameters.

Keywords: anaerobic digestion, lignocellulose, archaeal and bacterial community structure, straw, cow manure, anaerobic cellulose-degrading bacteria, glycoside hydrolase family 5 and 48.

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To my family

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 Risberg, K., Sun, L., Levén, L., Horn, S.J. & Schnürer, A. (2013). Biogas production from wheat straw and manure – Impact of pretreatment and process operating parameters. *Bioresource Technology* 149, 232-237.

II Sun, L., Müller, B. & Schnürer, A. (2013). Biogas production from wheat straw: community structure of cellulose-degrading bacteria. *Energy, Sustainability and Society* 3, 15.

III Sun, L., Pope, P.B., Eijsink, V.G. & Schnürer, A. (2015). Characterisation of microbial community structure during continuous anaerobic digestion of straw and cow manure. *Microbial Biotechnology* (doi:10.1111/1751-7915.12298).

IV Sun, L., Liu, T., Müller, B. & Schnürer, A. Straw and cellulose degradation efficiency in industrial biogas plants in Sweden and correlation to microbial community structure (manuscript).

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The contribution of the author to the papers included in this thesis was as follows:

I Participated in planning the study and performed part of the laboratory work. Co-author of the manuscript.

II Participated in planning the study and performed the majority of the laboratory work. Main writer of the manuscript.

III Participated in planning the study and performed the majority of the laboratory work. Main writer of the manuscript.

IV Participated in planning the study and performed half the laboratory work. Main writer of the manuscript.

In addition to Papers I-IV, the author contributed to the following papers within the timeframe of this thesis work:

Müller, B., Sun, L. & Schnürer, A. (2013). First insights into the syntrophic acetate-oxidizing bacteria – a genetic study. *MicrobiologyOpen* 2(1), 35-53.

Sun, L., Müller, B., Westerholm, M. & Schnürer, A. (2014). Syntrophic acetate oxidation in industrial CSTR biogas digesters. *Journal of Biotechnology* 171, 39-44.

Abbreviations

AD	Anaerobic digestion
BMP	Biochemical methane potential
CDB	Cellulose-degrading bacteria
Cel48	Glycoside hydrolase family 48
Cel5	Glycoside hydrolase family 5
CSTR	Continuously stirred tank reactor
DNA	Deoxyribonucleic acid
HRT	Hydraulic retention time
NGS	Next-generation sequencing
OLR	Organic loading rate
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
SAO	Syntrophic acetate oxidation
SRT	solid retention time
T-RF	Terminal restriction fragment
T-RFLP	Terminal restriction fragment length polymorphism
TS	Total solids
UASB	Upflow anaerobic sludge blanket
VFA	Volatile fatty acids
VS	Volatile solids

1 Introduction

Biogas is produced through anaerobic digestion (AD) of organic materials. Nowadays, this technology is increasingly used worldwide and related research activities are of growing importance. A likely explanation for this rapid expansion is the multi-functional nature of the technology (Weiland, 2010; Holm-Nielsen *et al.*, 2009). Its functions include: (1) Renewable energy production; methane, the main component of biogas, can be used as an alternative energy source to replace fossil fuels, either by direct combustion to generate heat and electricity or though upgrading to be used as vehicle fuel or injected into the gas grid; (2) waste management; the AD technology provides a method for stabilisation of a variety of organic waste fractions; and (3) production of an organic fertiliser; during the degradation process, nutrients in the organic waste are retained in the residue (digestate), which can be use as fertiliser on agricultural land, which reduces the need for mineral fertiliser and thus the associated use of fossil energy in mineral fertiliser manufacture.

Biogas can be produced from different types of organic materials, such as industrial wastewater, food waste, sewage sludge and agricultural waste (Sawatdeenarunat *et al.*, 2015). Materials rich in lignocellulose, such as plant residues, represent the most promising renewable organic feedstock for biogas production, as their production does not compete for arable land (Sawatdeenarunat *et al.*, 2015; Chandra *et al.*, 2012). Unfortunately, the nature of lignocellulose materials limits their application in anaerobic digestion. For example: (1) the insolubility and resistant structure prevent lignocellulose from being efficiently degraded; and (2) the low nutrient content (nitrogen, phosphorus, trace elements *etc*.) makes lignocellulosic materials a poor nutrient source for degrading microorganisms (Sawatdeenarunat *et al.*, 2015; Monlau *et al.*, 2013; Tsavkelova & Netrusov, 2012; Wang *et al.*, 2009a).

Anaerobic digestion of organic materials is a complex microbiological process requiring synchronised activity by several groups of microorganisms with different metabolic capacities. In the presence of complex lignocellulosic material, the first step, hydrolysis, is suggested to be rate-limiting (Lynd *et al.*, 2002; Noike *et al.*, 1985). Many attempts have been made to increase the efficiency of biogas processes using lignocellulosic materials as substrate, including *e.g*. different pre-treatments (Monlau *et al.*, 2013; Tsavkelova & Netrusov, 2012; Taherzadeh & Karimi, 2008) and co-digestion with more nutrient-rich materials (Wang *et al.*, 2010a; Wu *et al.*, 2010; Lehtomäki *et al.*, 2007; Mshandete *et al.*, 2004). However, despite decades of effort dedicated to biogas research, there is still insufficient information available concerning the microorganisms responsible for cellulose degradation during biogas production. Moreover, even less effort has been devoted to improving the process efficiency from a microbiological point of view.

1.1 Aim of the thesis

The aim of this thesis was to expand knowledge on the microbial community structure in anaerobic digestion of lignocellulosic biomass, with the focus on cellulose-degrading communities; and to examine potential correlations between operating parameters and community structures.

Specific objectives were to:

1. Determine the biogas production potential of cellulose and straw in laboratory- and industrial-scale biogas reactors (**I**, **II**, **III** and **IV**).
2. Evaluate biogas production from straw during co-digestion with manure at different operating temperatures and C/N ratios (**I**).
3. Analyse the microbial community structure in the studied AD processes by targeting functional genes, *i.e*. glycoside hydrolase family 5 and 48, and phylogenetic gene, *i.e*. 16S rDNA (**II**, **III** and **IV**).

2 Biogas production

Biogas is generated through the microbial degradation of organic materials in anaerobic conditions. During this complex process, methane (CH_4) is formed as the main product (55-70%), together with carbon dioxide (CO_2) (30-45%) and small amounts of other gases, such as hydrogen sulphide (H_2S) (Deublein & Steinhauser, 2008). Methane, the main energy carrier, is generated in the last step of the anaerobic degradation processes, which is called methanogenesis. In natural environments, methane is produced in anoxic environments such as peat and marshland, sediments and the rumen, which contribute a large proportion of global methane emissions (Lowe, 2006). It is worth noting that methane is a greenhouse gas with an ability for absorbing infrared radiation 20-fold higher than that of carbon dioxide (Wuebbles & Hayhoe, 2002). Through anaerobic digestion of organic materials in constructed biogas digesters, it is possible to produce energy and at the same time provide cost-effective waste management and reduce the amount of methane otherwise released to the atmosphere, for instance during storage of manure (Appels *et al.*, 2011).

Figure 1. Straw baling after harvest in Sweden.

2.1 Biogas production in Europe and Sweden

Within the European Union (EU), more than 14 500 biogas plants were in operation in 2014, with total production of 158.4 TWh biogas per year (European Biogas Association, 2015; EurObserv'ER, 2014). Different materials are used for biogas production, such as energy crops, landfill leachate, sewage sludge and agricultural waste, with different materials dominating in different countries. Usage of biogas also varies in different countries, depending *e.g.* on country-specific legislation and subsidies. Germany, the United Kingdom (UK) and Italy have the largest primary production of biogas, corresponding to 79.5, 21.6 and 21.5 TWh per year, respectively (EurObserv'ER, 2014). In Germany, around 60% of biogas is produced from maize, while the rest comes from other energy crops (16%), slurry (12%) and miscellaneous organic waste (8%). Only 4% originates from harvest residues (EurObserv'ER, 2014). In the UK, biomethane is mainly produced from landfill (84%) and sewage sludge (16%) (EurObserv'ER, 2014). In 2010, biogas from landfill also constituted the majority (69%) of total production in Italy (EurObserv'ER, 2012). However, by 2013 co-digestion plants processing organic waste such as agricultural waste and municipal solid waste had become more common in Italy (EurObserv'ER, 2014).

Sweden has decades of experience in the use of anaerobic digestion technology, since anaerobic digestion was first introduced at wastewater treatment plants in the 1960s. The initial objective was to reduce the sludge volume, but after the energy crisis in the 1970s the objective also became energy production and reduction of the dependency on fossil fuels (SGC, 2012; Berglund, 2006). To date, almost all the large-scale municipal wastewater treatment plants in Sweden, in total 137 facilities, have installed anaerobic digesters to produce biogas (Swedish Energy Agency, 2014). The total potential for biogas production in Sweden is estimated to be around 15 TWh/year, of which 8.1 TWh/year are derived from agricultural residues and manure. Around 69% (5.8 TWh) of the potential from agricultural residues is represented by using straw as substrate. However, it should be noted that dedicated energy crops were excluded from those calculations (SGC, 2012). Thus the agricultural sector, or more specifically the straw material it produces, represents huge potential for biogas production in Sweden and also in other countries in Europe and worldwide (Monlau *et al.*, 2013; Chandra *et al.*, 2012).

2.2 The microbiology of anaerobic digestion

The efficiency and stability of the AD process is dependent upon the concerted and syntrophic activity of different microorganisms (Vanwonterghem *et al.*, 2014). The anaerobic digestion of organic materials to produce biogas consists of four steps (Angelidaki *et al.*, 2011): Hydrolysis, fermentation, anaerobic oxidation and methanogenesis. In the first step, complex organic material such as polysaccharides, proteins and lipids are converted to soluble oligomers and monomers (*e.g.* sugars, amino acids and long-chain fatty acids). When cellulose-rich materials are converted to biogas, this hydrolysis step is suggested to be the rate-limiting step (Lynd *et al.*, 2002; Noike *et al.*, 1985). The cellulose-degrading community is responsible for the hydrolysis of cellulose into soluble compounds (**II** and **IV**) (Lynd *et al.*, 2002). In the second step of degradation, fermentative bacteria degrade the products from the hydrolysis reactions into intermediate products, which are used to generate acetate, carbon dioxide and hydrogen by syntrophic fermentative bacteria in the third step (Schink, 1997). As in general, the degradation of these intermediates, such as fatty acids, alcohols and propionate, is endergonic, *i.e.* thermodynamically unfavourable under standard conditions. As a consequence, the removal of hydrogen and acetate by methanogens is essential for these reactions to proceed (Schink, 1997). In the last step, methane is produced by two main groups of methanogens: hydrogenotrophic and aceticlastic methanogens (**III**) (De Vrieze *et al.*, 2012; Angelidaki *et al.*, 2011). The

hydrogenotrophic methanogens mainly use hydrogen or formate as an electron donor to reduce carbon dioxide to methane (Angelidaki *et al.*, 2011; Schink, 1997). The aceticlastic methanogens cleave acetate directly into a methyl and a carboxyl group, and then the methyl group is further converted to methane (Zinder & Koch, 1984). In the presence of inhibitor such as ammonia, methanogenesis can alternatively be carried out by the syntrophic acetate oxidation (SAO) pathway, which consists of a two-step reaction: i) Oxidation of acetate to H_2 and CO_2 by syntrophic acetate-oxidising bacteria, followed by ii) reduction of CO_2 to methane by hydrogenotrophic methanogens (Schnürer & Nordberg, 2008; Zinder, 1984).

2.3 Process parameters

To secure a stable biogas process with high efficiency, it is important that the process is managed in a way that allows growth of the different groups of microorganisms engaged in the whole process. The process factors described in this section include some important monitoring parameters (pH, VFA, ammonium/ammonia, sulphate content and methane yield) and some operating parameters (HRT, OLR, temperature and substrate composition) for guiding the process towards stable performance. The monitoring parameters can serve as indicators of the stability of the whole process, while the operating parameters affect the growth of the microorganisms within the anaerobic digester, which eventually affect the overall performance and stability of the biogas process. In this thesis, changes or dynamics of the microbial communities in response to a change in the operating parameters were studied (**I, II** and **III**). The change of operating parameters includes using straw as a substrate, pre-treatment of straw used as a substrate and altering the operating temperature.

2.3.1 Parameters for process monitoring

The pH within the AD process is generally within the range 6.0-8.5. The methanogens grow optimally at around neutral pH and a pH value outside this range can inhibit their growth, thus resulting in unstable digester performance and sometimes even process failure (Chandra *et al.*, 2012).

Volatile fatty acids (VFA) are intermediate products formed during degradation of complex organic materials (McCarty & McKinney, 1961). The levels of VFA within an anaerobic digester has been reported to be a useful indicator for monitoring the stability of the digestion process (Ahring *et al.*, 1995). Accumulation of VFA can be caused by, for example, an overload of substrate or inhibition of methanogens (Nielsen *et al.*, 2007). The methanogens

are more sensitive to pH drop and a pH below 6.2 is toxic, while the acidogenic bacteria can adapt to pH as low as 4.5-5.0 (Chandra *et al.*, 2012). Thus, accumulation of VFA can decrease the pH and inhibit the methanogens and thus also result in a more rapid pH drop. The level of propionate and/or propionate:acetate ratio has been suggested as key parameters for process control and optimisation (Nielsen *et al.*, 2007; Marchaim & Krause, 1993).

Alkalinity is also important for monitoring the process, as it is an indicator of the buffering capacity within the reactor. A high and constant alkalinity is able to maintain the pH within the neutral or slightly above neutral range, even in case of acid accumulation (Chandra *et al.*, 2012). For monitoring, alkalinity changes prior to pH and can be used as an early indicator of the risk of acidification (Drosg, 2013).

Ammonium (NH_4^+) is released during the degradation of proteins. Ammonium is in equilibrium with free ammonia (NH_3), which is a well-known inhibitor of the AD process (Chen *et al.*, 2014; Rajagopal *et al.*, 2013; Chen *et al.*, 2008). In the process, methanogens have been suggested to be less tolerant to ammonia and the inhibition is believed to be caused by a proton imbalance and/or a potassium deficiency within the cell (Niu *et al.*, 2014; Chen *et al.*, 2008). Under the ammonia stress, an alternative pathway, syntrophic acetate oxidation, takes over the methanogenesis (Sun *et al.*, 2014; Westerholm, 2012; Schnürer & Nordberg, 2008). A slow increase in ammonia concentration and a long acclimation period are suggested to be required for this shift to occur without process imbalance (Westerholm, 2012). The ammonium/ammonia equilibrium is affected by the pH and temperature, with a shift towards free ammonia with increasing temperature and pH. Ammonia and temperature have been suggested to be the main parameters clustering the AD bacterial community (De Vrieze *et al.*, 2015). The survey of 10 industrial-scale biogas plants within this thesis also identified free ammonia as a strong parameter for shaping the bacterial community (**III**).

Hydrogen sulphide can either be produced from the amino acids cysteine and methionine or by sulphate-reducing bacteria reducing sulphate present in the process (Moestedt *et al.*, 2013). Hydrogen sulphide within the AD process can cause inhibition, either directly or indirectly by precipitate trace metals (Ramírez *et al.*, 2011; Stams *et al.*, 2003). Trace metals are needed for the enzymes involved in methanogenesis (Glass & Orphan, 2012) and limited availability may affect the overall process performance (Demirel & Scherer, 2011; Lebuhn *et al.*, 2008). In addition, hydrogen sulphide is corrosive and causes a bad odour, all of which makes hydrogen sulphide a highly undesirable compound present in the digester and in the biogas. In practice, iron can be

supplied to the process and reduce the level of hydrogen sulphide by precipitation (Nordell *et al.*, 2015).

2.3.2 Parameters for process operation

Hydraulic retention time (HRT) is the average time that the slurry remains in a biogas digester (Yadvika *et al.*, 2004). For a continuously stirred tank reactor, the hydraulic retention time is the same as solid retention time. In general, the minimum HRT should be longer than the doubling time of the microorganisms to avoid washout (Yadvika *et al.*, 2004). On the other hand, a longer retention time requires a larger volume of reactor at a particular organic loading rate (Yadvika *et al.*, 2004). A typical HRT is 15-30 days under mesophilic conditions and slightly shorter under thermophilic conditions (Mao *et al.*, 2015; Braun *et al.*, 2010).

The organic loading rate (OLR) is defined as the amount of organic material (volatile solids, VS) fed daily per litre of digester volume (g VS/L/day). When sewage sludge is used as the main substrate, the OLR typically ranges from 1.2 to 8.9 g VS/L/day (Mata-Alvarez *et al.*, 2014). During co-digestion of cattle manure with other organic wastes, such as municipal waste or crude glycerol, OLR up to 5.5-7.3 g VS/L/day have been reported (Mata-Alvarez *et al.*, 2014). For mono- or co-digestion of lignocellulosic materials, reported OLR are typically lower, around 1.5-3.5 g VS/L/day (**I**) (Lucas *et al.*, 2015; Lebuhn *et al.*, 2014; Ziganshin *et al.*, 2013). An increase in OLR usually results in an increase in total methane yield, however, if a certain OLR value is exceeded, the process can be unstable and process failure may even occur (Mata-Alvarez *et al.*, 2014).

In natural environments, activity of methanogens has been detected within a wide temperature range, from nearly 0 °C to over 100 °C (Kurr *et al.*, 1991; Zeikus & Winfrey, 1976). Constructed digesters are commonly run either at mesophilic temperature (~37 °C) or thermophilic temperature (~55 °C). In the thermophilic temperature range (45-55 °C), the reaction typically proceeds much faster than under mesophilic conditions (25-40 °C), in general allowing higher OLR compared with digesters operating at lower temperature (Van Lier *et al.*, 1996). However, in this thesis work, an increase in operating temperature from 37 to 52 °C in a laboratory-scale digester resulted in similar process performance, *i.e*. similar methane yield and degree of degradation (**I**). Furthermore, at higher temperatures pathogens are removed at higher efficiency, which leads to a more sanitary end product (Sahlström, 2003). On the other hand, mesophilic digestion requires lower energy input for heating and is commonly more stable and less affected by inhibition, *e.g*. ammonia inhibition (Chen *et al.*, 2008).

Various substrates used for anaerobic digestion have their own advantages and disadvantages, for instance digestion of animal manure or slaughterhouse waste may lead to ammonia inhibition due to the high nitrogen content. Plant residues, on the other hand, lack nitrogen and are also low in trace elements (Sawatdeenarunat *et al.*, 2015; Mata-Alvarez *et al.*, 2014). Therefore co-digestion has been proposed as a solution in this regard. By carefully selecting the co-substrates and blending them in a ratio, it is possible to achieve synergies in the process, dilute harmful compounds and optimise methane production and digestate quality (Mata-Alvarez *et al.*, 2014). In one of the studies included in this thesis, wheat straw was evaluated as a substrate in co-digestion with cattle manure, with wheat straw in different ratios (**I**). Wheat straw has a C/N ratio of almost 100, which is far from the 15-30 suggested as optimal for anaerobic digestion (Chandra *et al.*, 2012). Co-digestion with cow manure gave a more optimal substrate mix in this regard but, interestingly, a stable process was observed even with a C/N ratio as high as 75 (**I**).

3 Biogas from lignocellulosic materials

Plant material is the most abundantly produced lignocellulosic biomass in the terrestrial ecosystems (Chandra *et al.*, 2012; Leschine, 1995). Within the agriculture sector, energy crop and agricultural residues are the most important feedstock for renewable energy production (Chandra *et al.*, 2012). Biogas produced from agriculture residues and energy crops cultivated on land not suitable for feed or food production is regarded as second-generation biofuel, as there is no direct competition for arable land (Monlau *et al.*, 2013; Chandra *et al.*, 2012).

The cellulose, hemicellulose and lignin within the plant cell wall form a complex structure, which greatly limits its biodegradability. The degree of polymerisation and crystallinity of cellulose, the structure of hemicellulose, the content and composition of lignin, the pectin content as well as the accessible surface area and pore volume have previously been identified as the main factors influencing the biodegradability (Monlau *et al.*, 2014; Zheng *et al.*, 2014; Monlau *et al.*, 2013). Moreover, as previously mentioned, lignocellulosic materials such as energy crops and plant residues are often rich in carbon, but low in nutrient content (nitrogen, phosphorus, trace elements *etc*.) (Sawatdeenarunat *et al.*, 2015). A carefully selected co-substrate that compensates for the drawbacks of lignocellulosic biomass may achieve a stable and efficient process (Mata-Alvarez *et al.*, 2014).

3.1 Structure of lignocellulosic biomass

Cellulose, hemicellulose and lignin are the three major fractions of lignocellulosic biomass, but the composition of each fraction varies among different plant materials and harvesting times (Sawatdeenarunat *et al.*, 2015; Monlau *et al.*, 2013; Weiland, 2010). Cellulose is typically found to be most abundant (30-70%), while hemicellulose and lignin represent 15-30% and 10-

25% of the biomass, respectively (Monlau *et al.*, 2013). Cellulose is a linear biopolymer consisting of repeating glucose units, joined by β-1,4 bonds. Parallel cellulose chains are then assembled by hydrogen bonds and van der Waals forces into so-called microfibrils. These microfibrils are often embedded in a matrix of other polymers, such as hemicellulose and lignin (Leschine, 1995). Hemicellulose is a heterogeneous polysaccharide composed of a series of random branched polysaccharides (Zheng *et al.*, 2014; Taherzadeh & Karimi, 2008), of which the short branch chains usually contain various polymers such as xylose, mannose, galactose, rhamnose and arabinose (Zheng *et al.*, 2014; Monlau *et al.*, 2013). In addition, hemicellulose interacts with cellulose, pectin and lignin to make this network structure resistance to hydrolysis (Zheng *et al.*, 2014; Monlau *et al.*, 2013). Lignin is a large, complex aromatic heteropolymer present in cell walls that is made of different phenylpropane alcohols. The content and composition of lignin also vary between plant species, but they all serve to increase the strength of plant cell wells. The degradation of lignin under strictly anaerobic conditions is slow (Zheng *et al.*, 2014; Dittmar & Lara, 2001; Dinsdale *et al.*, 1996).

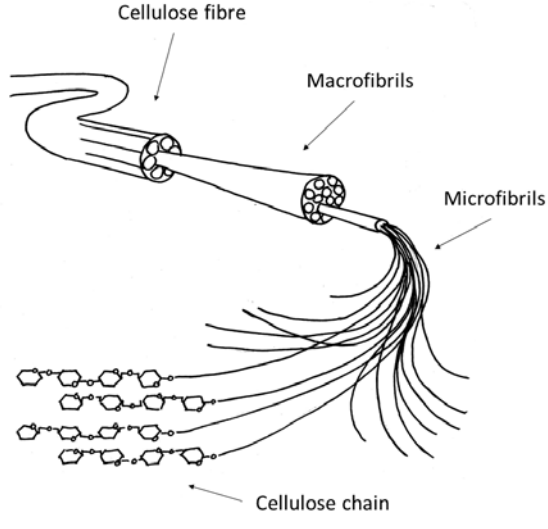

Figure 2. Structure of lignocellulose.

3.2 Pre-treatment methods

Different pre-treatment methods have been investigated in order to improve the AD process efficiency when using lignocellulosic biomass as substrate (**I**) (Zheng *et al.*, 2014; Krishania *et al.*, 2013). The various pre-treatment methods currently available can be summarised as: 1) physical pre-treatment, such as comminution, steam explosion, liquid hot water hydrothermolysis and irradiation; 2) chemical pre-treatment, such as acid pre-treatment, alkaline pre-treatment and catalysed steam explosion; and biological pre-treatment with enzymatic hydrolysis and white-rot fungi (Zheng *et al.*, 2014; Monlau *et al.*, 2013). Each strategy has its own advantages and drawback, but in general, pre-treatment can increase the accessible surface area, modify the lignin structure, reduce the cellulose crystallinity and polymerisation, and reduce the degree of hemicellulose acetylation (Zheng *et al.*, 2014; Monlau *et al.*, 2013). However, pre-treatment also increases energy consumption, which can compromise the economic feasibility of the biogas plant (Zheng *et al.*, 2014; Hendriks & Zeeman, 2009).

In this thesis, steam explosion was used as a pre-treatment for wheat straw (**I** and **II**). During this treatment, raw material is first heated up rapidly at a relatively high pressure, followed by a rapid pressure drop, which opens up the plant fibre structure (Horn *et al.*, 2011). The result of steam explosion depends on several parameters, including temperature, pressure and time, and using this method as a pre-treatment for substrate has shown different results. For example, a 20% increase (from 276 to 331 N mL/g VS) and a 30% increase (from 275 to 361 N mL/g VS) in methane potential has been observed after steam explosion of wheat straw at 180°C, 15 min, 20 bar and 170°C, 10 min, 10 bar, respectively (Bauer *et al.*, 2010; Bauer *et al.*, 2009). A 27% increase in methane potential (from 233 to 296 N mL/g VS) using wheat straw has also been reported following treatment at 200°C for 5 min (Ferreira *et al.*, 2014). However, a non-significant change in methane potential (from 276 to 287 N mL/g VS) has been observed after pre-treatment of wheat straw at 140°C for 60 min (Theuretzbacher *et al.*, 2015), although the rate of degradation improved in that study. In this thesis, pre-treatment of wheat straw at 210°C for 10 min did not result in any increase in methane potential or degradation rate (**I**). Moreover, using similarly pre-treated straw in laboratory-scale continuously stirred tank reactor (CSTR) did not result in increase of gas yield compared with untreated straw and the overall performance was not affected (**I**). However other positive effects, such as improved blending properties compared with no-treated straw and fewer problems with floating layers within the digester were observed (**I**).

During pretreatment, the partial degradation of the lignin-carbohydrate matrix may result in release of by-products such as furfural and soluble phenolic compounds (Monlau *et al.*, 2014; Monlau *et al.*, 2013; Horn *et al.*, 2011). The formation of such by-products has been reported to have a negative effect on ethanol production (Monlau *et al.*, 2014). In the AD process, phenols have been reported as having an inhibitory effect on aceticlastic methanogens (Levén *et al.*, 2012). However, by allowing an adaptation period the furfural and phenolic compounds can be degraded, as long as they are present at low levels (Monlau *et al.*, 2014).

3.3 Co-digestion

As mentioned previously, lignocellulosic materials are characterised as carbon-rich, poor in buffering capacity and deficient in nutrients (Mata-Alvarez *et al.*, 2014). Mono-digestion of lignocellulosic materials often results in a slow process and low methane yield (Sawatdeenarunat *et al.*, 2015). This limitation can be overcome by using a co-substrates, such as animal manure, can be used together with lignocellulosic biomass to supplement it with macro- and micronutrients and buffering capacity (Mata-Alvarez *et al.*, 2014). For instance, co-digestion has been investigated for wheat straw with dairy and chicken manure (Wang *et al.*, 2012), rice straw with kitchen waste and pig manure (Ye *et al.*, 2013) and oat straw with cattle manure (Lehtomäki *et al.*, 2007). These studies report higher methane yields (approximately 200-400 mL/g VS) compared with when using straw alone (~120-200 mL/g VS), as a consequence of the higher energy content of the co-digestion materials and their complementary properties. However, complementing manure with lignocellulosic materials do not always result in a higher specific yield as shown in this thesis work (**I**). But the high VS content of plant based materials can allow an increase in OLR without reduce the HRT, and thus increase the volumetric biogas production (Møller *et al.*, 2004). Beside macronutrients, lignocellulosic materials such as agricultural resides are often low in trace elements such as iron, nickel, cobalt, molybdenum, selenium and tungsten, which are required for microbial enzyme activity (Demirel & Scherer, 2011). In a long-term mono-digestion of maize silage for biogas production, trace element deficiency was suggested as the cause of acidification observed after around 8 months of operation (Lebuhn *et al.*, 2008). In a study by Wall *et al.* (2014), the specific methane yield of mono-digestion of grass silage in a laboratory-scale (CSTR) was increased by 12%, to 404 mL/g VS, by supplementation with trace elements.

3.4 Types of biogas digesters

Different types of digesters can be used for anaerobic digestion and they are often classified as: (1) Liquid or solid state process; (2) batch or continuous process; (3) single- or two-stage process.

Based on the total solids (TS) content within the anaerobic digester, the AD process can be divided into two types: liquid AD process (L-AD), with a TS content of less than 15%; and solid-state AD process (SS-AD), with a TS content of 15% or higher (Yang *et al.*, 2015; Brown *et al.*, 2012; Li *et al.*, 2011). The L-AD type is more suitable for substrates with a high moisture content, such as wastewater streams (Sawatdeenarunat *et al.*, 2015). The most common anaerobic reactor type for these more diluted materials at the present time is the CSTR. In this process, the material is typically pumped into the digester continuously or semi-continuously, while digestion residues are simultaneously removed. Solid materials can be fed into a CSTR directly at the top without pumping, but typically this reactor type is not optimal for handling lignocellulosic materials. Moreover, some feedstock such as wheat straw and grass silage may form a float layer on the top of the liquid phase (**I**). The upflow anaerobic sludge blanket (UASB) reactor is another type of single-stage reactor, which is designed to process high-rate sewage wastewater streams (Chong *et al.*, 2012). The configuration of the UASB reactor allows separation of the solid retention time (SRT) from the HRT, which minimises washout of microbes (*e.g.* the hydrolysing group of bacteria and methanogens) as a consequence of short HRT (Bal & Dhagat, 2001). The SS-AD, on the other hand, is better for processing high solids content feedstock such as lignocellulosic materials. Compared with L-AD, SS-AD requires less process water and lower energy input for heating and mixing (Li *et al.*, 2011). Moreover, for the same solid loading rate, SS-AD requires less reactor volume and achieves a higher volumetric methane yield (Brown *et al.*, 2012). The plug flow digester is one common type of SS-AD process, in which the substrate is fed from a feeding port and moved as a plug through the reactor to the exit. This process requires heavy equipment that can handle dry, viscous material (Li *et al.*, 2011).

The batch digester is another process that is suitable for the lignocellulosic materials. In such process the digester is filled once or on several occasions and the material is allowed to be degraded before being taken out of the digester. For large-scale applications, a series (at least three) of garage-type batch digesters with percolation and without mechanical mixing can be applied for mono-fermentation of energy crops (Weiland, 2010). Using four boxes of digesters, running with a substrate mixture of maize silage, poultry manure and digested material from the previous run, Heiermann *et al.* (2007) reported a

specific methane yield of 0.34 L/g of VS added. Laboratory-scale reactors of different designs can also be used to evaluate different substrates and substrate combinations. Batch digester is the type most commonly used to determine the biochemical methane potential (BMP), giving information on the methane yield and digestibility of a substrate (Sawatdeenarunat *et al.*, 2015; Chandra *et al.*, 2012). For the three most commonly available crop residues, maize crop waste, wheat straw and rice straw, the average potential is reported to be 0.34, 0.29 and 0.30 L CH_4/g VS respectively (Chandra *et al.*, 2012). In this thesis work, the BMP of straw and cellulose was evaluated using inoculum from laboratory-scale digesters and different industrial-scale biogas plants. The results showed similar level of methane potential for both wheat straw and cellulose (**I** and **IV**), however a lower potential when laboratory-scale reactor inoculum was used to initiate the process (**I**).

For a two-stage process, the microbiological degradation steps are separated into two digesters. The first digester is used for hydrolysis/ acidogenesis/acetogenesis and the second digester for methanogenesis (Kothari *et al.*, 2014). As the ideal pH ranges for hydrolysis (5.5-6.5) and methanogenesis (6.8-7.2) are different, this separation has been shown to achieve better hydrolysis of solid organic compounds (Kothari *et al.*, 2014; Weiland, 2010).

Figure 3. Laboratory-scale semi-continuous stirred tank reactor (CSTR).

3.5 Bioaugmentation

As mentioned above, the hydrolysis step in anaerobic digestion has been suggested to be the rate-limiting step. Efforts have thus been made to enhance the degradation of lignocellulose in biogas processes by inoculation with effective cellulose-degrading bacteria or bacterial consortia. For instance, bioaugmentation of *Clostridium cellulolyticum* during batch digestion of wheat straw achieved higher methane potential compared with a non-inoculated control (Peng *et al.*, 2014). In another batch cultivation, where brewery spent grain was used as substrate, an increase in the methane potential and a change in microbial community was observed by addition of various hydrolytic bacteria (Čater *et al.*, 2015). Moreover, in a two-phase continuous system digesting maize processing waste, bioaugmentation with a cellulolytic culture dominated by *Clostridium* achieved increasing substrate hydrolysis and methane production (Martin-Ryals *et al.*, 2015). Successful bioagumentation has also been shown using a non-hydrolytic, fermentative bacterium, *Acetobacteroides hydrogenigenes* (Zhang *et al.*, 2015). Adding this bacterium during biogas production from maize straw resulted in a 19-23% increase in methane yield. *Acetobacteroides hydrogenigenes* can degrade a number of substrates, including yeast extract, tryptone, arabinose, glucose, galactose, mannose, maltose, lactose, glycogen, pectin and starch (Su *et al.*, 2014). Acetate, hydrogen and carbon dioxide are the main fermentation products (Su *et al.*, 2014). Those results suggested that it is possible to improve the hydrolysis rate, *e.g.* cellulose and hemicellulose degradation rate, by bioaugmentation with a fermentative instead of hydrolytic bacterium. However, contradictory results have also been reported, *e.g.* bioaugmentation with an anaerobic fungus in a two-stage process using maize silage and cattail for combined bio-hydrogen and biogas production did not result in an increase in energy yield (Nkemka *et al.*, 2015). In summary, bioaugmentation of the hydrolytic group of microbes requires further investigation, while detailed investigation of microbial community dynamics during the bioaugmentation process is also needed.

4 Microbial community diversity

4.1 Methods for investigating the community structure of cellulose-degrading bacteria

As mentioned in the previous section, lignocellulosic materials are available in large amounts, but the intricate structure and imbalanced nutrient content limit the efficiency of AD of these materials. Although different strategies such as pre-treatment and co-digestion strategies have been investigated and applied, there is no conclusive solution for improving the biodegradability at a reasonable cost. Moreover, the microbiology of the AD process is still somewhat of a 'black box'. The microbial community, especially the cellulolytic community, needs further investigation.

4.1.1 Culture-dependent methods

Enrichment, isolation and cultivation of microorganisms in pure culture is a labour- and time-consuming task, but is also an essential step in studying the morphology, physiology and genetics of specific microorganisms. Furthermore, available pure culture makes the development of molecular tools feasible, based on genomic information. In order to cultivate anaerobic microorganisms, special equipment and techniques are required to provide an anaerobic environment, and the agar shake or role tube method is typically used for isolation (Schnürer *et al.*, 1996; Hungate & Macy, 1973).

In this thesis, in order to isolate cellulose-degrading bacteria, cellulose or cellobiose was used as the sole carbon source during the whole isolation procedure. The isolation started with enrichment of the bacterial consortia in a reduced mineral medium (Schnürer *et al.*, 1994), with the purpose of enriching the bacteria able to metabolise cellulose/cellobiose. Serial dilution of the enrichment cultures was then performed in the same mineral medium (Schnürer *et al.*, 1994). For the highest dilution at which growth (visual)

occurred, the agar shake method was applied for picking single colonies (Schnürer *et al.*, 1996) and cultivation in pure culture. The industrial biogas plants in Paper **IV** were used as the inoculum source for isolation and from this, two cellulose-degrading bacteria were isolated (unpublished data). The 16S rDNA sequence revealed the two isolates to be closely related to *Clostridium straminisolvens* CSK1 (1368 bp, 98% identity) and *Clostridium clariflavum* DSM 19732 (1500 bp, 97% identity) respectively (unpublished data). Other cellulose-degrading bacteria have been isolated in a similar way, including *Clostridium cellulolyticum* ATCC 35319 (Petitdemange *et al.*, 1984), *Clostridium cellulovorans* 743B (Sleat *et al.*, 1984), *Clostridium papyrosolvens* (Madden *et al.*, 1982), *Clostridium populeti* (Sleat & Mah, 1985) and *Clostridium stercorarium* (Madden, 1983).

Figure 4. Agar shake with single colonies.

Figure 5. The cellulose-degrading bacteria strains Bc1 and Dc1 cultivated in liquid medium.

4.1.2 Culture-independent methods

The majority of microorganisms in the AD process have not yet been cultivated, and it is estimated that 5% or less of the microbial diversity in the biosphere is cultivable using standard cultivation techniques (Curtis *et al.*, 2002; Amann *et al.*, 1995). Consequently, understanding of the microbial ecology and physiology associated with AD is most likely incomplete and biased. There are many factors that co-exist in this complex environment and affect microbial activity which cannot be studied when using culture-based methods. Moreover, functions related to competition and interaction between microorganisms are difficult to determine when using isolated microorganisms (Vanwonterghem *et al.*, 2014). Based on available genomic data, a variety of molecular methods have been invented and developed for use in further investigating the microbial community structures within AD processes.

Clone library is a culture-independent method that enables investigation of DNA extracted from an environmental sample by cloning and subsequent sequencing (Chouari *et al.*, 2005). In this thesis, clone libraries were constructed using products generated after polymerase chain reaction (PCR) amplification of genes encoding glycosidase hydrolase family 5 and 48 (**II** and **IV**). This technique has also been used to target 16S rDNA of the microbial community in various anaerobic digesters, such as digesters processing beet silage (Krakat *et al.*, 2011), crops and cow manure (Wang *et al.*, 2009b), grass

silage (Wang *et al.*, 2010a), pig manure (Liu *et al.*, 2009) and organic solid waste (Sasaki *et al.*, 2011).

Terminal restriction fragment length polymorphism (T-RFLP) is a method based on PCR technology where a selected target gene is amplified with PCR using the total DNA extracted from digester samples, However, different from clone library method, the primer in the PCR reaction is labelled with a fluorescent dye such as 6-carboxyfluorescein (FAM). In a second step, the amplicon generated from the PCR is digested with a selected restriction enzyme appropriate for the sequence of interest. The digestion products, which are called fluorescently labelled terminal restriction fragments (T-RFs) are separated and detected using capillary electrophoresis. The T-RFLP profile is visualised as the relative abundance of each T-RF at a specific length. As different sequences possibly have different restriction sites, in the assay each T-RF could represent a unique sequence, but occasionally the same T-RF can be represented by two different organisms (**II** and **IV**). Differences in the T-RFLP profile indicate the differences in structure between the communities. This method is usually combined with a clone library. Once the sequence for the environmental sample is available, the restriction site of each sequence can be analysed *in silico* and thus the corresponding sequence for each T-RF can be decided. This technique has been used in combination with clone libraries in several previously mentioned studies (Wang *et al.*, 2010b; Wang *et al.*, 2009b), as well as in this thesis work (**II** and **IV**).

Real-time quantitative PCR (qPCR) is another commonly used method for analysis of the microbial community and it can be applied to study, detect and quantify a targeted DNA sequence such as 16S rDNA or the functional gene. For quantification the real-time PCR technique is used, together with an intercalating dye such as SYBR green that fluoresces with double-stranded DNA. The fluorescence signal recorded increases as the double-stranded DNA increases after each amplification cycle. A melting curve analysis is performed following the PCR programme to inspect possible false positive signals such as primer-dimer and amplification errors (VanGuilder *et al.*, 2008). By comparison against the standard curve, usually a dilution of a known amount of target DNA cloned in the plasmid, the absolute amount of the gene of interest can be calculated (VanGuilder *et al.*, 2008). In this thesis, qPCR was used to quantify the relative abundance of *cel48* (**II**). This method has also been used successfully to quantitatively analyse other bacterial populations in biogas processes (Moestedt *et al.*, 2013; Westerholm *et al.*, 2011).

The development of next-generation sequencing (NGS), such as 454-pyrosequencing, has enabled cost-effective massive parallel sequencing of environmental samples with comparatively high coverage of the community

(Zakrzewski *et al.*, 2012; Schlüter *et al.*, 2008). The amplicon sequencing approach enables sequencing of PCR products without an extra clone step, thus eliminating the clone bias. Moreover, the multiplex technique allows the integration of barcode onto the primer, which enables processing of a considerable number of samples at the same time. Rapidly increasing numbers of studies are using amplicon sequencing targeting the 16S rDNA of samples from AD processes, including both laboratory-scale and industrial-scale biogas digesters. These include to date: laboratory-scale processes digesting straw and manure (**III**), industrial-scale digesters operating with various substrates (**IV**) (De Vrieze *et al.*, 2015; Sundberg *et al.*, 2013), a full-scale plant processing energy crops (Lucas *et al.*, 2015), batch cultivation of wheat straw and swine manure (Li *et al.*, 2014) and laboratory-scale digesters processing straw and hay (Lebuhn *et al.*, 2014). Metagenomic shotgun sequencing is another NGS approach, which directly sequences a library of sheared DNA fragments. Unlike the clone library or amplicon approach, the shotgun approach sequences random DNA fragments resulting from microbial genomes. Thus this method not only generates the sequences of phylogenetic genes, but also provides functional insights into the microbial community (Sharpton, 2014). A number of AD processes have been investigated using this metagenomic approach and it has generated information leading to a specific understanding of the hydrolysis step in the AD process (Hanreich *et al.*, 2013; Yan *et al.*, 2013; Schlüter *et al.*, 2008).

4.1.3 Limitation of the methods

Both the culture-dependent and culture-independent methods have their particular limitations that need to be considered and for optimal results these methods can be combined. With the culture-based approach, as previously mentioned, the culture might be biased. For instance, the enrichment step might enrich the fast-growing microbes but miss those most important for maintaining the functional stability of the whole community. For example, the isolated strain Dc1, targeted by T-RFLP analysis of the glycosidase gene *cel48* (T-RF 296), was present at very low levels in the industrial biogas plants from where it was isolated (under detection limit) in this thesis (**IV**). However, this bacterium increased to a level of 41.4% at the end point after batch digestion with straw, illustrating its ability to thrive in batch cultivation mode (**IV**). For the DNA extraction step used in all the molecular-based methods, the yield and purity vary between different extraction methods and this can affect the results of downstream analysis (Li *et al.*, 2013; Bergmann *et al.*, 2010). In addition, PCR bias and artefacts generated during PCR amplification, such as bias caused by differences in primer binding energy or the generation of

heteroduplexes and chimera, may provide incorrect population data (Kanagawa, 2003). To analyse the whole microbial community, primers for PCR have been designed to cover as many species as possible, but there is no "universal" primer that can target all bacteria (Klindworth *et al.*, 2013; Baker *et al.*, 2003) and different primer sets may amplify different fractions of a whole community (Fredriksson *et al.*, 2013; Rajendhran & Gunasekaran, 2011). Moreover, the cloning step within the clone library method may introduce bias, *e.g*. some sequences/fragments might be less cloned, in which regard GC content is suggested to be one influencing factor (Rajendhran & Gunasekaran, 2011; Morgan *et al.*, 2010). The metagenomic approach is able to eliminate the cloning bias, but the sequencing length is relatively short compared with the clone library approach (Kumar *et al.*, 2011; Morgan *et al.*, 2010; Kröber *et al.*, 2009). For the T-RFLP approach, the selection of restriction enzyme is critical in order to separate the target amplicons generated from a complex community. Furthermore, the star activity of restriction enzyme is another practical issue in this approach, giving false T-RFs (Wei *et al.*, 2008). For the NGS approach, horizontal transfer may lead to misleading inference in phylogenetic trees (Zarraonaindia *et al.*, 2013). With current sequencing techniques, the sequencing depth covers the dominant/more abundant microbes, but may still miss targeting the low-abundance taxa (Zarraonaindia *et al.*, 2013). These rare taxa may represent organisms with critical functions for the whole community, such as preserving novel genetic materials and the resilience ability of the community (Zarraonaindia *et al.*, 2013).

Another common conclusion regarding NGS data is that only a small fraction of sequencing reads can be classified to lower taxonomic levels such as genus (**III** and **IV**) (Li *et al.*, 2014; Smith *et al.*, 2014; Lu *et al.*, 2013; Zakrzewski *et al.*, 2012; Schlüter *et al.*, 2008). This indicates that current knowledge on genomic and physiological data is limited and more efforts should be made at isolation and cultivation (**III** and **IV**) (Curtis *et al.*, 2013; Narihiro & Sekiguchi, 2007).

4.2 Cellulose degradation in anaerobic environments

4.2.1 Anaerobic cellulose-degrading bacteria

Globally, around 5-10% of cellulose is degraded in anaerobic environments (Leschine, 1995). Anaerobic cellulose degradation occurs in various environments, such as soil, aquatic and animal gut environments (Morrison *et al.*, 2009; Lynd *et al.*, 2002; Leschine, 1995). Cellulosic biomass degradation in anaerobic environments can be performed by physiologically diverse taxa of

microorganisms (Lynd *et al.*, 2002; Schwarz, 2001; Leschine, 1995). For example, cellulose-degrading bacteria have been found within the genera *Clostridium*, *Ruminococcus*, *Caldicellulosiruptor*, *Acetivibrio*, *Butyrivibrio*, *Halocella*, *Fibrobacter*, *Bacteroides* and *Spirochaeta* (Azman *et al.*, 2015; Tsavkelova & Netrusov, 2012). In the rumen, *Fibrobacter*, *Ruminococcus*, *Butyrivibrio*, *Prevotella* and *Eubacterium* have been identified as the dominant cellulolytic bacterial genera, with *Clostridia* a minor player (Ransom-Jones *et al.*, 2012; Koike & Kobayashi, 2009; Leschine, 1995). In municipal waste landfill sites, *Clostridium* is the most commonly reported genus (Burrell *et al.*, 2004; Van Dyke & McCarthy, 2002). However, new evidence has shown the importance of bacteria from the genera *Fibrobacter* for cellulose degradation at landfill sites (McDonald *et al.*, 2012). Within anaerobic digesters, the class *Clostridium*, belonging to the phylum Firmicutes, is commonly found and is suggested to be involved in the hydrolysis of cellulosic materials (Lebuhn *et al.*, 2014; Hanreich *et al.*, 2013; Wang *et al.*, 2009b; Krause *et al.*, 2008; Klocke *et al.*, 2007). Bacteria belonging to this class were also detected in the laboratory-scale digesters using mono-digestion of manure or co-digestion with wheat straw in Paper **II**. In the same series of digesters, Bacteroidetes was identified as the dominant bacterial phylum, with its members possibly engaged in cellulose degradation, while the genus *Ruminococcus* was a minor group. However, *Ruminococcus* has previously also been identified in some anaerobic digesters operating with plant-based materials and/or manure (Wirth *et al.*, 2012; Schlüter *et al.*, 2008).

To date, only limited numbers of bacteria capable of degrading cellulose have been isolated from anaerobic digestion processes and these mainly belong to the genus *Clostridium*, but also include some members of the *Bacteroides*.

Clostridium aldrichii was first isolated from a wood-fermenting anaerobic digester and is able to utilise cellulose, xylan and cellobiose at temperatures between 20 and 45 °C (optimum 35 °C) (Yang *et al.*, 1990). Acetate, propionate, butyrate, isobutyrate, isovalerate, lactate, succinate, hydrogen and carbon dioxide are products of cellobiose fermentation.

Clostridium celerecrescens was first isolated from a methanogenic cellulose-enrichment culture (originating from a cow rumen) (Palop *et al.*, 1989). The fermentation products from cellulose or cellobiose are ethanol, acetate, formate, butyrate, isobutyrate, isovalerate, caproate, lactate, succinate, carbon dioxide and hydrogen. Optimal growth temperature is between 30 and 37 °C.

Clostridium clariflavum, isolated from a methanogenic reactor fed wastepaper, uses cellulose and cellobiose as its sole carbon and energy sources. Growth has been observed within the temperature range 45-65 °C, with the

optimal range 55-60 °C. The fermentation products from cellulose include carbon dioxide, hydrogen, acetate, lactate, ethanol and a small amount of formate (Shiratori *et al.*, 2009; Shiratori *et al.*, 2006).

Clostridium cellulovorans was first isolated from a batch fermentation of poplar wood. This bacterium has a temperature optimum for growth at 37 °C, but growth can occur from 20 to 40 °C. Hydrogen, carbon dioxide, butyrate, formate and ethanol are produced as the main fermentation products, but acetate and lactate are also produced in lower amounts (Sleat *et al.*, 1984).

Clostridium populeti, isolated from an anaerobic digester processing woody biomass, utilises cellulose, cellobiose, xylose and other sugars, while producing hydrogen, carbon dioxide, acetate, butyrate and lactate. Optimal growth is at 35 °C, but growth can occur at 20-40 °C (Sleat & Mah, 1985).

Acetivibrio cellulolyticus, isolated from municipal sewage sludge, grows on cellulose, cellobiose and salicin, but cannot use simple sugars such as glucose, fructose or xylose. The growth temperature range is 20-40 °C (optimum 35 °C) (Patel *et al.*, 1980).

The strains Bc1 and Dc1 isolated within this thesis can both utilise cellulose, cellobiose, xylose, ribose and glucose. In addition, Bc1 is able to ferment fructose and sorbitol. When cellulose was used as substrate, acetate, butyrate and glucose were detected as major fermentation products in the liquid phase. Growth was observed within the temperature range 25-54 °C for Bc1 and 25-51 °C for Dc1 (unpublished data).

Bacteroides cellulosolvens was first isolated from sewage sludge and has been shown to ferment only cellulose and cellobiose. Acetic acid, CO_2, H_2, ethanol and small amounts of lactic acid are produced when either substrate is used (Murray *et al.*, 1984). It has been suggested that this organism be reclassified as a member of Clostridium III (Horino *et al.*, 2014).

Ponpium *et al.* (2000) reported a *Bacteroides* sp. strain P-1 isolated from an anaerobic digester that is able to grow on cellulose, with ethanol and acetic acid as the main fermentation products.

4.2.2 Enzyme system of anaerobic cellulose-degrading bacteria

In nature, the enzymatic degradation of cellulose is generally a slow and incomplete process. The degradation proceeds through the action of extracellular enzymes and, due to the heterogeneity of native cellulose, multiple cellulolytic enzymes are required in order to achieve efficient degradation (Bayer *et al.*, 2004; Lynd *et al.*, 2002). Thus many anaerobic cellulose-degrading bacteria possess an extracellular multi-enzyme complex, called cellulosome (Lynd *et al.*, 2002; Leschine, 1995). This distinguishes

them from the aerobic cellulose-degrading microorganism, which instead secrete numerous individual extra-cellular enzymes (Schwarz, 2001).

The cellulosome is a large extracellular enzyme complex (including catalytic modules containing enzymes such as glycoside hydrolases). In fact, it is probably the largest enzyme complex in nature, with a molecular weight ranging from 650 000 Da to 2.5 MDa (Doi *et al.*, 2003). It has been observed in various anaerobic bacteria, such as *Clostridium*, *Acetivibrio*, *Bacteroides* and *Ruminococcus* (Doi *et al.*, 2003). The common structure of the cellulosome consists of large non-catalytic scaffolding proteins (also called scaffoldins) and numerous catalytic modules. The scaffolding proteins usually contain a carbohydrate-binding module (CBM), surface layer homology (SLH) modules and a number of cohesin domains (Bae *et al.*, 2013). The catalytic module consists of the catalytic domain, which exhibits hydrolytic activity, and the dockerin domain, which interacts with the cohesin domain. This interaction plays an important role in assembly of the cellulosome (Doi *et al.*, 2003). The number of cohesins on the scaffoldins is limited compared with the number of enzyme subunits present in the cellulosome, which suggests a heterogeneous population of cellulosomes. This heterogeneous population contributes to the efficient degradation of plant materials in nature (Murashima *et al.*, 2002; Pohlschröder *et al.*, 1994).

The draft genomes of both strains isolated within this thesis work, *i.e.* Bc1 and Dc1, were recovered by *de novo* sequencing using Pacific Biosciences RSII system. Bioinformatic analysis of the genomes revealed that one of the strains isolated contained the complete set of CBM and SLH modules and dockerin and cohesion domains for a cellulosome structure, but the other strain was lacking the SLH module in the genome, which suggested that it either contained unidentified SLH module or a non-cellular attached cellulosome. Information on the complete genome is still lacking, so the finding of a missing SLH module could also be an artefact (unpublished data).

Glycoside hydrolases (GH) catalyse hydrolysis of the glycosidic linkage of glycosides. Classification of glycoside hydrolases into families based on amino acid sequence similarity was proposed by Henrissat (1991), as the number of glycoside hydrolases identified was increasing rapidly at that time. The majority of glycoside hydrolases produced by anaerobic bacteria belong to three families: 5, 9 and 48 (Pereyra *et al.*, 2010). However, the glycoside hydrolase family may also contain hydrolases with multiple substrate specificities (Izquierdo *et al.*, 2010; Henrissat & Davies, 1997). The draft genomes of the two strains isolated from the industrial-scale biogas plants in Paper **IV** both contained the glycoside hydrolase families 5, 9, 26 and 48 (unpublished data)

4.3 Bacterial community within anaerobic digestion processes

Lignocellulosic biomass has a complex structure, which requires a range of enzymes and microorganisms working in a synergistic way to achieve effective degradation (Kostylev & Wilson, 2012). Various culture-independent studies have been used to investigate the general bacterial community structures in biogas digesters and have revealed dominance of the phyla Firmicutes and Bacteroidetes (**III**) (Sundberg *et al.*, 2013; Riviere *et al.*, 2009). When lignocellulosic materials are specifically included in the substrate, Clostridiales from the phylum Firmicutes and Bacteroidales from the phylum Bacteroidetes are commonly found as two dominant orders, *e.g.* in: A production-scale biogas plant fed maize, green rye and chicken manure (Schlüter *et al.*, 2008); batch fermentation of straw and hay (Hanreich *et al.*, 2013); batch fermentation of cellulose (Lu *et al.*, 2013); a hydrolysis/acidogenesis reactor of a two-stage AD process fed straw and hay (Lebuhn *et al.*, 2014); and laboratory-scale reactors degrading manure and straw (**II**, **III**).

In addition to identification of Clostridiales and Bacteroidetes in biogas processes operating with lignocellulose material, one or both of these taxa have also been shown specifically to be responsible for cellulose degradation in different AD processes: (1) Batch fermentation of ^{13}C-labelled cellulose and glucose, where members of Clostridiales and Bacteroidetes were dominant in the heavy fraction resulting from ^{13}C-labelled cellulose and glucose, respectively (Li *et al.*, 2009); (2) batch cultivation processing straw and hay, in which the relative abundance of Clostridiales was higher on day 5 than day 30, when the lignocellulosic material was depleted, while the Bacteroidales presented in the opposite way (Hanreich *et al.*, 2013); (3) this thesis, where a higher level of Bacteroidales was recorded in a digester fed wheat straw and cattle manure than in a digester mono-digesting cattle manure (**III**); (4) studies using culture-dependent techniques revealing that bacteria isolated from different AD processes and belonging to Clostridiales and Bacteroidales, are capable of degrading crystal cellulose (Koeck *et al.*, 2014; Lynd *et al.*, 2002).

The microbial community can be affected by various process parameters, among which the substrate composition has a pronounced effect. In this thesis, 10 industrial-scale biogas plants were used as inoculum sources for batch cultivation using cellulose and straw as substrate (**IV**). By targeting the *cel5* and *cel48* gene, it was found that the post-digestion communities within batch reactors fed cellulose were different from those in reactors fed straw for six out of 10 biogas digesters for *cel5* and nine out 10 biogas digesters for *cel48* (**IV**). This clearly demonstrates the impact of substrate on the community structure potentially involved in cellulose degradation. Moreover, within this thesis, the bacterial community (**III**) and the *cel5* community (**II**) within laboratory-scale

digesters co-digesting cattle manure and wheat straw were different from the communities within laboratory-scale digesters mono-digesting manure. Despite this difference in community structure, overall process performance (*e.g*. methane yield per unit VS) was not affected (**I**, **II** and **III**). The effect of substrate has also been reported in another study including mono- or co-digestion of cattle manure with various agricultural wastes, including chicken manure, dried distiller's grains with soluble (DDGS), maize silage and maize straw. In that study, the bacterial communities were similar in the digester using cattle manure, alone or in co-digestion with straw or silage, but more distantly separated from those in the digester co-digesting manure with DDGS (Ziganshin *et al.*, 2013).

The HRT and OLR are two other important operating parameters that may affect the community structure during degradation of lignocellulose-rich substrates. For example, in a laboratory-scale study including co-digestion of cattle manure with grass silage or oat straw, the bacterial 16S rDNA T-RFLP profiles in both digesters changed when the OLR was increased from 2 to 3 g VS/L/day (HRT decreased from 20 to 18 days) (Wang *et al.*, 2009b).

Various pre-treatments have also been investigated in order to enhance the AD process performance, but a limited number of studies have monitored the microbial community change associated with pre-treatment *per se*. In one of the few published studies, thermal pre-treatment of the solid fraction of cattle and swine manure at 140 °C for 40 min was reported to increase the methane productivity in CSTR compared with the non-treated manure (Mladenovska *et al.*, 2006). The associated bacterial community was analysed with T-RFLP targeting the 16S rDNA gene and this revealed a change in the abundance, but not the composition, of the T-RF profile (Mladenovska *et al.*, 2006). In this thesis, co-digestion of steam-exploded straw and cattle manure in laboratory CSTR resulted in similar process performance (*e.g*. methane yield) compared with the digester fed untreated straw and manure (**I**). The T-RFLP profile (*i.e*. the abundance and composition of T-RFs) of the *cel48* gene was changed in response to the steam explosion pre-treatment (**II**). More specifically, an uncultured bacterium (68.7% identity to *Ruminococcus flavefaciens*) emerged in the digester receiving the pre-treated straw (**II**). Interestingly, however, no change was seen for the *cel5* gene community.

Temperature is another parameter that strongly influences the bacterial community structure (De Vrieze *et al.*, 2015). The species richness of bacteria has in several studies been shown to decrease in parallel with increasing process operating temperature from mesophilic to thermophilic range (**III**) (Sundberg *et al.*, 2013; Levén *et al.*, 2007). Moreover, in several studies Clostridia have been found to increase in relative abundance with increasing

process operating temperature, for instance in a reactor digesting cattle manure (Ziganshin *et al.*, 2013) and in the hydrolysis/acidogenesis reactor of a two-stage process fed straw and hay (Lebuhn *et al.*, 2014). In this thesis, this trend was also observed by using both amplicon sequencing targeting the 16S rDNA gene (**III**) and a functional approach (T-RFLP) targeting the glycosidase *cel5* and *cel48* genes (**II**).

Free ammonia is a well-known inhibitor of biogas production if present at high levels. However, ammonia inhibition is not a common problem within AD processes fed lignocellulosic materials, but can still arise during co-digestion with nitrogen-rich materials such as swine manure or kitchen waste (Ye *et al.*, 2013; Xie *et al.*, 2011). In this thesis, two inoculum samples (CD01 and CD02) originating from industrial-scale biogas plants processing protein-rich materials resulted in long lag phases when used for batch fermentation using cellulose or straw as substrate, but managed to reach a similar level of final methane potential (**IV**). This confirmed previous findings that ammonia not only impacts on acidogenesis and methanogenesis, but also has an inhibitory effect on the hydrolysis step (Niu *et al.*, 2014). To achieve a stable AD process, co-digestion of carbon-rich lignocellulosic materials with substrate with a higher nitrogen level is typically required. Considering the results from this thesis and those reported by Niu et al. (2014), it should clearly be taken into consideration that the ratio of the substrates may have to be adjusted to avoid ammonia inhibition of the hydrolysis step.

4.4 Methanogens and the methanogenic pathway

The methanogens metabolise the intermediate products resulting from the hydrolysis/acidogenesis step and thus operating practices that alter these intermediate products might potentially affect the methanogenic community. Such practices include changing the process operating parameters, such as temperature, OLR, HRT and substrate composition, as well as process environmental conditions such as pH and level of ammonia and VFA. The pre-treatment strategy chosen for the substrate may also impact on the methanogenic community, as some pre-treatment methods can result in the release of inhibitors or methanogenic substrates, such as furfurals or acetate. It is worth considering that many reactions in the AD process are influenced by each other and that the methanogenic community shaped by the operating parameters might influence the overall degradation of the substrate.

In this thesis, the methanogenic community within digesters fed cattle manure and wheat straw was affected by increasing the operating temperature, with an increase in the relative abundance of *Methanobacterium* and

Methanoculleus being observed at 52 °C compared with 37 °C (**III**). However, the methane yield and the degradation efficiency were not affected by this change in process temperature, even though changes in both the bacterial and methanogenic communities occurred (**I**, **II** and **III**). In a study examining mono-digestion of maize silage, the dominant methanogen was Methanobacteriales in a thermophilic digester, while at mesophilic conditions the process was dominated by Methanomicrobiales (Bauer *et al.*, 2008). During mono-digestion of cattle manure, an increase in *Methanoculleus* has been observed with a temperature rise from 38 to 55 °C, whereas in the same process the level of *Methanosarcina* decreased (Ziganshin *et al.*, 2013). However, the increase in operating temperature in that study eventually resulted in accumulation of VFA and an unstable process. Within the same study, one digester fed cattle manure and maize silage was subjected to an increase in OLR, which resulted in an unstable process and an increase in the abundance of *Methanosarcina* (Ziganshin *et al.*, 2013).

The laboratory-scale reactor in this thesis used for processing cattle manure as the sole substrate was dominated by methanogens belonging to the genus *Methanosarcina* (**I** and **III**). Including straw as co-substrate did not affect the dominance of this organism (**III**). Similarly, in work by Ziganshin (2013), *Methanosarcina* was dominant during both mono- and co-digestion of cattle manure with maize straw. *Methanosarcina*-related methanogens have also been reported as most commonly present in other studies, including cattle manure in co-digestion with other organic waste (St-Pierre & Wright, 2014; Karakashev *et al.*, 2005). The dominance of *Methanosarcina* is most likely due to the fact that members of this genus are: (1) Able to use both the hydrogenotrophic and aceticlastic pathway for methane formation; (2) tolerant to ammonium up to 7000 mg/L; (3) able to grow at short retention time; and (4) tolerant to Na^+ concentrations up to 18 000 ml/L (De Vrieze *et al.*, 2012). Moreover, repeated pulse feeding has been reported to select for this genus rather than *Methanosaeta* (Conklin *et al.*, 2006).

In addition to *Methanosarcina*, other methanogens have been found in digesters operating with lignocellulosic materials, such as: (a) Methanobacteriales, found as the dominant methanogens during mono-digestion of maize silage at mesophilic temperature range (Munk *et al.*, 2010); and (b) Methanomicrobiales, shown to be dominant in a full-scale plant co-digesting cattle manure and maize silage under mesophilic conditions (Nettmann *et al.*, 2008); a full-scale biogas plant processing maize silage, green rye and chicken manure at 41 °C (Krause *et al.*, 2008); and five out of six mesophilic full-scale plants fed various combinations of substrate, *e.g.*

maize silage, grass silage, pig manure and cattle manure (Nettmann *et al.*, 2010).

The aceticlastic methanogen *Methanosaeta* has also been found to be the dominant methanogen in, for instance, co-digestion of cattle manure and maize silage (Ziganshin *et al.*, 2013) and co-digestion of cattle manure, maize silage, grass silage, cattle manure and small amounts of grain (Nettmann *et al.*, 2010). Demirel (2013) suggested that the hydrogentrophic pathway may be the dominant pathway for methane produced from energy crops. However, in this thesis and in other studies using lignocellulosic materials as substrate, both aceticlastic and hydrogentrophic methanogens were found. In general, the thermophilic process was dominated by hydrogentrophic methanogens, while the acetoclastic methanogens dominated in processes with low ammonia levels.

5 Conclusions and Perspectives

Lignocellulosic biomass represents a highly abundant feedstock for biogas production. However, obstacles to utilising this kind of substrate efficiently still remain. This thesis investigated the potential for using wheat straw, one of the most widely available lignocellulosic materials, with particular focus on the microbial community structures under variations in co-substrate, pre-treatment, process parameters and inoculum sources. The results represent novel information on the bacterial and archaeal communities involved in AD processes operating with lignocellulosic materials.

Results from the laboratory-scale study showed that wheat straw can be used as a co-substrate for biogas production, even at rather high C/N ratio. Including wheat straw in the digester changed the profile of the overall bacterial community, as well as the potential cellulose-degrading bacteria. The community also changed according to the character of straw (*i.e.* mechanically chopped or steam exploded) and to the operating temperature. However, the methane yield was not changed and remained at a level that is non-profitable using current available technology. Apparently, a change in operating condition results in a change in the community structure, but does not necessary affect process performance. However, this practice of altering the operating conditions generated valuable information on microbial community response to environmental change.

The free ammonia level was identified as one potential factor affecting both the bacterial diversity and the taxonomic composition of the microbial community within the industrial-scale biogas plants investigated. The ammonia level was also found to be negatively correlated with the methane production efficiency from both straw and cellulose. Interestingly, with the *cel5* community T-RFLP profile, it was possible to identify two dominant T-RFs positively correlated with methane production efficiency. However, only

assumptions rather than conclusions can be made on the importance of these organisms for degradation based on currently available data.

The microbial community analysis revealed a large fraction of uncultured bacteria in all digesters investigated, which suggests that in the future more efforts are needed in isolation work in order to understand the microbial function and its role in the metabolic network. The two strains isolated during this thesis work represent one source of new information regarding cellulose degradation in biogas processes, but further characterisation is needed in order to gain insights into their physiology and their capacity for degradation of lignocellulosic materials. Moreover, direct application of these isolates and other isolated bacteria can potentially be used to boost the degradation by bioaugmentation. Another interesting and promising future research topic in this area is fungi. Aerobic fungi have been used for pre-treatment of lignocellulosic materials to enhance the biogas production, and lately anaerobic fungi have shown promising results for enhanced degradation in the digester. However, at present only a limited number of studies cover the fungal community and thus another future research perspective would be to investigate the fungal community in an AD process fed lignocellulosic materials.

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