Enhancing biogas production from recalcitrant lignocellulosic residue

Tsapekos, Panagiotis; Angelidaki, Irini; Kougias, Panagiotis

Publication date:
2017

Document Version
Publisher's PDF, also known as Version of record

Citation (APA):
Tsapekos, P., Angelidaki, I., & Kougias, P. (2017). Enhancing biogas production from recalcitrant lignocellulosic residue. Kgs. Lyngby: Technical University of Denmark, DTU Environment.

Enhancing biogas production from recalcitrant lignocellulosic residues

Panagiotis Tsapekos

PhD Thesis
February 2017

DTU Environment
Department of Environmental Engineering

Enhancing biogas production from recalcitrant lignocellulosic residues

Panagiotis Tsapekos

PhD Thesis, February 2017

Address: DTU Environment
Department of Environmental Engineering
Technical University of Denmark
Miljoevej, building 113
2800 Kgs. Lyngby
Denmark

Phone reception: +45 4525 1600
Fax: +45 4593 2850

Homepage: http://www.env.dtu.dk
E-mail: reception@env.dtu.dk

Cover: GraphicCo

Preface

This PhD thesis, entitled “Enhancing biogas production from recalcitrant lignocellulosic residues”, comprises the research carried out at the Department of Environmental Engineering, Technical University of Denmark from December 01, 2013 to November 30, 2016. Professor Irini Angelidaki and researcher Panagiotis Kougias were supervisor and co-supervisor, respectively.

The thesis is organized in two parts: the first part puts into context the findings of the PhD in an introductive review; the second part consists of the papers listed below. These will be referred to in the text by their paper number written with the Roman numerals **I-VIII**.

I Tsapekos, P., Kougias, P.G., Angelidaki, I., 2015. Biogas production from ensiled meadow grass; effect of mechanical pretreatments and rapid determination of substrate biodegradability via physicochemical methods. Bioresource Technology 182, 329–335.

II Tsapekos, P., Kougias, P.G., Angelidaki, I., 2015. Anaerobic Mono- and Co-digestion of Mechanically Pretreated Meadow Grass for Biogas Production. Energy & Fuels 29, 4005–4010.

III Tsapekos, P., Kougias, P.G., Frison, A., Raga, R., Angelidaki, I., 2016. Improving methane production from digested manure biofibers by mechanical and thermal alkaline pretreatment. Bioresource Technology 216, 545–552.

IV Tsapekos, P., Kougias, P.G., Treu, L., Campanaro, S., Angelidaki. I., 2017. Process performance and comparative metagenomic analysis during co-digestion of manure and lignocellulosic biomass for biogas production. Applied Energy 185, 126–135.

V Tsapekos, P., Kougias, P.G., Larsen, U., Pedersen, J., Trénel, P., Angelidaki. I., Mechanical pretreatment at harvesting increases the bioenergy output from marginal land grasses. Submitted to Renewable Energy. September 06, 2016

VI Tsapekos, P., Kougias, P.G., Larsen, U., Pedersen, J., Trénel, P., Angelidaki. I., 2016. Improving the energy balance of grass-based anaerobic digestion through combined harvesting and pretreatment. Anaerobe. doi: org/10.1016/j.anaerobe.2016.12.00505

VII Tsapekos, P., Kougias, P.G., Vasileiou, S.A., Lyberatos, G., and Angelidaki. I., 2017. Effect of microaeration and inoculum type on the biodegradation of lignocellulosic substrate. Bioresource Technology 225, 246–253.

VIII Tsapekos, P., Kougias, P.G., Vasileiou, S.A., Treu, L., Campanaro, S., Lyberatos, G., and Angelidaki. I., Bioaugmentation with hydrolytic microbes to improve the anaerobic biodegradability of lignocellulosic agricultural residues. Submitted to Water Research. December 02, 2016

In addition, the following publications, not included in this thesis, were also concluded during this PhD study:

Kougias, P.G., Campanaro, S., Treu, L., Tsapekos, P., Angelidaki, I., Behind the mechanism of lignocellulosic degradation in anaerobic digestion as revealed by genome-centric metagenomics. Manuscript. 2016

Awais, M., Alvarado-Morales, M., Tsapekos, P., Gulfraz, M., Angelidaki, I., 2016. Methane production and kinetic modeling for co-digestion of manure with lignocellulosic residues. Energy & Fuels 30, 10516–10523.

Morales, A.-M., Tsapekos, P., Awais, M., Gulfraz, M., Angelidaki, I., 2016. TiO_2/UV based photocatalytic pretreatment of wheat straw for biogas production. Anaerobe. doi: 10.1016/j.anaerobe.2016.11.002.

In this online version of the thesis, paper **I-VIII** are not included but can be obtained from electronic article databases e.g. via www.orbit.dtu.dk or on request from DTU Environment, Technical University of Denmark, Miljoevej, Building 113, 2800 Kgs. Lyngby, Denmark, info@env.dtu.dk.

Acknowledgements

This PhD thesis was conducted under the supervision of Professor Irini Angelidaki and the co-supervision of researcher Panagiotis Kougias, both of whom I wish to thank first. I would like to thank my supervisor for her guidance during these three years and offered me greatest freedom and unlimited inspirations on the scientific field. Likewise, I thank Panos who I collaborated extensively with, for inspiring and guiding me when needed, for his supervision and support. Also, for giving me extra responsibilities and trusting me with various tasks that have added another dimension to my PhD project.

Great thanks are extended to Alessandro Frison, Aristotelis Vasileiou, Laura Treu, Stefano Campanaro and Merlin Alvarado-Morales for working together. I wish to express my gratitude to the project partners from Kverneland and AgroTech for a nice collaboration. Hector and Hector thank you for the technical assistance during the experiments.

I wish to give thanks to all of the officemates that I met in room 072 and to Yifeng Zhang for the accompanying in the same office during the last two years. Dear colleagues and members of Bioenergy group thanks for assisting me working in the laboratory.

Last but not least; I want to thank my whole family in Greece for all their support and understanding. Additionally, I would like to thank my friends for the well-being in Copenhagen and those who persuaded me to start the PhD journey. Special thanks are going to Eirini; thank you for daily understanding and supporting.

This PhD thesis is dedicated to everybody who helped me and has been by my side all over these three years.

Summary

Lignocellulosic substrates are abundant in agricultural areas around the world and lately, are utilized for biogas production in full-scale anaerobic digesters. However, the anaerobic digestion (AD) of these substrates is associated with specific difficulties due to their recalcitrant nature which protects them from enzymatic attack. Hence, the main purpose of this work was to define diverse ways to improve the performance of AD systems using these unconventional biomasses. Thus, mechanical and thermal alkaline pretreatments, microaeration and bioaugmentation with hydrolytic microbes were examined. The studied substrates were fresh and ensiled meadow grass, regularly cultivated ensiled grass, digested manure fibers and wheat straw.

AD of lignocellulosic substrates is time demanding and an extended incubation period is often needed. Initially, diverse analytical methods were used (i.e. electrical conductivity, soluble chemical oxygen demand and enzymatic hydrolysis) as a rapid way to predict the methane production. However, the precision of methane yield prediction was not high ($R^2 < 0.68$) and thus, the biochemical methane potential (BMP) test is concluded to be the most precise method to estimate the biomethanation process.

Various mechanical pretreatments were examined on ensiled meadow grass biodegradability by applying shearing forces. Preliminary results showed that the methane production of ensiled meadow grass can be efficiently increased up to 25% compared to untreated samples. Hence, the most efficient method was further applied on the same substrate, focusing on different age of vegetation under mono- and co-digestion with livestock manures (i.e. poultry, mink and cattle manure). The differences on biomass' chemical composition were also determined in order to demonstrate the effect of vegetation stage. Clear alterations were revealed due to late harvest time and specifically, the lignin content was markedly augmented (~30% of dry matter) with advancing age, implying the need of pretreatment. Mechanically pretreated biomass of increased maturity was co-digested with diverse livestock manures in order to define the optimum silage/manure ratio in the feedstock. Results showed that the ideal lignocellulose/manure contribution differs among the examined substrates and that the chemical characteristics of the feedstock mixture significantly influenced the biomethanation process.

The application of shearing forces was also examined on the hardly degradable fraction of digested manure fibers. However, limited efficacy was

observed on biomethanation and the remaining volatile solids (VS) were not highly utilized. Conversely, the well-studied thermal alkaline pretreatments using sodium hydroxide as a catalyst promoted the yield from approximately 42 $mLCH_4/gVS$ to 170 $mLCH_4/gVS$. Furthermore, the positive results were validated in the co-digestion of biofibers with cattle manure under continuous mode operation. Mechanical and thermal alkaline pretreatment (6% NaOH at 55 °C for 24 h) had an effect of 7% and 26% respectively, without provoking process inhibition.

Focusing on full-scale practices, the application of simple and efficient treatment methods is generally suggested. Accordingly, the reduction of supply chain steps prior to AD could eventually improve the energy budget and subsequently, process profitability. Hence, the integration of mechanical pretreatment at harvesting step was examined as a solution to scale-up the used mechanical method in real-life applications. On this topic, an innovative Disc-mower (named as Excoriator) was studied in order to simultaneously harvest and pretreat fresh meadow grass through the application of shearing forces. Kinetic studies showed that the lag phase was decreased, the methane production rate was increased and finally, the methane yield was significantly enhanced by up to 27% under optimal conditions. Further investigations on full-scale experiments mowing regularly cultivated grass confirmed the positive effect due to the selection of the most appropriate harvester. The modern harvester poses the ability improve the energy balance and subsequently, the sustainability of lignocellulose-based AD.

The co-digestion of pig manure and lignocellulosic silage was assessed in continuous stirred tank reactors (CSTR). Addition of mechanically pretreated silage in the feedstock positively affected the methane yield (+16%) and in parallel, reduced the risk of ammonia inhibition compared to mono-digestion of pig manure. Furthermore, metagenomic analysis was performed to determine differences among the microbial communities in CSTRs operating under mono- and co-digestion. Species similar to *Clostridium thermocellum*, with increased cellulolytic activity, were detected to be adherent to the solid fraction of digested feedstock and concluded to be key players for lignocellulose's disintegration.

Moreover, various microaeration strategies were applied in order to elucidate the effect of oxygen load (O_2), pulse repeatability and treatment period on the AD of wheat straw. The results obtained from this study demonstrated a 7.2% increase in methane yield after a 3 days microaeration period, using 5 mL

O_2/gVS served by once. In addition, an optimisation study was conducted and the analysis indicated that the methane yield could have been increased by 9%, if 7.3 mL O_2/gVS were injected. It was indicated that microaeration can be an alternative solution for augmented biomass solubilization without causing inhibition to the mandatory anaerobic methanogenic community.

Based on the initial microbial analysis, the bioaugmentation with the typically abundant in AD systems *C. thermocellum* was examined in biogas reactors fed with wheat straw. Bioaugmentation with the hydrolytic strain had immediately a remarkable result on methane production. Nevertheless, the long term monitoring showed that routine bioaugmentation is needed to retain a positive effect of approximately 7%. Moreover, it was indicated that the bioaugmentation with *C. thermocellum* can be periodically applied in biogas reactors in order to extract the residual methane from the amassing materials and avoid potential accumulation. Additionally, the facultative anaerobic *Melioribacter roseus* was inoculated in a replicate CSTR following different bioaugmentation strategies, either strictly anaerobic or micro-aerobic conditions. Nevertheless, the novel strain did not enhance the biomethanation process and the metagenomic analysis revealed that the inoculated strain did not adapt in the biogas reactor.

The results obtained confirm that lignocellulose-based AD can lead to high biogas yield. At lab-scale experiments, the bioenergy production can be further improved using micro-aeration, bioaugmentation with *C. thermocellum*, thermal-alkaline or mechanical pretreatments. Further insights into AD microbiome can improve and optimize the used processes. Among the examined pretreatments, only mechanical methods were evaluated in full-scale operation due to their easiness in application. On this topic, modern harvesting technology simulating the process applied in lab-scale could generate similar enhancement under full-scale trials. Machineries orientated to pretreat biomass using simplified techniques can positively affect the industrial applications.

Dansk sammenfatning

Lignocellulosiske substrater til biogasproduktion findes overalt i verden, i form af restprodukter fra landbrugshøst, og er på det seneste anvendt i fuldskala-reaktorer. Her er de særlige udfordringer ved den anaerobe nedbrydning (AN), at lignocellulosiske substraters komplekse struktur beskytter dem mod ”enzymatisk angreb”. Afhandlingens hovedformål har således været at finde alternative forbehandlingsmetoder til at forbedre AN af lignocellulosisk biomasse. De i projektet undersøgte substrater var nyhøstet og ensileret enggræs, kultiveret ensileret græs, hvedestrå, ensileret enggræs i samudrådning med gylle. Derudover restfibre fra omsat gylle.

Anaerob nedbrydning af lignocellulosisk biomasse er generelt ekstremt tidskrævende med forlænget inkubationstid. Initialt blev det undersøgt, om man kunne anvende hurtigere metoder til at forudsige methan-potentialet for denne type biomasse. De tre metoder var hhv. elektrisk konduktivitet, opløseligt kemisk iltbehov og enzymatisk hydrolyse. Præcisionen ved disse metoder var ikke høj ($R^2 < 0.68$), hvorfor BMP (biomechanical methan potential) valgtes som den foretrukne metode til at vurdere det potentielle methan-udbytte.

Forskellige former for mekanisk forbehandling med forskydningskræfter blev undersøgt ved nedbrydning af ensileret enggræs. De indledende resultater viste, at methan-produktionen effektivt kunne øges op til 25% sammenlignet med ubehandlet substrat. Af betydning for methan-udbyttet er ligeledes høsttidspunktet, med højst ligninindhold i sent høstet enggræs (laveste methan-udbytte).

Samudrådning af mekanisk forbehandlet modent enggræs med forskellige slags gylle (kylling, mink, ko), blev derpå undersøgt (batch) med formål at fastsætte optimale blandingsforhold. Ud fra de meget varierende resultater konkluderedes ’vigtigheden af forudbestemmelse af substraternes kemiske karakteristika’.

Samme type mekanisk forbehandling forsøgt på svært nedbrydeligt ”omsat gyllefibermasse” i batch-eksperimenter havde en begrænset effekt på biogasproduktionen (60 mL CH_4/gVS). Derimod gav termisk alkalisk forbehandling (6% NaOH ved 55 °C i 24 timer) et betydeligt forøget udbytte i batch (fra 42 mL CH_4/gVS til 170 mL CH_4/gVS). I kontinuerligt omrørt tank reaktor (CSTR) viste mekanisk og termisk alkalisk forbehandling at have lignende effekt, med en øgning i methan-produktionen på henholdsvis 7% og 26% (uden at provokere processinhibering).

Med henblik på udvidelse til daglig applikation i landbruget blev ”integration af mekanisk forbehandling under selve høsten” undersøgt i fuldskala: Friskt enggræs blev høstet ved hjælp af en Excoriator, som samtidig forbehandlede biomassen ved brug af forskydningskræfter. Den kinetiske undersøgelse viste, at lagfasen (tiden for opstart af methan-produktionen) blev kortere, methanproduktionshastigheden større, og endeligt var methan-udbyttet væsentligt højere (op til 27 % højere under optimale forhold) i fuldskala biogasreaktor.

Samudrådning af svinegylle med lignocellulose ensilage blev vurderet i en CSTR: Tilsætning af mekanisk forbehandlet ensilage til reaktorens gyllefødestrøm påvirkede methan-udbyttet positivt (+16%) og reducerede samtidig risikoen for ammoniakhæmning, sammenlignet med AN af svinegylle alene (monosubstrat). Desuden viste metagenomics-analyser, at *Clostridium thermocellum* lignende bakterier, som er kendt for cellulolytisk aktivitet, havde hæftet sig til den faste fraktion af den omsatte gylle. Dette kunne tyde på, at de har en central rolle for lignocelluloses disintegration.

Strategier for mikro-iltning blev evalueret for at belyse effekten på AN af hvedehalm. Hvis udført uden samtidigt at forårsage hæmning af de strengt anaerobe methanogene mikroorganismer, kan opnås positive resultater på methan-udbyttet (+ 9%). Dette indikerer, at mikroiltning kan være en alternativ vej til effektivitet, hvis udført med en vis forsigtighed.

Baseret på initial mikrobiel analyse, blev to sideløbende forsøg med ’bioaugmentation’ udført med hhv. den fakultativt anaerobe *Melioribacter roseus* og den typisk tilstedeværende*C. thermocellum*. Begge undersøgt i CSTR med AN af hvedestrå. *Melioribacter roseus* blev podet i en, ud fra forskellige bioaugmentationsstrategier, enten strengt anaerobt eller under mikroaerobe forhold. Denne hidtil ukendte bakteriestamme viste sig ikke i stand til at forbedre biogasproduktionen, og den metagenomiske analyse afslørede, at den podede stamme ikke tilpassede sig i biogasreaktoren. Til gengæld var den omgående forøgelse i methan-produktionen med *C. thermocellum* bemærkelsesværdig (+7%), om end målinger over længere tid viste, at rutinemæssig gentagen bioaugmentation er nødvendig for at fastholde den positive effekt.

Resultaterne fra dette studie viser, at anaerob nedbrydning af lignocellulosiske affaldsprodukter leder til højt biogasudbytte, og at dette kan øges væsentligt ved at applicere mekanisk forbehandling med forskydningskræfter ved brug af moderne høstmaskiner. Lovende batchresultater peger på yderligere biogasudbytte, med en alkalisk behandling, inden substratet føres til biogasreaktoren. Gentagen bioaugmentation med *C. thermocellum* vil kunne anvendes periodisk i biogasanlæg for at trække den resterende methan ud af restbiomassen og dermed undgå en potentiel akkumulering. Yderligere indsigt i det anaerobe microbiom vil kunne optimere biogasprocessen. Samlet vil de, i denne afhandling undersøgte metoder, kunne gøre lignocellulose-baseret biogasproduktion både udbytterigt og bæredygtigt.

.

Table of contents

Abbreviations

AD	Anaerobic digestion
BMP	Biochemical methane potential
CSTR	Continuous stirred tank reactors
DMF	Digested manure fibers
EC	Electrical conductivity
EH	Enzymatic hydrolysis
E_{in}	Energy input
E_{out}	Energy output
EU	European Union
g	Gram
Ha	Hectare
HRT	Hydraulic retention time
J	Joule
L	Litre
LCA	Life cycle assessment
LCFA	Long chain fatty acids
sCOD	Soluble chemical oxygen demand
SEM	Scanning electron microscopy
TKN	Total Kjeldahl nitrogen
TS	Total solids
V	speed
VFA	Volatile fatty acids
VS	Volatile solids

1 Introduction

1.1 Background

In 2015, 86% of the global primary energy consumption was originated from fossil fuels and specifically, oil, coal and natural gas accounted for 32.9%, 29.2% and 23.8%, respectively (British Petroleum, 2016). However, the dependence on fossil fuels is associated with remarkably adverse impacts, for example, increased levels of air pollution, depletion of natural landscapes and finally, climate change (IPCC, 2013).

On the contrary, alternative sources of energy are available and also, more environmentally friendly. Hence, the solid growth of renewables is considered as an advantageous way to partly replace the extended use of fossil fuels. Therefore, renewables accounted for a record 2.8% of world's energy consumption in 2015 (Figure 1). Additionally, European Union (EU) intends on achieving 20% share of renewable energy in overall energy consumption until 2020, leading to increased share in the forthcoming years. Following this concept, the anaerobic digestion (AD) is already roared as another sustainable solution to efficiently satisfy the needs of the growing humanity with respect to the environment.

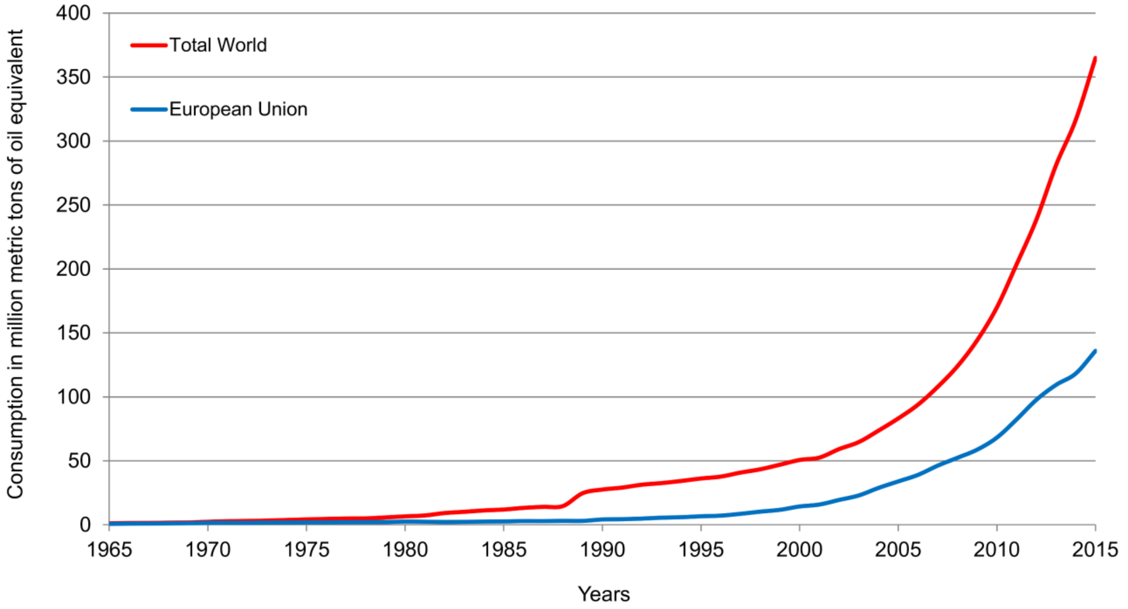

Figure 1. Energy consumption from renewable energy sources (i.e. wind, geothermal, solar, biomass and waste) during the years 1965-2015. The energy carriers are calculated assuming a modern thermal plant with 38% conversion efficiency (British Petroleum, 2016) (British Petroleum, 2016).

The AD process results in methane-rich biogas which is subsequently utilized as energy source. Methane can be produced from a huge variety of organic residues through AD and especially, industrial wastewater, livestock manure, food waste and lignocellulosic residues are the most common substrates (Sawatdeenarunat et al., 2015). Among them, lignocellulosic residues pose some unique characteristics that can further improve the economic viability of the AD plants. First of all, they are plentiful in nature and for example in EU-28 more than $200*10^6$ Ha of grasslands, meadows and agricultural areas are available for exploitation (Faostat, 2016). As a result of their abundance, the lignocellulosic residues are additionally considered as a cheap biomass source. Nevertheless, the usage for industrial scale applications is still narrow. Specifically, their usage is generally connected with limited efficiency as their structure and especially, the lignin component acts as a physical barrier to the enzymatic attack (Čater et al., 2014; Zeng et al., 2014).

Therefore, surface disruption is a mandatory action in order to efficiently be accomplished the lignocellulose-based AD. Many researchers studied the application of different pretreatments in order to boost substrates' biodegradability (Monlau et al., 2013; Taherzadeh and Karimi, 2008; Zheng et al., 2014). However, there is still a need of finding or optimizing treatment methods, as the existed approaches can be cost-demanding (i.e. milling), time consuming (i.e. fungi), have difficulties in full-scale applications (i.e. biological treatments) or are associated with the production of inhibitors to the AD microbiome (i.e. acid pretreatments) (Hendriks and Zeeman, 2009; Kratky and Jirout, 2011; Monlau et al., 2013; Zheng et al., 2014). Hence, the problem of lignocellulose deconstruction should be addressed without deteriorating the feasibility of AD system. Additionally, deeper insights of the microbial populations can provide important knowledge in order to improve the overall process efficiency of lignocellulose-based AD.

1.2 The biogas process

In the absence of oxygen, a huge variety of organic substrates are metabolized mainly into two molecules: methane and carbon dioxide (trace amounts of other gases are produced e.g. ammonia, hydrogen, hydrogen sulphide). AD is a well-studied process and it is widely known that it is dictated by bacteria and archaea (Luo et al., 2015); however, due to microbiome's complexity and interactions among the species, a tremendous number of still unexplored microbes is presented in AD systems (Treu et al., 2016b). This biological process can be quickly categorized in four stages:

hydrolysis, acidogenesis, acetogenesis and methanogenesis (Angelidaki et al., 2011). The specific steps are depicted in Figure 2.

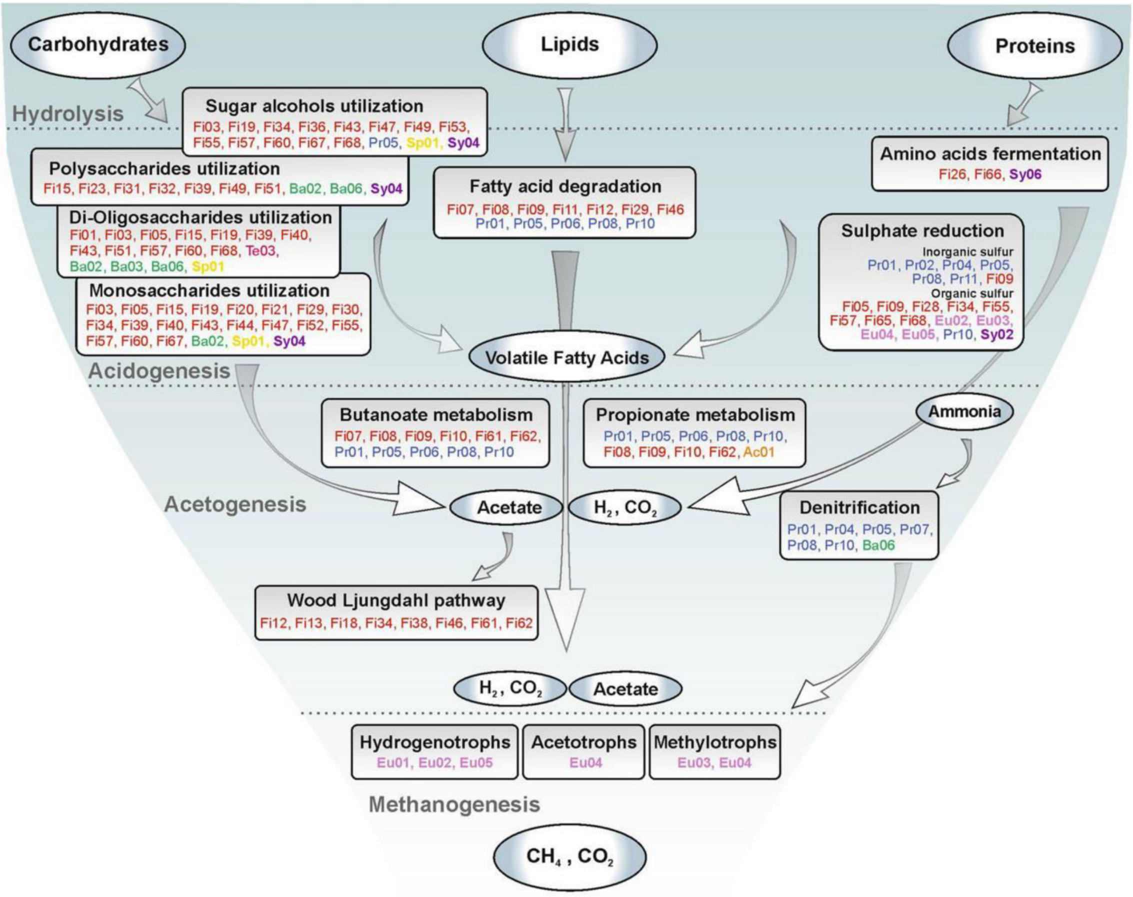

Figure 2. Major steps of AD process accompanied by the more relevant Genome bins involved. [Adapted from (Campanaro et al., 2016)]

1.2.1 Hydrolysis

Organic substrates are mainly consisting of carbohydrates, proteins and lipids; macromolecules which in anaerobic environment are initially broken down to monosaccharides, amino acids, long chain fatty acids (LCFA) and glycerol, respectively. This step is basically an enzymatic process and thus, the efficiency is based on the presence and action of hydrolytic and fermentative microbes to excrete extracellular enzymes.

A variety of enzymes are mandatory for the deconstruction of each macromolecule. For instance, hydrolytic enzymes (e.g. cellulase, β-glucosidase, xylanase) or complex enzyme systems (e.g. cellulosome) attack on polysaccharides, protease degrade protein and lipase are suitable for lipids (Azman et al., 2015; Mshandete et al., 2005).

The role of individual communities to conduct the initial AD step is widely studied. For instance, lipids are degraded by anaerobic lipolytic microbes (Angelidaki et al., 1999) and more specifically, species belonging to genus *Clostridium* are able to hydrolyse this energy rich fraction into glycerol and LCFA (Cirne et al., 2006). Secondly, the proteolytic activities are equally important for the deconstruction of recalcitrant substrates. For example, species similar to *Coprothermobacter proteolyticus* are highly involved in the synthesis of extracellular proteases (Lü et al., 2014). Furthermore, their presence and role is also connected with the degradation of polysaccharides. Specifically, these genera are known to interact with members of high cellulolytic activity (Lü et al., 2014a). Our recent study validated the co-presence of *Clostridium thermocellum* strains along with *C. proteolyticus* on lignocellulose-based AD (Paper IV).

Interestingly, lately research on AD microbiome revealed that hydrolysis is mediated by a markedly increased amount of Genome Bins (microorganisms) compared to the following steps of biogas production (Campanaro et al., 2016). Results showed, that a diversity of microbes originated from different phyla act and interact together in order to accomplish polymers' breakdown; thus, further investigation is majorly needed in order to decipher the specific roles and relationship among the biogas members.

In AD systems fed with lignocellulosic substrates, hydrolysis is considered to be the rate-limiting step (Sträuber et al., 2012), due to the presence of lignin which forms, along with cellulose and hemicellulose units, a rigid three-dimensional complex. This physical barrier protects the biomass from the enzymatic attack. Thus, the existence of microbes with augmented cellulytic activity is mandatory for an efficient decomposition.

1.2.2 Acidogenesis

The hydrolysed products of the long macromolecules are subjected to the fermentation step following different metabolic pathways to produce volatile fatty acids (VFA), hydrogen, carbon dioxide and alcohols. In the second step, sugars and amino acids are the major substrates. Results of glycerol fermentation are propionate production and biomass generation (Angelidaki et al., 1999):

$$C_{57}H_{104}O_6 + 3H_2O \rightarrow C_3H_8O_3 + 3C_{18}H_{34}O_2 \qquad (1)$$

A coupled oxidation-reduction reaction is occurring in pairs for amino acids acidogenic fermentation releasing NH_3 (Angelidaki et al., 2011). In the socalled Stickland reaction, different amino acids act either as an electron donor or as an electron acceptor. Although, uncoupled acidogenic conversion can also occurr for amino acids, as for example the glutamate degradation (Buckel, 2001) or when hydrogen partial pressure is low and energetics are appropriate (Stams, 1994).

Hydrolysed sugars are transformed through the Emben–Meyerhof–Parnas (EMP) or Entner Doudoroff (ED) pathway (Angelidaki et al., 2011). Lactate and propionate are produced through EMP pathway. Acetate, butyrate and caproate are fermented through acetyl-CoA. In contrast to amino acids, glucose can act both as electron acceptor for oxidation (e.g. acetate) and donor for reduction (e.g. propionate, ethanol etc.). Fermentative strains of glucose have branched metabolisms. Thus, they can metabolise the available monosaccharide through different pathways leading to different amounts of energy and products. Different glucose fermentation products (i.e. acetate, propionate, butyrate, lactate, ethanol) are presented below (Schink, 1997; Thauer et al., 1977):

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \tag{2}$$

$$3C_6H_{12}O_6 \rightarrow 4CH_3CH_2COOH + 2CH_3COOH + 2CO_2 + 2H_2O \tag{3}$$

$$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2 \tag{4}$$

$$C_6H_{12}O_6 \rightarrow 2CH_3CHOHCOOH \tag{5}$$

$$C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2 \tag{6}$$

Regarding the microbial consortium responsible for the mediation of acidogenesis, fermentative microbes can be found among different phyla. However, *Firmicutes* are deciphered to be dominant in biogas microbiome (Treu et al., 2016b); hence, there is a variety of members belong to this phylum that are able to degrade oligosaccharides into the aforementioned products. Some examples could be found in microbes similar to *Clostridium propionicum* for propionate (Buckel, 2001), *Clostridium thermocellum* for acetate and ethanol (Lamed et al., 1988), *Clostridium butyricum* for butyrate (Schink, 1997) or *Lactobacillus* species for lactate production (De Francisci et al., 2015). Process characteristics, as pH, feedstock composition and hydrogen pressures significantly influence the biogas microbiome (Rodriguez et al., 2006). Thus, it is clear that the specific microbial community differs among the various AD systems.

1.2.3 Acetogenesis

During acetogenesis, acetate is formed by different microbial members, either the hydrogen-producing acetogens or the hydrogen-utilizing acetogens. The products of acidogenesis (i.e. VFA and alcohols) are utilized by hydrogen-producing acetogens, using carbon dioxide and hydrogen ions as electron acceptors (e.g. *Syntrophomonas wolfei*). This bioconversion process is not exergonic and thus, a syntrophic relationship with methanogens is mandatory to maintain the H_2 partial pressure low for acetogenic reactions to be energetic favourable (Treu et al., 2016a). For instance, acetogens and methanogenic archaea should co-operate for the degradation of propionate and butyrate which are oxidized through the methyl-malonyl-CoA pathway producing acetate, H_2 and CO_2 (De Bok et al., 2004) and through β-oxidation to acetate (Batstone et al., 2003), respectively. The share of available energy during the syntrophic fermentation is crucial (Kougias et al., 2016). Additionally, sulphate reducers consume hydrogen and improve hydrogen concentrations for the acetogenesis process. On the other hand, hydrogen-utilizing acetogens (e.g. *Acetobacterium* sp.) use the acetyl-CoA pathway to form acetate by the reduction of CO_2 (Drake, 1994). These microbial members compete with the hydrogenotrophic methanogens for the utilization of hydrogen, methanol and formate (Batstone et al., 2006).

Moreover, acetate and hydrogen is also produced from lipids decomposition, as the LCFA undergo to β-oxidation (Kim et al., 2004; Treu et al., 2016a):

$$CH_3(CH_2)_nCOOH + 2H_2O \rightarrow CH_3(CH_2)_{n-2}COOH + CH_3COOH + 2H_2 \qquad (7)$$

Strains playing important roles in acetogenesis process can be found among various strains; for example, *Clostridium*, *Lactobacillus*, *Bacillus* and *Bacteroides* are markedly involved in this step (Snell-Castro et al., 2005).

1.2.4 Methanogenesis

Methanogenesis is the last step of AD, in which the strict anaerobic methanogenic archaea convert mainly acetate and H_2/CO_2 to CH_4 and CO_2; however, to less extent, substrates as formate, methyl and alcohols are also used (Schink, 1997; Stams, 1994). The larger portion of methane is derived from the conversion of acetate and the rest is primarily produced from H_2/CO_2 and formate (Angelidaki et al., 2011). Extended methane production can be conducted via the hydrogenotrophic pathway based on process characteristics (i.e. temperature, feedstock characteristics etc.) (Campanaro et al., 2016; Wirth et al., 2012).

The aceticlastic and syntrophic acetate oxidation (SAO) are the two potential pathways for methanogenesis consuming acetate. In the first pathway, the aceticlastic methanogens consume acetate and produce methane and carbon dioxide (Angelidaki et al., 2011):

$$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^- \qquad \Delta G^{0'} = -31.0 \text{ kJ/mol} \qquad (8)$$

Regarding the SAO pathway, initially, the syntrophic acetate oxidation bacteria (SAOB) convert acetate into hydrogen and carbon dioxide and subsequently, these products are taken from hydrogenotrophic methanogens and convert them to methane (Kougias et al., 2016; Zinder and Koch, 1984):

$$CH_3COO^- + 4H_2O \rightarrow 2HCO_3^- + 4H_2 + H^+ \qquad \Delta G^{0'} = +104.6 \text{ kJ/mol} \qquad (9)$$

$$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O \qquad \Delta G^{0'} = -135.6 \text{ kJ/mol} \qquad (10)$$

Methanosarcinaceae spp. and *Methanosaetaceae* spp. are able to perform the aceticlastic methanogenesis (Fotidis et al., 2013). Conversely, SAOB can perform the reverse Wood-Ljungdahl pathway followed by hydrogenotrophic methanogens *Methanomicrobiales* spp., *Methanobacteriales* spp. and *Methanococcales* spp. (Campanaro et al., 2016; Karakashev et al., 2006).

1.3 Objectives and thesis structure

1.3.1 Specific objectives

The main objective of this PhD study was to improve the sustainability of lignocellulose-based biogas production applying a variety of treatment methods on fresh and ensiled meadow grass, regularly cultivated ensiled grass, digested manure fibers and wheat straw. Thus, mechanical and chemical pretreatments, microaeration and bioaugmentation with hydrolytic bacteria were elucidated as solutions to improve the biogas production. Specific objectives were:

- Explore the existence of analytical methods able to be used as BMP prediction tools.
- Characterize the chemical composition of lignocellulosic substrates and identify alterations among species.
- Apply different mechanical pretreatment methods using shearing forces on grass biodegradability.
- Evaluate various co-digestion mixtures of grass silage and livestock manures in order to boost the methane production of agricultural residues.

- Examine the effect of implementing mechanical pretreatment at the harvesting step at industrial scale applications.
- Assess if the combination of two overall process steps (i.e. harvesting and pretreatment) can lead to positive energy balance for a sustainable grass-based biogas production.
- Evaluate mechanical and thermal alkaline pretreatment methods on partially degraded manure fibers in order to boost the energy output.
- Test microaeration as a tool to boost lignocellulose deconstruction and subsequently, improve methane production.
- Define differences among the microbiome of manure mono-digestion and the unattached or firmly attached communities of reactors co-digesting manure and grass silage.
- Elucidate the bioaugmentation with hydrolytic microbes to increase the methane productivity of agricultural residues.
- Define the changes in microbial communities before and after the bioaugmentation.

1.3.2 Structure of the thesis

In Chapter 2, the main chemical components of lignocellulosic substrates are presented. Additionally, advantages and limitations of selected physicochemical methods to predict the methane production are highlighted.

In Chapter 3, diverse strategies to increase the bioconversion of ensiled grass and digested manure fibers are investigated. Co-digestion strategies, mechanical and thermal alkaline pretreatments are investigated under lab and full-scale applications.

In Chapter 4, the injection of limited amounts of oxygen is examined into AD reactors filled with a mixture of inocula, containing obligate and facultative anaerobic microorganisms. Diverse micro-aeration strategies are noted.

In Chapter 5, the changes on microbial diversity and dynamicity of co-digestion reactors are highlighted. Moreover, the idea of bioaugmentation with hydrolytic strains to improve the biodegradability of lignocellulosic substrates is presented. Different bioaugmentation approaches are followed based on strains' characteristics. Conclusions and future perspectives follow.

2 Lignocellulosic substrates

Biofuels represent a potential solution to decrease the environmental impacts derived from petroleum-based energy sources (Chandra et al., 2012a). Feedstocks for biofuels are available in aquaculture, forestry, agricultural-, industrial- and domestic- sectors (Cherubini, 2010); and among these material sources, lignocellulosic biomass is an abundantly available carbon-rich and land-based feedstock, which can improve the independency on gas and oil (Pickett et al., 2008).

Valorisation of plant material for biogas production gained increased attention during the last decades as it is energetically more efficient compared to alternatively liquid biofuels (Frigon and Guiot, 2010; Samson et al., 2008). A variety of lignocellulosic materials is already examined as input streams into biogas reactors (e.g. energy crops, silages or fresh biomass, straw etc.). However, bioenergy purposes should not deteriorate the battle for land usage, as the increased demand for food production is worldwide acknowledged. Hence, only the wastes and residues are currently considered as suitable solution for AD and in this framework, huge amounts of fibrous leftovers are available for exploitation (Guerriero et al., 2016).

The major fractions of lignocellulosic biomass are cellulose, hemicellulose and lignin corresponding to approximately 90% of the total dry matter (Figure 3). Apart from the three major components, some other compounds as ash, pectin and proteins are also presented in smaller amounts. Table 1 presents the major chemical characteristics of the used lignocellulosic substrates.

Table 1. Main chemical characteristics of the examined lignocellulosic biomasses

Biomass	Glucan, %TS	Xylan, %TS	Arabinan, %TS	Lignin, %TS	TKN, %TS	C:N
Ensiled Meadow grass	27.4±6.4	16.0±4.5	3.2±0.8	23.1±6.4	1.9±0.2	22.5±2.8
Ensiled Cultivated grass	31.2±3.2	13.1±1.4	4.1±0.5	9.3±3.5	2.9±0.2	13.6±0.4
Wheat straw	42.0±0.7	27.9±0.4	2.8±0.1	26.7±2.4	0.4±0.1	103.0±4.8
Digested manure fibers	22.6±0.1	10.9±0.1	0.8±0.0	31.2±0.7	0.9±0.0	45.0±2.3

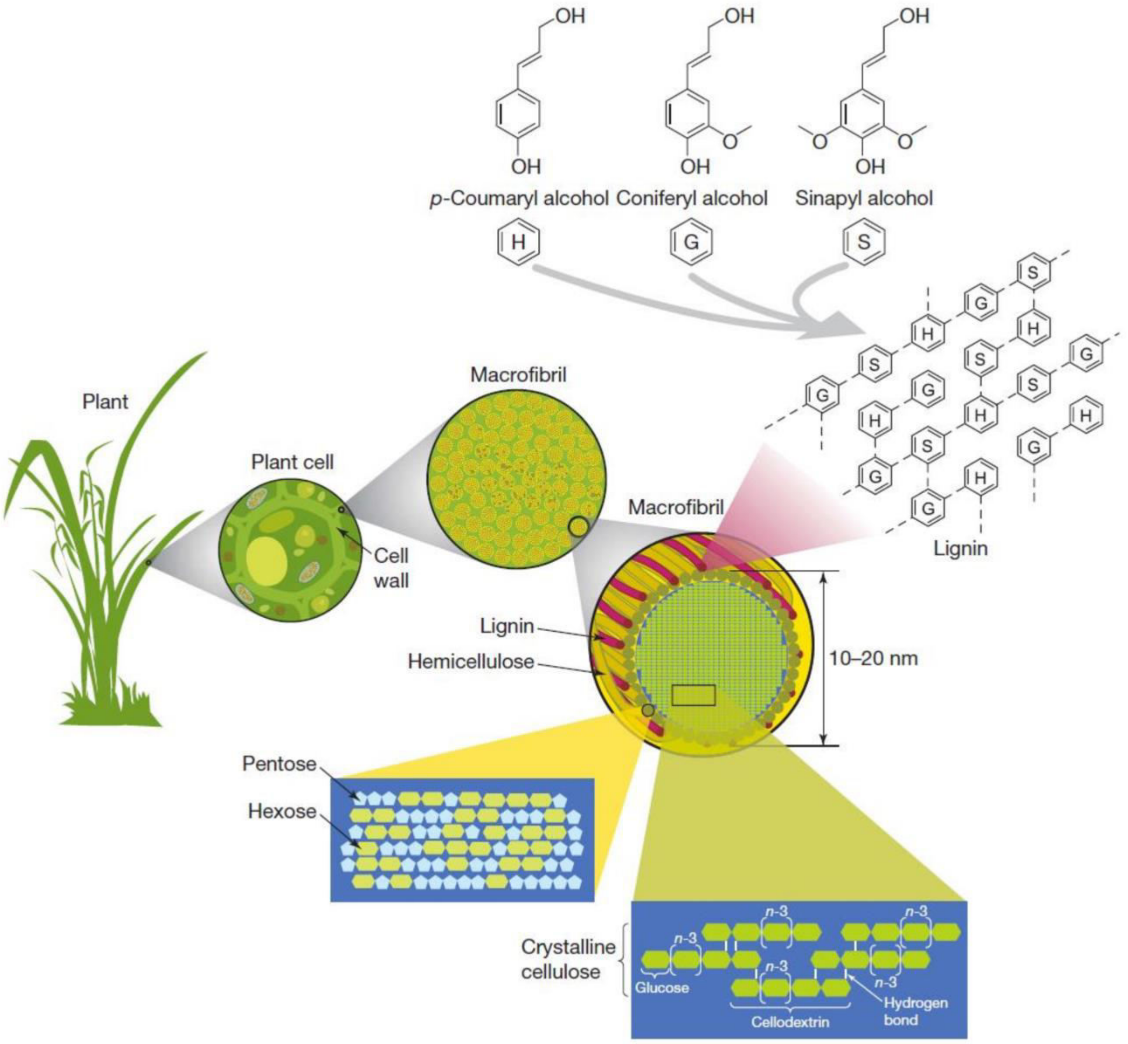

Figure 3. Structure of lignocellulose. [Adapted from (Rubin, 2008)]

2.1 Chemical composition

2.1.1 Cellulose

The major component of lignocellulose cell walls representing 17-50% of the total organic matter is the cellulose polymer (Gnansounou and Dauriat, 2010; Mutschlechner et al., 2015). It is a linear polysaccharide joined by D-glucose subunits, linked by β-1,4-glycosidic linkages (Fengel and Wegener, 1984). The biopolymers are linked by hydrogen bonds and van der Waals interactions, resulting in packed and non-soluble microfibrils (Guerriero et al., 2016). The hydrophobic surface of crystalline cellulose increases the resistance of plant cell wall to the microbial attack (Jørgensen et al., 2007). The anaerobic depolymerization of cellulose is conducted by hydrolytic bacteria and fungi strains, which produce cellulolytic enzymes in order to degrade the polymer into cellobiose and glucose units (Gnansounou and Dauriat, 2010; Procházka et al., 2012).

2.1.2 Hemicellulose

In contrast to cellulose, hemicellulose is not entirely consisted of one monosaccharide. A variety of C_6 sugars (e.g. D-glucose, D-mannose, D-galactose, L-rhamnose), C_5 sugars (e.g. D-xylose, D-arabinose) and sugar acids (e.g. D-glucuronic acid, 4-*O*-methyl-D-glucuronic acid) are the dominant polymers (Hendriks and Zeeman, 2009; Straathof, 2014; Zheng et al., 2014). Due to increased heterogeneity, a broad variety of enzymes are needed to conduct hemicellulose breakdown (Azman et al., 2015). However, characteristics as the short length, low molecular weight and amorphous shape make hemicellulose units the easiest hydrolysed components compared to cellulose and lignin (Fengel and Wegener, 1984).

2.1.3 Lignin

The most abundant non-polysaccharide organic matter and commonly the second most abundant organic polymer in lignocellulosic biomass is the lignin fraction (Jørgensen et al., 2007; Zheng et al., 2014). It is a complex aromatic and hydrophobic network consisted of phenylpropane monomers (e.g. p-coumaryl, coniferyl and sinapyl alcohol) linked by alkyl-aryl, alkyl-alkyl, and aryl-aryl ether bonds into a three-dimensional structure (Kumar et al., 2009; Rubin, 2008). This amorphous heteropolymer cross-links among polysaccharides and creates an impermeable and resistant structure acting as the main barrier for biomass deconstruction. Although its oligomeric and polymeric components can be partially degraded under anaerobic conditions, lignin is generally considered as the non-degradable organic matter in organic wastes (Angelidaki and Sanders, 2004; Monlau et al., 2013).

2.2 Prediction of methane production

The methane production of lignocellulosic substrates is significantly affected by various parameters as chemical composition, conservation conditions, specie variety and stage of development (Dandikas et al., 2015). In this context, usage of substrates with low biodegradability will lead to limited profitability. Hence, rapid methods which can efficiently predict the methane potential of a substrate would be extremely helpful in order to improve the selection process of energy rich substrates and subsequently, maximize the energy output of full-scale AD plants.

For this purpose, physicochemical methods based on biomass' chemical characteristics would be advantageous as they are inexpensive, quick and easily applicable. More specifically, the results from the analytical methods can be simply correlated using regression analyses with databases of BMP values to predict the biogas production. Among the available methods, near infrared spectroscopy (NIRS) is lately considered as a highly efficient tool to forecast the biomethanation (Triolo et al., 2014). Accordingly, methane production of herbaceous phytomass was predicted using partial least squares regression with coefficient of determination (R^2) equal to 0.93 and residual prediction deviation (RPD) of 3.77 (Wahid et al., 2015). Moreover, component composition analysis of the biomass can also give an adequate approximation of biogas production (Triolo et al., 2011). Taking into account the acid detergent lignin and hemicellulose content of various energy crops, a multiple linear regression was developed with a promising R^2 of 0.83 (Dandikas et al., 2014). However these methods demand the structural change of biomass in powder form; an action that is considered as pretreatment step and indeed, positively affects the anaerobic degradation (Kratky and Jirout, 2011). Hence, a more direct way to predict the biogas yield without affecting biomass characteristics is preferable.

Therefore, electrical conductivity (EC), soluble Chemical Oxygen Demand (sCOD) and enzymatic hydrolysis (EH) were evaluated as alternative prediction tools of ensiled meadow grass AD (Paper I). However, prediction statistics found to be unsatisfactory (i.e. R^2=0.39-0.68, RMSEP=29.36-40.38 and RPD=1.29-1.77). Specifically, these measurements are based on the release of different quantities of ions (i.e. EC) and organic matter (i.e. sCOD and EH) due to damages on biomass surface (Koegel and Kraus, 1996; Lesteur et al., 2010). Thus, they do not take into account the unattached molecules such as intact cellulose that will contribute later in the methane production. Additionally, the poor calibration statistics can be explained by the fact that the substrate was extremely heterogeneous, regarding species composition and morphology. The used biomass was originated from meadows that were never been plowed and thus, was composed of a huge variety of different grass species. Hence, improved homogeneity using only one substrate could increase prediction capability.

AD is a complex process conducted by a complex microbiome and hence, it is challenging to predict the capacity of methane production under rapid methods. Through these alternative methods, a quick and rough estimation can be achieved. However, the BMP test is still the most suitable way to precisely assess the biodegradability of a substrate.

3 Pretreatment methods

Identifying pretreatment methods which are appropriate for lignocellulose-based AD is of high importance for the feasibility of a biogas plant. Pretreatments can be roughly categorized as physical, chemical and biological (Zheng et al., 2014). Physical methods intend to improve the access to the degradable organic matter by alternating biomass size (Kratky and Jirout, 2011). The efficiency of chemical methods is mainly based on the characteristic of specific compounds to change the properties of lignocellulose's components (Zheng et al., 2014). Regarding the biological pretreatments, the action of selected microbial members is taken into advantage in order to improve biomass deconstruction (Čater et al., 2014). Hence, a huge variety of pretreatments is available and therefore, there is always a need to carefully identify and apply the most appropriate method based on the operational characteristics (e.g. feedstock composition, temperature, reactor configuration). Table 2 lists a few treatment methods that are used to improve the biomethanation process of lignocellulosic substrates and comparable of them used in the present PhD thesis.

Table 2. Applied methods to improve the biodegradability of lignocellulosic substrates. Comparable strategies were examined in the present PhD study.

Methods	Substrate	Conditions	CH_4 Increase	Reference
Mechanical pretreatment				
Milling	Wheat straw	Size reduction from 5 to 0.2 cm	80%	(Menardo et al., 2012)
Grinding	Ley crop silage	Size reduction from 1-16 mm to 0.1- 2.0 mm	59%	(Lindmark et al., 2012)
Chemical pretreatment				
Alkaline	Biofibers	6% CaO w/w, 15 °C, 10 days	66%	(Bruni et al., 2010)
Thermal alkaline	Wheat straw	4% NaOH (g/g TS), 37 °C, 5 days	112%	(Chandra et al., 2012b)
Bioaugmentation				
Clostridium cellulolyticum	Wheat straw	33% of the working volume	13%	(Peng et al., 2014)
Pseudobutyrivibrio xylanivorans Mz5^T	Brewery spent grain	5% of the total volume	18%	(Čater et al., 2015)
Micro-aeration				
Oxygen	Corn straw	12.5 mLO_2/L_R/day	17%	(Fu et al., 2016)
Oxygen	Sugarcane bagasse	10 mLO_2/gVS	17%	(Fu et al., 2015)

3.1 Mechanical pretreatment

The mechanical methods are generally accepted to be suitable for full-scale applications due to their easiness of application and to the absence of inhibitors release (Kratky and Jirout, 2011). Conversely, their drawback derives from the increased energy consumption that is often demanded for an efficient disintegration (Hidaka et al., 2013; Rodriguez et al., 2016).

Application of shearing forces is already considered as an effective way to disrupt biomass and prepare it for AD (Hartmann et al., 2000). Hence, in Paper I the effect of shearing forces was examined using a simple mechanism in order to simultaneously macerate and pretreat ensiled meadow grass. The commercial available metal plates managed to improve substrate's biodegradability in the range of 8% to 25%. Specifically the combination of two mesh grating plates with coarse surface was the most efficient, as 377 ± 34 mLCH$_4$/gVS were produced by the mechanically pretreated meadow silage. The superiority compared to other alternatives was indirectly observed by the result on length reduction. In this context, 43% of grass particles had average length less than 10 cm. In contrast, after the less efficient pretreatment (+8% biogas increase), 45% of total silage samples had average length higher than 15 cm. The positive effect was additionally verified from Scanning Electron Microscopy (SEM) pictures, in which distinct structural damages in silage's longitudinal direction were observed (Paper I). As a result from the aforementioned positive outcomes, the combination of coarse metal plates was further investigated in Paper II.

As a next step, the co-digestion of diverse livestock manures with mechanically pretreated ensiled meadow grass, harvested during the late stage of development, was examined. The chemical composition of mature grass implied higher need for pretreatment, as the plant tissue was significantly more lignified (~30% TS) compared to samples harvested at the early development stage (~15% TS). Mink, poultry and cattle manure were examined as co-substrates under different manure to silage VS$_{contribution}$: 100:0, 80:20, 60:40, 40:60 and 20:80. Mink manure was favoured by the highest silage share in the feedstock (348 ± 45 mLCH$_4$/gVS) compared to its limited BMP under mono-digestion (239 ± 5 mLCH$_4$/gVS). Conversely, when the share of meadow silage was 40% and 60% in the feedstock, the highest methane production was achieved in co-digestion with poultry and cattle manure, respectively.

It is generally accepted that co-digestion is an efficient way to treat animal slurry with organic wastes in full-scale AD with benefits for every substrate (Ahring et al., 1992; Yangin-Gomec and Ozturk, 2013). For example, manure normally contains low C:N ratio which will be adjusted closer to the optimal by the addition of carbon-rich lignocellulose (Nielfa et al., 2015). Also, manures are rather diluted samples, affecting negatively the volumetric methane production. This obstacle is significantly diminished through co-digestion with lignocellulosic substrates obtaining a considerably thicker feedstock (Møller et al., 2004). In the meantime, livestock manure can assist the digester with high buffer capacity and the necessary amount of trace elements for long term operation (Thamsiriroj et al., 2012). So, the co-digestion process can positively affect the biogas production and consequently, the feasibility of industrial applications. On this topic, the knowledge of feedstock characteristics is crucial to define the optimum manure to lignocellulose contribution and achieve the predetermined targets.

However, due to the intricacy of AD process, the typically performed BMP experiments alone do not illustrate reliably the outcomes of full-scale applications. Thus, as a next step, continuous lab-scale experiments need to be monitored in order to simulate more efficiently the real-life biogas plants. Hence, a typical nitrogen rich substrate (i.e. pig manure) was co-digested with a relatively high carbon rich substrate (i.e. either untreated or mechanically pretreated grass silage) under continuous mode operation (Paper IV). Interestingly, the findings of the first two studies were validated to some extent. Specifically, the CSTR fed with pretreated biomass had 6.4% improved biomethanation ($p > 0.05$) than the untreated operation, confirming the positive effect of mechanical pretreatment (Paper I). Accordingly, semi-continuous trials examining the mono-digestion of grass proved that simply decreasing plant's length had minor effect on methane production (Wall et al., 2015). Thus, it can be deduced that the action that positively enhances biomass biodegradation is the enhanced surface's damage by the application of frictional forces (Paper I). Moreover, the improved performance due to the efficient pretreatment was observed by the rest process characteristics. Specifically, the remaining sugars in the effluent and on the other hand, the free ammonia concentrations during AD were both decreased.

The preliminary co-digestion experiment implied that feedstock's enrichment with dissimilar substrates positively affects the biogas production (Paper II). Similarly, the addition of untreated and pretreated meadow silage in the influent significantly enhanced ($p < 0.05$) the biogas production by ~9% and

~16% compared to pig manure mono-digestion, respectively. Co-digestion is accepted as possible solution to counteract ammonia inhibition and enhance the bioconversion efficiency (Chen et al., 2008). Hence, the positive effect of the co-digestion strategy was presented either with untreated or pretreated meadow grass silage.

Nevertheless, the operation of AD plants fed with livestock manure and lignocellulosic substrates is often associated with poor energy output due to the limited biodegradation levels. Hence, a substantial amount of organic matter is discarded in the post-storage tank (Angelidaki et al., 2005). The further exploitation of the remaining biomass can improve the overall efficiency.

In Paper III, the performance of mechanical pretreatments was further examined on Digested Manure Fibers (DMF) obtained from the solids fraction of AD effluent. The used organic fraction was already undergone an initial digestion process and thus, was consisted from hardly degradable lignocellulose. Nevertheless, the metal plates significantly affected the biodegradability ($p < 0.05$) under BMP experiments in a range of 15 to 45% compared to the untreated DMF (42 ± 8 $mLCH_4/gVS$). Specifically, the usage of metal plates covered by sandpaper was connected with the highest methane yield (60 ± 10 $mLCH_4/gVS$) and subsequently, this mechanical pretreatment method was examined in continuous mode experiments (Figure 4). However, the final improvement (+7%) was significantly lower compared to the effect in batch assays. This result is comparable to previous findings in the literature (8–9.3%) regarding mechanical pretreatments on digested lignocellulosic residues (Bruni et al., 2010; Lindner et al., 2015). However, in long-term AD, the more desirable operational characteristics compared to control reactor (i.e. accumulation of TS and VFA, and limited degradation of carbohydrates and VS) indicated the positive impact of applied pretreatment. Hence, observations made in this study indicated that despite the limited biogas production, mechanical pretreatment can be used as an efficient method to maximize the energy output from unconventional substrates.

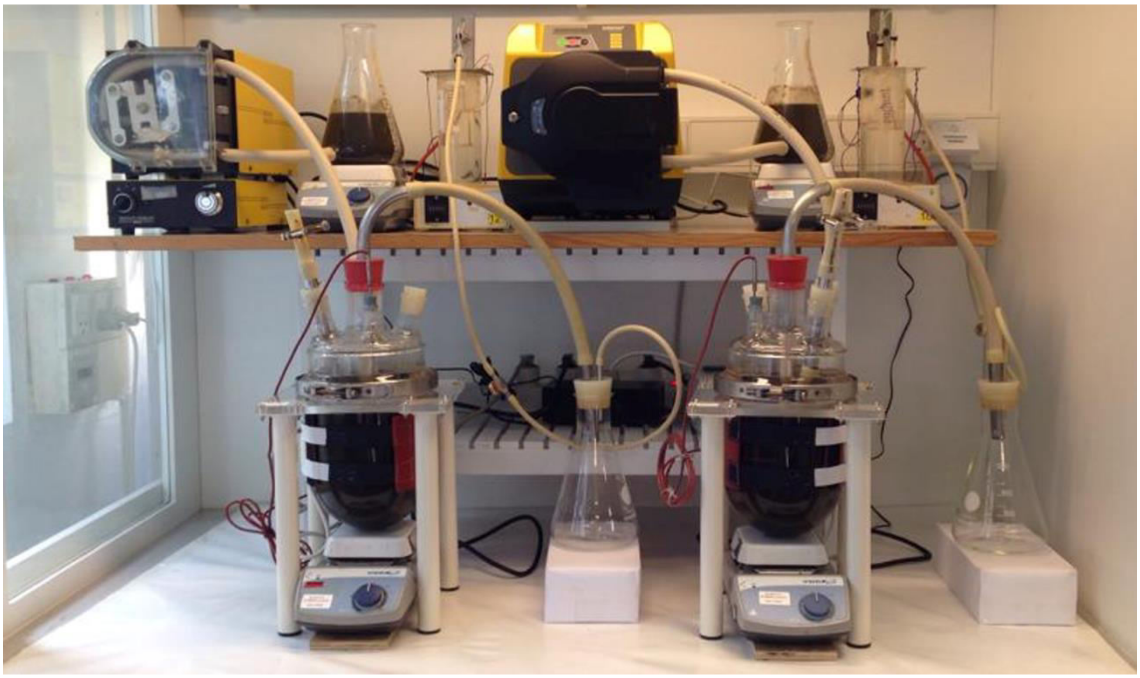

Figure 4. System set up for the AD of mechanically and thermal alkaline pretreated digested manure fibers.

3.2 Chemical pretreatment

Apart from the mechanical pretreatments that were presented in the previous chapter, the chemical pretreatments pose also the ability to succeed in an feasible AD (Zheng et al., 2014). Alkaline, acid, wet oxidation, catalysed steam-explosion and ionic liquids methods are included in this category. In general, the efficiency of these pretreatments is based on the capability of chemical compounds to disrupt the lignocellulosic polymers and specifically, the most widely studied chemical pretreatments examined the usage of acids or bases.

Acid pretreatments are known to solubilise hemicellulose units and break the bonds of lignin structure. However, they do not dissolve lignin and are typically applied in high temperature levels and thus, generate inhibitors as furfural and hydroxymethylfurfural (HMF) (Zheng et al., 2014). Conversely, alkali pretreatments can boost the saponification and induce the disruption of lignin-carbohydrate bonds and form less severe inhibitors to methanogenesis (Hendriks and Zeeman, 2009). Additionally, the efficacy can be enhanced if catalyst’s usage is combined with application of thermal energy and more specifically, the thermochemical methods are considered to be among the most appropriate for lignocellulose treatment (Biswas et al., 2012).

In this context, sodium hydroxide was used in several concentrations and temperatures as an alternative method to improve the biodegradability of DMF (Paper III). Results obtained in this study showed that the efficiency was primarily defined by the concentration of the catalyst; the greater the chemical agent, the more promising the biomethanation. Thus, the highest methane yield was achieved using either 6% NaOH – 55 °C (168 ± 9 $mLCH_4/gVS$) or 6% NaOH – 121 °C (173 ± 34 $mLCH_4/gVS$) under batch assays. Beyond the very promising findings, questions still can be raised about the result of alkaline pretreatment in a more realistic application, due to the limited knowledge on continuous reactor operation (Angelidaki and Ahring, 2000; Sambusiti et al., 2013). Interestingly, CSTRs monitoring revealed that 4% NaOH – 121 °C affected the biomethanation (+25%) in similar level with the highest catalyst dosage (+26%). Additionally, no process inhibition was defined by the augmented sodium concentration (Chen et al., 2008).

3.3 Integration of mechanical pretreatment at harvesting

As a continuation of the lab scale experiments the perspective of applying shearing forces was assessed in full-scale practices. The reduction of supply chain steps could potentially improve the energy balance of the overall AD process. Thus, the hypothesis of integrating the mechanical pretreatment into harvesting step was examined.

Within the framework of the present study, three commercially available machines were examined as means of mainly improving the energy output per hectare and affecting kinetics parameters (Paper V). Based on literature, two suitable machines were used (i.e. Disc-mower and a Chopper) to harvest non-cultivated fields (Boscaro et al., 2015). Additionally, a developed model of Disc-mower, named as "Excoriator", equipped with a number of rough barbs was elucidated, simulating the mechanism of the coarse metal plates (Paper I).

Results showed that the Excoriator significantly promoted ($p < 0.05$) the bioenergy production by approximately 20% compared to Disc-mower, which did not provoke any damage to the grass surface. Promising results were also presented through chopping, as the methane production was augmented by 11%. The positive effect of harvesters was initially observed by the increased dry matter measurements compared to the untreated fresh grass. Accordingly, a feedstock with higher solids content can lead in side benefits to the overall process, as the transportation and logistics costs will be decreased due to the partially drying of the biomass (Gunnarsson et al., 2008).

Furthermore, more than 90% of the final methane yield was produced until the 15th incubation day, which is particularly interesting as a typical AD plant is operated with similar or longer hydraulic retention time (HRT) (Karakashev et al., 2005). In conjunction with the increased biomethanation, Excoriator's superiority was also observed through the kinetic modelling. More specifically, reduction of lag phase and increase of methane production rate were favoured by the most modern harvesting technology (Paper V). Indeed, the lignocellulose-based AD is a time consuming process and in this concept, the examined machinery showed to be capable of diminishing the demanded time frame.

Biogas utilization using either a CHP unit (i.e. electrical and thermal energy generation) or an upgrading unit for biomethane production (i.e. transport fuel or injection into the gas grid) are the most widely applied pathways to improve the independence from fossil fuels. In this context, the potential energy output due to harvesting with the alternative machines was calculated. In the developed case study, the Danish grasslands were selected as the reference area (i.e. $229*10^3$ ha). In fact, the Excoriator treatment could annually boost the energy generation with extra 16 million m^3 CH_4 or alternatively, 8 kt crude oil equivalents (COE) compared to harvesting with a classical Disc-mower (Paper V).

Also, a further detailed assessment was conducted focusing on the efficiency of harvesting machines to improve the energy balance (Paper VI). Different types of silages, mowed on different vegetation stages revealed quite similar results on the biomethanation process. During full-scale trials, high biomethanation was achieved for both harvesting machines mowing different types of grass (298-372 $mLCH_4/gVS$). The values are in the range of previous studies examining similar substrates (Lehtomäki and Björnsson, 2006; Mähnert et al., 2005; Raju et al., 2011; Søndergaard et al., 2015), indicating a well-performing AD process. In fact, the biodegradability of meadow and regularly cultivated grass was increased up to 10%, due to the shearing forces of Excoriator. In comparison to this result, different models of commercially available harvesters lead to similar effect in AD process, increasing the biogas yield up to 13% (Herrmann et al., 2012a).

Among the overall aims of the present PhD study was to define applicable solutions in real life that can reduce the energy loss. On this topic, the preliminary technical analysis revealed that the energy output can be optimized using the prototype harvester equipped with the set of a rotating drum and a fixed shell, both armed with aggressive barbs. Taking into account the corresponding energy demand for harvester's operation per hectare and the subsequent, energy produced from AD as input ($\boldsymbol{E_{in}}$) and output ($\boldsymbol{E_{out}}$) variables, respectively; it was calculated that the balance can be improved by 0.87-1.55 GJ/ha, based on the different harvesting speeds ($\boldsymbol{V}$) (Table 3). However, for the widespread establishment of grass usage in the feedstock of full-scale biogas plants, further energy inputs should be considered (i.e. ensiling process, storage, transportation to the biogas plant, electricity supply and heat demand, operation of biogas plant etc.) to define the actual energy benefit. In addition, it would be particularly interesting to examine the level that the examined harvesters affect the economic profitability of a biogas plant. Similarly, a previous detailed cost and revenues assessment of lignocellulose based-AD, including the harvesting step, proved that the economics can be improved by the optimal treatment at the field (Herrmann et al., 2012b).

Table 3. Energetic analysis of harvesting machines operated under different conditions [Adapted from Paper VI]

Harvesting machine	V, km/h	E_{in}, GJ/ha	E_{out}, GJ/ha	E, GJ/ha	Excoriator Effect, GJ/ha
Disc-mower	20.0	0.03±0.01	15.77±2.09	15.74±2.09	
Excoriator	4.0	0.08±0.01	16.68±2.19	16.60±2.19	0.87±1.00
Excoriator	7.5	0.06±0.01	16.91±1.28	16.86±1.27	1.12±1.04
Excoriator	11.0	0.04±0.01	17.34±1.56	17.29±1.56	1.55±1.09

4 Impact of micro-aeration

The delignification of recalcitrant substrates is among the main objectives of pretreatments in order to improve the anaerobic degradability. Thus, the oxidation of the phenolic skeletal structure of lignin barrier can increase the biodegradability. Lignin oxidation occurs in natural environments, for example in the compost facilities where fungi or other bacteria excrete enzymes (e.g. laccases, peroxidases) to break down the phenolic hydroxyl groups (Brown and Chang, 2014; Jurado et al., 2015).

Hence, the enrichment of AD effluent derived from a biogas plant with inoculum obtained from a composting plant was examined as an alternative treatment (Paper VII). Indeed, previous findings indicated the positive impact of similarly enriched microbiome in lignocellulose-based AD (Scherer and Neumann, 2013). However, in our study the mixed inoculum did not influence the AD process of wheat straw and the effect on biogas production was negligible (+1%) compared to the samples operated with AD effluent as sole inoculum type.

In spite of the non-enhanced biomethanation due to the mixture of inocula in the preliminary trials, their combination was further elucidated under micro-aerobic conditions (Paper VII). Indeed, the introduction of limited amounts of oxygen could promote the activities of the aforementioned microorganisms that are present in the enriched microbiome.

Nevertheless, micro-aeration should be carefully applied and controlled in AD systems in order to avoid augmented aerobic oxidation of holocellulose and on the other hand, do not provoke inhibition to methanogenic archaea that are extremely sensitive to oxygen exposure. Hence, diverse micro-aeration strategies were thoroughly investigated by examining three variables in three levels: a) oxygen load (i.e. 5, 10 and 15 mLO_2/gVS), b) pulse repeatability (i.e. 1, 2 and 3 injections) and c) micro-aeration period (i.e. 1, 2 and 3 days). During this study, the measurements of pH, sCOD, VFA accumulation and finally, methane yield were used as parameters to evaluate the effect of the various micro-aeration strategies.

Although the desired effect of oxygen was initially detected by the accumulated acetate content in the micro-aerated samples; finally, statistically significant ($p > 0.05$) enhancements were not observed by means of cumulative methane production (Figure 5). The highest increment (+7.2%) was achieved by introducing 5 mLO_2/gVS by one pulse for 3 days. In contrast, the maximum negative impact (-12.7%) was presented injecting the highest the oxygen concentration (i.e. 15 mLO_2/gVS) distributed in three pulses in the minimum micro-aeration period (i.e. 1 day).

Among the three variables, the analysis of BMP results indicated that the most significant parameter to affect the anaerobic degradation was the oxygen load (Paper VII). Moreover, the numerically optimisation study suggested an alternative micro-aeration strategy to be followed. The injection of 7.3 mLO_2/gVS, distributed in equally shared volume and conducted into 47 hours, was calculated to result in 9.0% higher BMP compared to the non-aerated wheat straw. Interestingly, Lim and Wang (2013) reached similar enhancement by applying micro-aerobic conditions in batch experiments using brown water and food waste as substrates. However, their results are not directly comparable to ours, as these substrates are more susceptible to AD compared to wheat straw and additionally, a different micro-aeration strategy was followed.

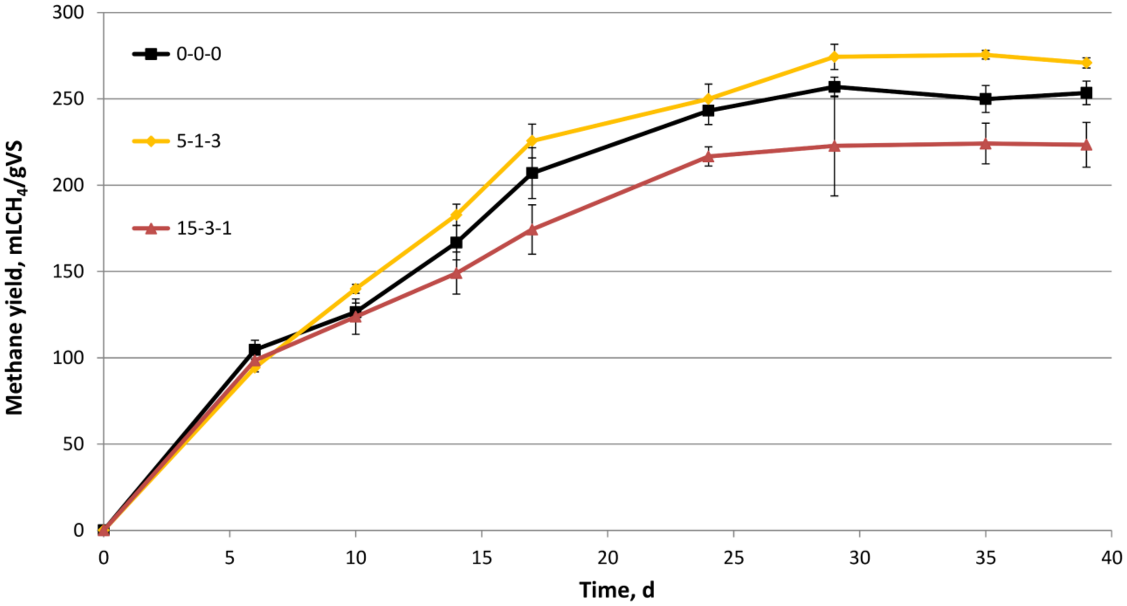

Figure 5. Methane development plotted against time for batch reactors digesting untreated wheat straw (i.e. 0–0–0) and treated with oxygen addition. The treatments are named with the volume of oxygen load (mLO_2/gVS) – pulse repeatability – micro-aeration period (i.e. 5–1–3 and 15–3–1)

5 Insights into microbiome of lignocellulose based-AD

Despite the fact that AD systems are known to be consisted of a "core microbiome" indispensable for methane production, the majority of biogas community is still comprised of unknown microbes at species level (Treu et al., 2016b; Tuan et al., 2014). In parallel, and as mentioned above, the lignocellulosic residues are commonly co-digested with livestock manure, in order to overcome specific obstacles originated from the alternative mono-digestion of both substrates' categories. Thus, the deeper exploration of both specialized (i.e. during mono-digestion) and enriched (i.e. during co-digestion) biogas-producing microbiome can markedly contribute to achieve the target of increased efficiency of AD systems.

Regarding the decomposition of lignocellulosic substrates, the knowledge about the development and distribution of bacterial and archaeal genera, in both solid and liquid phase of a biogas reactor, can significantly fill gaps in the literature. Thus, unassembled shotgun genomic sequences analysis was performed in AD reactors operating with pig manure and lignocellulosic silage, to reveal differences based on feedstock composition (i.e. mono-digestion against co-digestion) and distribution in the reactor (i.e. firmly against loosely attached microbes to lignocellulose) (Paper IV).

Subsequently, findings from the comparative metagenomic analysis could indicate the most important members for the deconstruction of lignocellulosic materials. Hence, inoculating selected microbes into lignocellulose-based biogas reactors could contribute on the further improvement of biomethanation process. Thus, bioaugmentation with selected hydrolytic strains was applied in the co-digestion of cattle manure with wheat straw (Paper VIII). The microbial changes prior and after bioaugmentation were assessed using 16S rRNA gene sequencing analysis. The results of both next generation sequencing analyses are presented in the following subchapters.

5.1 Development of microbial communities

Based on previous phylogenetic assignments, *Firmicutes* and *Proteobacteria* are among the most abundant phyla in manure based-AD systems (Bassani et al., 2015); result that was also revealed in our study (Paper IV). *Firmicutes* are extremely important for the degradation of lignocellulosic biomass, as species belonging to this phylum are well-known producers of either multiple cellulolytic enzymes or cellulosome complex, utilized for adhesion and enzymatic purposes (Treu et al., 2016b). Indeed, the most abundant microbes firmly attached at the solid fractions were species similar to *Clostridium thermocellum* (Paper IV). Similarly, *Clostridia* species were previously found adherent to lignocellulosic fibers (Wang et al., 2010). More specifically, the abundance of strains related to *C. thermocellum* is originated from the existence of a specific type of module, the cellulose-binding domain responsible to anchor the cells at the cellulose polymers (Lamed et al., 1983; Shimon et al., 2000). Moreover, microbes similar to *Coprothermobacter proteolyticus* strains were also predominant at the same samples. Specifically, *C. proteolyticus* are known for the utilization of extracellular proteinaceous compounds and additionally, are useful for lignocellulose's deconstruction due to their abundant expression of glycoside hydrolase enzyme (Lü et al., 2014a). On the other hand, they are notable hydrogen producers and thus, their presence could establish an syntrophic association with hydrogenotrophic archaea (Lü et al., 2014b).

In contrast to the hydrolytic microbes, the majority of methanogenic population was mainly observed at the liquid samples of both mono- and co-digestion experiments (Paper IV). For example, only few members of *Methanosarcina* found to be abundant at the solid phase, probably being capable of inhabiting the biofilm around the polysaccharides (Song et al., 2005; Wang et al., 2010). However, clear differences were also revealed about the archaeal populations between mono- and co-digestion trials. Specifically, it is well-known that the composition of methanogenic communities is affected by the operational characteristics (i.e. feedstock, temperature) (Luo et al., 2016). For instance, the acetoclastic methanogens are more sensitive to inhibition compared to hydrogenotrophic methanogens during the AD of ammonia rich substrates (Fotidis et al., 2014). Hence, the archaeal diversity is significantly affected in the presence of nitrogenous compounds. Accordingly, various *Methanothermobacter* species were found in increased abundance in the mono-digestion of swine manure under thermophilic conditions (Tuan et al., 2014; Paper IV). Moreover, hydrogenotrophic archaea of the genera *Methanoculleus*, which are commonly dominant in AD systems operating with livestock manure (Campanaro et al., 2016; Treu et al., 2016b), revealed to be more abundant in the co-digestion compared to mono-digestion trials (~2.7 folds). Their dominance could have been favoured by the abundance of *C. thermocellum* providing the appropriate feedstock to the observed *Methanoculleus* *marisnigri* (Schlüter et al., 2008). However, versatile acetoclastic methanogens of the order *Methanosarcinales*, which were dominant at the solid lignocellulosic residues, could also favoured by the abundance of *Clostridium* genera (Fournier and Gogarten, 2008).

5.2 Bioaugmentation as a tool to improve process efficiency

In a well-performing biogas process treating agricultural wastes (e.g. livestock manure, lignocellulosic residues), the microbial community is composed of specific bacterial and archaeal genera; and as already mentioned, the "core microbial consortium" exists independently from the operational characteristics (e.g. temperature, feedstock composition, organic load) under steady state conditions. However, the AD process is known to be sensitive to process imbalances (e.g. temperature fluctuation, organic overload) and thus, the digesters are not always working under optimal steady-state conditions. For instance, problems can periodically occur in biogas plants (e.g. ammonia inhibition, VFA and solids accumulation), stressing or inhibiting specific members of the microbiome leading to a dramatically deteriorated profitability.

In this topic, the inoculation with suitable microbes is considered as a common solution in order to utilize their beneficial properties and thus, prevent or overcome the instabilities. The bioaugmentation with bacterial and/or archaeal strains aims to favour the action of selected strains and/or shift the digester towards specific metabolic pathways. However, despite the positive results that were observed through bioaugmentation with either bacterial (Čater et al., 2015) or archaeal strains (Fotidis et al., 2014), it is still unclear whether it is necessary to bioaugment a reactor with specific strains as the result is not always successful (Nielsen et al., 2007). In this concept, the need for bioaugmentation is still questionable, as a conflict opinion exists in the scientific community claiming that the microbiome will finally adapt in the system despite the suboptimal conditions and subsequently, result in adequate process efficiency (Chen et al., 2008).

In terms of lignocellulose based-AD, the bioaugmentation with hydrolytic pure or mixed cultures is considered as a potential way to improve lignocellulose's depolymerization and subsequently, methane production (Čater et al., 2015; Martin-Ryals et al., 2015; Peng et al., 2014). Nevertheless, a robust and reproducible method for bioaugmentation does not exist and thus, there is always a risk of failure. For example, insufficient adaptation of the inoculated strain, competition with existing microbiome and not adequate bioaugmented volume to prevent washout, are among the most commonly detected reasons of process failure.

Therefore, the bioaugmentation with hydrolytic microbes was tested in the current study under different approaches (Paper VIII). As it was introduced above (Chapter 5.1), *C. thermocellum* is among the most prevalent of known anaerobic hydrolytic microbes. Hence, the typically abundant cellulolytic strain was examined under co-digestion experiments of cattle manure with wheat straw, in different manure to lignocellulose ratio on VS_{basis}: a) 90:10 and b) 85:15. In contrast to the predominant in AD systems *C. thermocellum*, a generally scarce and also, never found in biogas process microbe was examined. Specifically, the facultative anaerobic strain of *Melioribacter roseus* was inoculated as the alternative cellulytic culture (Podosokorskaya et al., 2013). Accordingly, it has been proved that the excretion of hydrolytic enzymes is more intense in the presence of oxygen compared to obligate anaerobic conditions (Lim and Wang, 2013). Thus, it is implied that the bioaugmentation of facultative anaerobic bacteria with verified cellulolytic characteristics could lead to beneficial effects. In this concept, *M. roseus* was initially examined under strictly anaerobic environment and subsequently, under microaerobic conditions to thoroughly assess the efficiency of the bioaugmented microorganisms.

5.2.1 Effect on biochemical process characteristics

The results of both BMP and CSTR experiments demonstrated the efficient cellulolytic properties of *C. thermocellum* (Paper VIII). In fact, the replacement of 20% of the inoculum volume with the hydrolytic strain lead to significant yields' enhancement ($p < 0.05$) up to 34% and 16% compared to mono-digestion of wheat straw and co-digestion with cattle manure, respectively. In contrast, batch assays bioaugmented with *M. roseus* reached markedly limited increase, 11% and 8% ($p > 0.05$) respectively. The superiority of *C. thermocellum* was also observed from the more desirable kinetic parameters (i.e. lower lag phase and higher CH_4 rate). The BMP experiments are monitored in a closed system without the possibility of washout and thus, it was assumed that the critical biomass of *C. thermocellum* was enough in order to promote the biogas production (Fotidis et al., 2014). Conversely, the poor efficiency of *M. roseus* can be attributed to various reasons, as for example the limited acclimatization of the strain to the new environment due to suboptimal operational conditions or competition with indigenous microbiome.

Distinct differences on the performance of inoculated microbes were observed also by monitoring the continuous mode experiment (Figure 6). In fact, a remarkable efficiency of *C. thermocellum* was observed during both bioaugmentation periods reaching extraordinarily higher methane production up to 33% ($p < 0.05$), compared to non-bioaugmented period (Paper VIII). However, in the long run, the effect on the productivity was insignificant higher ($p > 0.05$) or in other words, approximately 7% increase was achieved in both co-digestion strategies. In contrast, the examination of *M. roseus* had no positive impact during both bioaugmentation and steady state periods. Apart from the negligible result on steady state conditions, it was also notable that the yield was deteriorated during the second bioaugmentation period with *M. roseus* under microaerobic conditions. In parallel, the performance of control reactor was also slightly worsened which can probably be attributed to the sensitivity of the archaeal community to the oxygen exposure (Botheju and Bakke, 2011; Jarrell, 1985). The extended adverse impact on the bioaugmented reactor showed that the facultative anaerobic inoculated bacterium could not adapt properly in the biogas reactor.

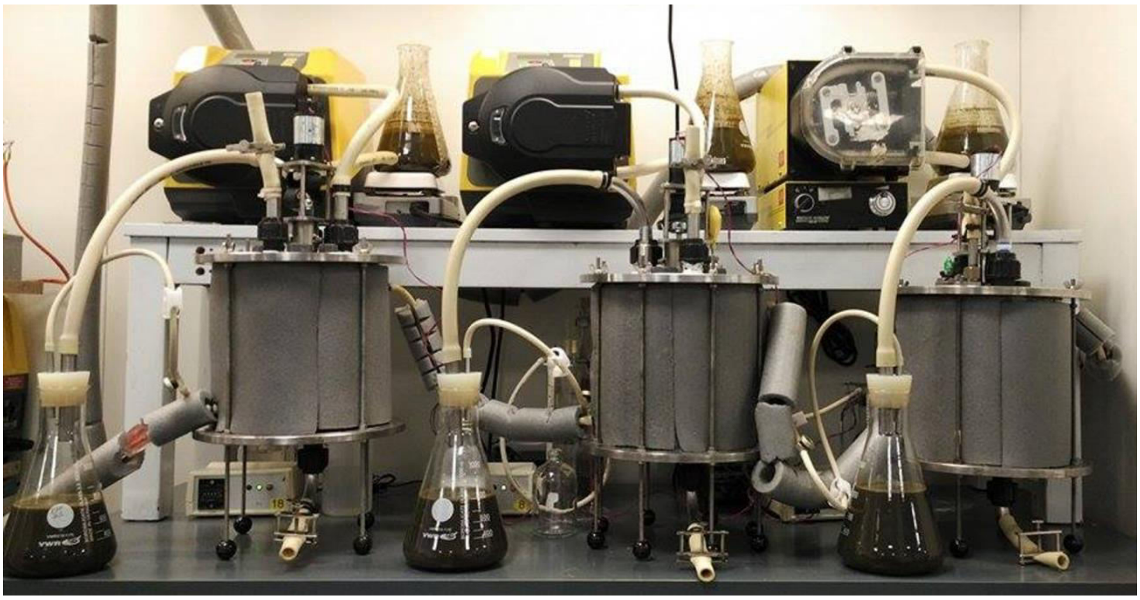

Figure 6. System set up for the bioaugmentation with hydrolytic microbes during the co-digestion of wheat straw with cattle manure.

5.2.2 Effect on bacterial and archaeal communities

The shifts of bioaugmentation on microbial populations were revealed targeting the 16S rRNA gene by metagenomic analysis (Paper VIII). Samples were taken from distinctly separated experimental phases in order to define the level that the various bioaugmentation strategies can affect the microbiome.

Regarding the bioaugmentation with *C. thermocellum*, a profound establishment of the inoculated strain was not revealed at species level. However, the relative abundance of a *Clostridium* genus was marginally increased after both bioaugmentation periods. Hence, improvements are still needed in order to succeed a more efficient cohabitation of the strain into biogas microbiome and subsequently, maintain the needed critical biomass (Fotidis et al., 2014). Additionally, the rest members of the AD community were not significantly affected due to bioaugmentation and generally, small changes in relative abundances were revealed (Paper VIII).

Likewise, strains similar to *M. roseus* were not found after the alternative bioaugmentation strategies, operated under strictly anaerobic and micro-aerobic conditions. Due to the fact that microbes related to *M. roseus* were never detected before in a biogas reactor (Azman et al., 2015) in combination with their total absence into microbial samples, it is implied that their residence along with the indigenous AD microbiota is very challenging. The poor acclimation could be attributed to predation or competition with the existing communities or non-ideal environmental conditions for their growth (Herrero and Stuckey, 2015).

In summary, despite the positive effect obtained on AD trials from the routine inoculation with *C. thermocellum*, the acclimation during the long term operation is still questionable. Hence, more studies are needed in order to define the minimum essential volume of inoculated bioculture and also, the proper time frame that is needed to conduct the periodically bioaugmentation. On the contrary, both bioaugmentation strategies with *M. roseus* had limited efficiency and thus, the usage of the examined strain is not considered as alternative solution to increase the biodegradability of lignocellulosic substrates.

6 Conclusions

This thesis focused on the optimisation of lignocellulose-based AD, assessing a variety of treatment techniques, co-substrates, and process parameters. Changes on the bacterial and archaeal communities during AD process were considered. The major contributions resulting of this thesis are summarised below.

- Physicochemical methods as EC, sCOD and EH proved to have limited applicability in predicting the BMP.
- Harvesting time and species composition affected markedly the chemical composition of lignocellulosic residues.
- Applying shearing forces on meadow grass as a mechanical pretreatment method resulted in improved biodegradability up to 25% in batch assays.
- The optimum silage to manure ratio in the feedstock is markedly affected by the chemical characteristics of livestock manures.
- In continuous mode operation, the mechanical methods improved the overall process in the range of 6-7% treating either digested manure fibers or ensiled meadow grass. The thermochemical pretreatment (6% NaOH – 55 °C) enhanced the methane yield of the biofibers in significantly higher level (+26%).
- The integration of mechanical pretreatment at the harvesting step, using an Excoriator as machinery, can improve the energy output of a full-scale biogas plant by 10%. Additionally, the methane production rate is increased and lag phase is decreased due to the shearing forces.
- The proper microaeration strategy can improve the biodegradability of recalcitrant biomass using a mixture of inocula obtained from the effluent stream of biogas plant and a compost facility. Results from digestion trials and optimisation case study revealed an increase of 7.2% and 9.0%, respectively.
- Distinct differences were detected between firmly and loosely attached microorganisms. The archaeal community was majorly found in liquid fraction. Conversely, bacteria were identified also in the solid fraction of biogas reactors. Specifically, species similar to *C. thermocellum* and *C. proteolyticus* were predominantly bounded in digested samples.

- The bioaugmentation with *C. thermocellum* boosted remarkably the hydrolysis and subsequently, the methane production of wheat straw. The examined bioaugmentation method can be periodically applied in a full-scale biogas plants in order to alleviate solids accumulation.
- The cohabitation of inoculated hydrolytic strains with the indigenous AD microbiota was not fully succeeded. Microbes of the genus *Clostridium* slightly increased their relative abundance. Conversely, strains related to *M. roseus* were not detected in microbial samples.

7 Future perspectives

The present PhD study showed that biomethanation of lignocellulosic residues can be increased by the application of different treatment methods. To further improve the efficiency of the real-world AD processes the following points are suggested:

- Detailed life cycle assessment (LCA) and cost-benefit analysis of the integration of pretreatment during harvesting. Environmental impacts and economic balance need to be assessed in order to reveal the actual efficiency of lignocellulose based-AD process.
- Mathematical modelling to simulate the conducted co-digestion experiments in order to increase the AD performance. Subsequently, the optimal scenarios for full-scale implementations can be suggested with respect to critical process parameters (e.g. yields' improvement and instabilities' avoidance).
- Optimisation of bioaugmentation with *C. thermocellum* to define the minimum demanding amount of inoculated bacteria. Tests using either alternative pure cultures of cellulolytic strains or microbial consortium providing metabolic diversity and robustness are also needed. Moreover, different reactors configuration (e.g. two-stage CSTR) could improve the efficiency of bioaugmentation.
- Enzymes responsible for lignin degradation are oxygen dependent. Next-generation sequencing will give a deeper insight in the microbial community of micro-aerated AD reactors. Deeper knowledge on oxygen's role at the excretion of enzymes liable for augmented lignocellulose's depolymerization is demanded.

8 References

Ahring, B.K., Angelidaki, I., Johansen, K., 1992. Anaerobic treatment of manure together with industrial waste. Water Science and Technology 25, 311–318.

Angelidaki, I., Boe, K., Ellegaard, L., 2005. Effect of operating conditions and reactor configuration on efficiency of full-scale biogas plants. Water science and technology : a journal of the International Association on Water Pollution Research 52, 189–94.

Angelidaki, I., Ellegaard, L., Ahring, B.K., 1999. A comprehensive model of anaerobic bioconversion of complex substrates to biogas. Biotechnology and Bioengineering 63, 363–372.

Angelidaki, I., Karakashev, D., Batstone, D.J., Plugge, C.M., Stams, A.J.M., 2011. Biomethanation and its potential, 1st ed, Methods in Enzymology. Elsevier Inc.

Angelidaki, I., Sanders, W., 2004. Assessment of the anaerobic biodegradability of macropollutants. Reviews in Environmental Science and Bio/Technology 3, 117–129.

Angelidaki, I., Ahring, B.K., 2000. Methods for increasing the biogas potential from the recalcitrant organic matter contained in manure. Water science and technology : a journal of the International Association on Water Pollution Research 41, 189–94.

Azman, S., Khadem, A.F., Van Lier, J.B., Zeeman, G., Plugge, C.M., 2015. Presence and role of anaerobic hydrolytic microbes in conversion of lignocellulosic biomass for biogas production. Critical Reviews in Environmental Science and Technology 3389, 00–00.

Bassani, I., Kougias, P.G., Treu, L., Angelidaki, I., 2015. Biogas Upgrading via Hydrogenotrophic Methanogenesis in Two-Stage Continuous Stirred Tank Reactors at Mesophilic and Thermophilic Conditions. Environmental Science & Technology 151001074540007.

Batstone, D.J., Picioreanu, C., van Loosdrecht, M.C.M., 2006. Multidimensional modelling to investigate interspecies hydrogen transfer in anaerobic biofilms. Water Research 40, 3099–3108.

Biswas, R., Ahring, B.K., Uellendahl, H., 2012. Improving biogas yields using an innovative concept for conversion of the fiber fraction of manure. Water Science and Technology 66, 1751–1758.

Boscaro, D., Pezzuolo, A., Grigolato, S., Cavalli, R., Marinello, F., Sartori, L., 2015. Preliminary analysis on mowing and harvesting grass along riverbanks for the supply of anaerobic digestion plants in north-eastern Italy. Journal of Agricultural Engineering 46, 100–104.

Botheju, D., Bakke, R., 2011. Oxygen Effects in Anaerobic Digestion – A Review. The Open Waste Management Journal 4, 1–19.

British Petroleum, 2016. BP Statistical Review of World Energy, June 2016. London, England.

Brown, M.E., Chang, M.C.Y., 2014. Exploring bacterial lignin degradation. Current Opinion in Chemical Biology 19, 1–7.

Bruni, E., Jensen, A.P., Angelidaki, I., 2010. Comparative study of mechanical, hydrothermal, chemical and enzymatic treatments of digested biofibers to improve biogas production. Bioresource Technology 101, 8713–8717.

Buckel, W., 2001. Unusual enzymes involved in five pathways of glutamate fermentation. Applied Microbiology and Biotechnology 57, 263–273.

Campanaro, S., Treu, L., Kougias, P.G., De Francisci, D., Valle, G., Angelidaki, I., 2016. Metagenomic analysis and functional characterization of the biogas microbiome using high throughput shotgun sequencing and a novel binning strategy. Biotechnology for biofuels 9, 26.

Čater, M., Fanedl, L., Malovrh, S., Marinšek Logar, R., 2015. Biogas production from brewery spent grain enhanced by bioaugmentation with hydrolytic anaerobic bacteria. Bioresource Technology 186, 261–269.

Čater, M., Zorec, M., Marinšek Logar, R., 2014. Methods for Improving Anaerobic Lignocellulosic Substrates Degradation for Enhanced Biogas Production. Springer Science Reviews 2, 51–61.

Chandra, R., Takeuchi, H., Hasegawa, T., 2012a. Hydrothermal pretreatment of rice straw biomass: A potential and promising method for enhanced methane production. Applied Energy 94, 129–140.

Chandra, R., Takeuchi, H., Hasegawa, T., Kumar, R., 2012b. Improving biodegradability and biogas production of wheat straw substrates using sodium hydroxide and hydrothermal pretreatments. Energy 43, 273–282.

Chen, Y., Cheng, J.J., Creamer, K.S., 2008. Inhibition of anaerobic digestion process: a review. Bioresource technology 99, 4044–64.

Cherubini, F., 2010. The biorefinery concept: Using biomass instead of oil for producing energy and chemicals. Energy Conversion and Management 51, 1412–1421.

Cirne, D.G., Björnsson, L., Alves, M., Mattiasson, B., 2006. Effects of bioaugmentation by an anaerobic lipolytic bacterium on anaerobic digestion of lipid-rich waste. Journal of Chemical Technology & Biotechnology 81, 1745–1752.

Dandikas, V., Heuwinkel, H., Lichti, F., Drewes, J.E., Koch, K., 2014. Correlation between biogas yield and chemical composition of energy crops. Bioresource technology 174, 316–20.

Dandikas, V., Heuwinkel, H., Lichti, F., Drewes, J.E., Koch, K., 2015. Correlation between biogas yield and chemical composition of Grassland Plant Species. Energy & Fuels 29, 7221–7229.

De Francisci, D., Kougias, P.G., Treu, L., Campanaro, S., Angelidaki, I., 2015. Microbial diversity and dynamicity of biogas reactors due to radical changes of feedstock composition. Bioresource Technology 176, 56–64.

Drake, H.L., 1994. Acetogenesis, 1994. Chapman & Hall, New York.

Faostat, 2016. Food and Agriculture Organization of the United Nations - Statistical Databases. <http://faostat.fao.org>.

Fengel, D., Wegener, G., 1984. Wood: Chemistry, Ultrastructure, Reactions. De Gruyter, Berlin.

Fotidis, I.A., Karakashev, D., Kotsopoulos, T.A., Martzopoulos, G.G., Angelidaki, I., 2013. Effect of ammonium and acetate on methanogenic pathway and methanogenic community composition. FEMS microbiology ecology 83, 38–48.

Fotidis, I.A., Wang, H., Fiedel, N.R., Luo, G., Karakashev, D.B., Angelidaki, I., 2014. Bioaugmentation as a solution to increase methane production from an ammonia-rich substrate. Environmental Science and Technology 48, 7669–7676.

Fournier, G.P., Gogarten, J.P., 2008. Evolution of acetoclastic methanogenesis in Methanosarcina via horizontal gene transfer from cellulolytic Clostridia. Journal of Bacteriology 190, 1124–1127.

Frigon, J.C., Guiot, S.R., 2010. Biomethane production from starch and lignocellulosic crops: A comparative review. Biofuels, Bioproducts and Biorefining 4, 447–458.

Fu, S., Shi, X., Wang, F., Yuan, X., Guo, R.-B., 2015. Comparison of thermophilic microaerobic and alkali pretreatment of sugarcane bagasse for anaerobic digestion. RSC Adv. 5, 63903–63908.

Fu, S.F., Wang, F., Shi, X.S., Guo, R.B., 2016. Impacts of microaeration on the anaerobic digestion of corn straw and the microbial community structure. Chemical Engineering Journal 287, 523–528.

Gnansounou, E., Dauriat, A., 2010. Technoeconomic analysis of lignocellulosic ethanol: A review. Bioresource Technology 4980–4991.

Guerriero, G., Hausman, J.F., Strauss, J., Ertan, H., Siddiqui, K.S., 2016. Lignocellulosic biomass: Biosynthesis, degradation, and industrial utilization. Engineering in Life Sciences 16, 1–16.

Gunnarsson, C., Vågström, L., Hansson, P.A., 2008. Logistics for forage harvest to biogas production-Timeliness, capacities and costs in a Swedish case study. Biomass and Bioenergy 32, 1263–1273.

Hartmann, H., Angelidaki, I., Ahring, B.K., 2000. Increase of anaerobic degradation of particulate organic matter in full-scale biogas plants by mechanical maceration. Water science and technology : a journal of the International Association on Water Pollution Research 41, 145–53.

Hendriks, A.T.W.M., Zeeman, G., 2009. Pretreatments to enhance the digestibility of lignocellulosic biomass. Bioresource technology 100, 10–8.

Herrero, M., Stuckey, D.C., 2015. Bioaugmentation and its application in wastewater treatment: A review. Chemosphere 140, 119–128.

Herrmann, C., Prochnow, A., Heiermann, M., Idler, C., 2012a. Particle Size Reduction during Harvesting of Crop Feedstock for Biogas Production I: Effects on Ensiling Process and Methane Yields. Bioenergy Research 5, 926–936.

Herrmann, C., Prochnow, A., Heiermann, M., Idler, C., 2012b. Particle Size Reduction During Harvesting of Crop Feedstock for Biogas Production II: Effects on Energy Balance, Greenhouse Gas Emissions and Profitability. Bioenergy Research 5, 937–948.

Hidaka, T., Arai, S., Okamoto, S., Uchida, T., 2013. Anaerobic co-digestion of sewage sludge with shredded grass from public green spaces. Bioresource technology 130, 667–72.

IPCC, 2013. Climate Change: The Physical Science Basis. Geneva, Switzerland.

Jarrell, K.F., 1985. Extreme Oxygen Sensitivity in Methanogenic Archaebacteria. Bioscience 35, 298–302.

Jurado, M.M., Suarez-Estrella, F., Lopez, M.J., Vargas-Garcia, M.C., Lopez-Gonzalez, J.A., Moreno, J., 2015. Enhanced turnover of organic matter fractions by microbial stimulation during lignocellulosic waste composting. Bioresource Technology 186, 15–24.

Jørgensen, H., Kristensen, J.B., Felby, C., 2007. Enzymatic conversion of lignocellulose into fermentable sugars : challenges and opportunities 119–134.

Karakashev, D., Batstone, D.J., Trably, E., Angelidaki, I., 2006. Acetate oxidation is the dominant methanogenic pathway from acetate in the absence of Methanosaetaceae. Applied and Environmental Microbiology 72, 5138–5141.

Karakashev, D., Batstone, D.J., Angelidaki, I., 2005. Influence of Environmental Conditions on Methanogenic Compositions in Anaerobic Biogas Reactors Influence of Environmental Conditions on Methanogenic Compositions in Anaerobic Biogas Reactors. Applied and environmental microbiology 71, 331–338.

Kim, S.-H., Han, S.-K., Shin, H.-S., 2004. Kinetics of LCFA Inhibition on Acetoclastic Methanogenesis, Propionate Degradation and β-Oxidation. Journal of Environmental Science and Health, Part A 39, 1025–1037.

Koegel, R., Kraus, T., 1996. Intensive Forage Conditioning Increase Value 35–42.

Kougias, P.G., Treu, L., Campanaro, S., Zhu, X., Angelidaki, I., 2016. Dynamic functional characterization and phylogenetic changes due to Long Chain Fatty Acids pulses in biogas reactors. Scientific Reports 6, 1-10.

Kratky, L., Jirout, T., 2011. Biomass Size Reduction Machines for Enhancing Biogas Production. Chemical Engineering & Technology 34, 391–399.

Kumar, P., Barrett, D.M., Delwiche, M.J., Stroeve, P., 2009. Methods for Pretreatment of Lignocellulosic Biomass for Efficient Hydrolysis and Biofuel Production. Industrial & Engineering Chemistry Research 48, 3713–3729.

Lamed, R., Setter, E., Bayer, E.A., 1983. Characterization of a cellulose-binding, cellulase-containing complex in clostridium thermocellum. Journal of Bacteriology 156, 828–836.

Lamed, R.J., Lobos, J.H., Su, T.M., 1988. Effects of Stirring and Hydrogen on Fermentation Products of Clostridium thermocellum. Applied and environmental microbiology 54, 1216–1221.

Lehtomäki, A., Björnsson, L., 2006. Two-stage anaerobic digestion of energy crops: methane production, nitrogen mineralisation and heavy metal mobilisation. Environmental technology 27, 209–18.

Lesteur, M., Bellon-Maurel, V., Gonzalez, C., Latrille, E., Roger, J.M., Junqua, G., Steyer, J.P., 2010. Alternative methods for determining anaerobic biodegradability: A review. Process Biochemistry 45, 431–440.

Lim, J.W., Wang, J.Y., 2013. Enhanced hydrolysis and methane yield by applying microaeration pretreatment to the anaerobic co-digestion of brown water and food waste. Waste Management 33, 813–819.

Lindmark, J., Leksell, N., Schnürer, A., Thorin, E., 2012. Effects of mechanical pretreatment on the biogas yield from ley crop silage. Applied Energy 97, 498–502.

Lindner, J., Zielonka, S., Oechsner, H., Lemmer, A., 2015. Effects of mechanical treatment of digestate after anaerobic digestion on the degree of degradation. Bioresource technology 178, 194–200.

Luo, G., De Francisci, D., Kougias, P.G., Laura, T., Zhu, X., Angelidaki, I., 2015. New steady-state microbial community compositions and process performances in biogas reactors induced by temperature disturbances. Biotechnology for Biofuels 8, 1–10.

Luo, G., Fotidis, I.A., Angelidaki, I., 2016. Comparative analysis of taxonomic, functional, and metabolic patterns of microbiomes from 14 full-scale biogas reactors by metagenomic sequencing and radioisotopic analysis. Biotechnology for biofuels 9, 51.

Lü, F., Bize, A., Guillot, A., Monnet, V., Madigou, C., Chapleur, O., Mazeas, L., He, P., Bouchez, T., 2014a. Metaproteomics of cellulose methanisation under thermophilic conditions reveals a surprisingly high proteolytic activity. ISME J. 8, 88–102.

Lü, F., Li, T., Wang, T., Shao, L., He, P., 2014b. Improvement of sludge digestate biodegradability by thermophilic bioaugmentation. Applied Microbiology and Biotechnology 98, 969–977.

Martin-Ryals, A., Schideman, L., Li, P., Wilkinson, H., Wagner, R., 2015. Improving anaerobic digestion of a cellulosic waste via routine bioaugmentation with cellulolytic microorganisms. Bioresource Technology 189, 62–70.

Menardo, S., Airoldi, G., Balsari, P., 2012. The effect of particle size and thermal pre-treatment on the methane yield of four agricultural by-products. Bioresource Technology 104, 708–714.

Monlau, F., Barakat, A., Trably, E., Dumas, C., Steyer, J.-P., Carrère, H., 2013. Lignocellulosic Materials Into Biohydrogen and Biomethane: Impact of Structural Features and Pretreatment. Critical Reviews in Environmental Science and Technology 43, 260–322.

Mshandete, A., Bjornsson, L., Kivaisi, A.K., Rubindamayugi, S.T., Mattiasson, B., 2005. Enhancement of anaerobic batch digestion of sisal pulp waste by mesophilic aerobic pre-treatment. Water Research 39, 1569–1575.

Mutschlechner, M., Illmer, P., Wagner, A.O., 2015. Biological pre-treatment: Enhancing biogas production using the highly cellulolytic fungus Trichoderma viride. Waste Management 43, 98–107.

Mähnert, P., Heiermann, M., Linke, B., 2005. Batch- and Semi-continuous Biogas Production from Different Grass Species. CIGR E-Journal VII, 1–11.

Møller, H.B., Sommer, S.G., Ahring, B.K., 2004. Methane productivity of manure, straw and solid fractions of manure. Biomass and Bioenergy 26, 485–495.

Nielfa, A., Cano, R., Fdz-Polanco, M., 2015. Theoretical methane production generated by the co-digestion of organic fraction municipal solid waste and biological sludge. Biotechnology Reports 5, 14–21.

Nielsen, H.B., Mladenovska, Z., Ahring, B.K., 2007. Bioaugmentation of a Two-Stage Thermophilic (68 C/55C) Anaerobic Digestion Concept for Improvement of the Methane Yield From Cattle Manure. Biotechnology and bioengineering 97.

Peng, X., Börner, R.A., Nges, I.A., Liu, J., 2014. Impact of bioaugmentation on biochemical methane potential for wheat straw with addition of Clostridium cellulolyticum. Bioresource Technology 152, 567–571.

Pickett, J., Anderson, D., Bowles, D., Bridgwater, T., Jarvis, P., Mortimer, N., Poliakoff, M., Woods, J., 2008. Sustainable biofuels: prospects and challenges, The Royal Society. UK. London.

Podosokorskaya, O.A., Kadnikov, V. V., Gavrilov, S.N., Mardanov, A. V., Merkel, A.Y., Karnachuk, O. V., Ravin, N. V., Bonch-Osmolovskaya, E.A., Kublanov, I. V., 2013. Characterization of Melioribacter roseus gen. nov., sp. nov., a novel facultatively anaerobic thermophilic cellulolytic bacterium from the class Ignavibacteria, and a proposal of a novel bacterial phylum Ignavibacteriae. Environmental Microbiology 15, 1759–1771.

Procházka, J., Mrázek, J., Štrosová, L., Fliegerová, K., Zábranská, J., Dohányos, M., 2012. Enhanced biogas yield from energy crops with rumen anaerobic fungi. Engineering in Life Sciences 12, 343–351.

Raju, C.S., Ward, A.J., Nielsen, L., Møller, H.B., 2011. Comparison of near infra-red spectroscopy, neutral detergent fibre assay and in-vitro organic matter digestibility assay for rapid determination of the biochemical methane potential of meadow grasses. Bioresource technology 102, 7835–9.

Rodriguez, C., Alaswad, A., Benyounis, K.Y., Olabi, A.G., 2016. Pretreatment techniques used in biogas production from grass. Renewable and Sustainable Energy Reviews 1–12.

Rodriguez, J., Kleerebezem, R., Lema, J.M., Van Loosdrecht, M.C.M., 2006. Modeling product formation in anaerobic mixed culture fermentations. Biotechnology and Bioengineering 93, 592–606.

Rubin, E.M., 2008. Genomics of cellulosic biofuels. Nature 454, 841–5.

Sambusiti, C., Ficara, E., Malpei, F., Steyer, J.P., Carrère, H., 2013. Benefit of sodium hydroxide pretreatment of ensiled sorghum forage on the anaerobic reactor stability and methane production. Bioresource Technology 144, 149–155.

Samson, R., Lem, C.H., Stamler, S.B., Dooper, J., 2008. Developing energy crops for thermal applications: Optimizing fuel quality, energy security and GHG mitigation. Biofuels, Solar and Wind as Renewable Energy Systems: Benefits and Risks 395–423.

Sawatdeenarunat, C., Surendra, K.C., Takara, D., Oechsner, H., Khanal, S.K., 2015. Anaerobic digestion of lignocellulosic biomass: Challenges and opportunities. Bioresource Technology 178, 178–186.

Scherer, P., Neumann, L., 2013. "Methano-compost", a booster and restoring agent for thermophilic anaerobic digestion of energy crops. Biomass and Bioenergy 56, 471–478.

Schink, B., 1997. Energetics of syntrophic cooperation in methanogenic degradation. Microbiology and molecular biology reviews : MMBR 61, 262–280.

Schlüter, A., Bekel, T., Diaz, N.N., Dondrup, M., Eichenlaub, R., Gartemann, K.H., Krahn, I., Krause, L., Krömeke, H., Kruse, O., Mussgnug, J.H., Neuweger, H., Niehaus, K., Pühler, A., Runte, K.J., Szczepanowski, R., Tauch, A., Tilker, A., Viehöver, P., Goesmann, A., 2008. The metagenome of a biogas-producing microbial community of a production-scale biogas plant fermenter analysed by the 454-pyrosequencing technology. Journal of Biotechnology 136, 77–90.

Shimon, L.J.W., Pages, S., Belaich, A., Belaich, J.P., Bayer, E.A., Lamed, R., Shoham, Y., Frolow, F., 2000. Structure of a family IIIa scaffoldin CBD from the cellulosome of Clostridium cellulolyticum at 2.2 A resolution. Acta Crystallographica Section D: Biological Crystallography 56, 1560–1568.

Snell-Castro, R., Godon, J.J., Delgenès, J.P., Dabert, P., 2005. Characterisation of the microbial diversity in a pig manure storage pit using small subunit rDNA sequence analysis. FEMS Microbiology Ecology 52, 229–242.

Song, H., Clarke, W.P., Blackall, L.L., 2005. Concurrent microscopic observations and activity measurements of cellulose hydrolyzing and methanogenic populations during the batch anaerobic digestion of crystalline cellulose. Biotechnology and Bioengineering 91, 369–378.

Stams, A.J.M., 1994. Metabolic interactions between anaerobic bacteria in methanogenic environments. Antonie van Leeuwenhoek 66, 271–294.

Sträuber, H., Schröder, M., Kleinsteuber, S., 2012. Metabolic and microbial community dynamics during the hydrolytic and acidogenic fermentation in a leach-bed process. Energy, Sustainability and Society 2, 13.

Straathof, A.J.J., 2014. Transformation of biomass into commodity chemicals using enzymes or cells. Chemical Reviews 114, 1871–1908.

Søndergaard, M.M., Fotidis, I.A., Kovalovszki, A., Angelidaki, I., 2015. Anaerobic Co-digestion of Agricultural Byproducts with Manure for Enhanced Biogas Production. Energy & Fuels acs.energyfuels.5b02373.

Taherzadeh, M.J., Karimi, K., 2008. Pretreatment of lignocellulosic wastes to improve ethanol and biogas production: a review., International journal of molecular sciences.

Thamsiriroj, T., Nizami, A.S., Murphy, J.D., 2012. Why does mono-digestion of grass silage fail in long term operation? Applied Energy 95, 64–76.

Thauer, R.K., Jungermann, K., Decker, K., 1977. Energy conservation in chemotrophic anaerobic bacteria. Bacteriological reviews 41, 100–180.

Treu, L., Campanaro, S., Kougias, P.G., Zhu, X., Angelidaki, I., 2016a. Untangling the Effect of Fatty Acid Addition at Species Level Revealed Different Transcriptional Responses of the Biogas Microbial Community Members. Environmental Science and Technology 50, 6079–6090.

Treu, L., Kougias, P.G., Campanaro, S., Bassani, I., Angelidaki, I., 2016b. Deeper insight into the structure of the anaerobic digestion microbial community; the biogas microbiome database is expanded with 157 new genomes. Bioresource Technology 216, 260–266.

Triolo, J.M., Sommer, S.G., Møller, H.B., Weisbjerg, M.R., Jiang, X.Y., 2011. A new algorithm to characterize biodegradability of biomass during anaerobic digestion: influence of lignin concentration on methane production potential. Bioresource technology 102, 9395–402.

Triolo, J.M., Ward, A.J., Pedersen, L., Løkke, M.M., Qu, H., Sommer, S.G., 2014. Near Infrared Reflectance Spectroscopy (NIRS) for rapid determination of biochemical methane potential of plant biomass. Applied Energy 116, 52–57.

Tuan, N.N., Chang, Y.C., Yu, C.P., Huang, S.L., 2014. Multiple approaches to characterize the microbial community in a thermophilic anaerobic digester running on swine manure: A case study. Microbiological Research 169, 717–724.

Wahid, R., Ward, A.J., Møller, H.B., Søegaard, K., Eriksen, J., 2015. Biogas potential from forbs and grass-clover mixture with the application of near infrared spectroscopy. Bioresource Technology 198, 124–132.

Wall, D.M., Straccialini, B., Allen, E., Nolan, P., Herrmann, C., O'Kiely, P., Murphy, J.D., 2015. Investigation of effect of particle size and rumen fluid addition on specific methane yields of high lignocellulose grass silage. Bioresource Technology 192, 266–271.

Wang, H., Vuorela, M., Keranen, A.L., Lehtinen, T.M., Lensu, A., Lehtomaki, A., Rintala, J., 2010. Development of microbial populations in the anaerobic hydrolysis of grass silage for methane production. FEMS Microbiology Ecology 72, 496–506.

Wirth, R., Kovács, E., Maróti, G., Bagi, Z., Rákhely, G., Kovács, K.L., 2012. Characterization of a biogas-producing microbial community by short-read next generation DNA sequencing. Biotechnology for Biofuels 5, 41.

Yangin-Gomec, C., Ozturk, I., 2013. Effect of maize silage addition on biomethane recovery from mesophilic co-digestion of chicken and cattle manure to suppress ammonia inhibition. Energy Conversion and Management 71, 92–100.

Zeng, Y., Zhao, S., Yang, S., Ding, S.-Y., 2014. Lignin plays a negative role in the biochemical process for producing lignocellulosic biofuels. Current Opinion in Biotechnology 27, 38–45.

Zheng, Y., Zhao, J., Xu, F., Li, Y., 2014. Pretreatment of lignocellulosic biomass for enhanced biogas production. Progress in Energy and Combustion Science 42, 35–53.

Zinder, S.H., Koch, M., 1984. Non-aceticlastic methanogenesis from acetate: acetate oxidation by a thermophilic syntrophic coculture. Archives of Microbiology 138, 263–272.

9 Papers

I Tsapekos, P., Kougias, P.G., Angelidaki, I., 2015. Biogas production from ensiled meadow grass; effect of mechanical pretreatments and rapid determination of substrate biodegradability via physicochemical methods. Bioresource Technology 182, 329–335.

II Tsapekos, P., Kougias, P.G., Angelidaki, I., 2015. Anaerobic Mono- and Co-digestion of Mechanically Pretreated Meadow Grass for Biogas Production. Energy & Fuels 29, 4005–4010

III Tsapekos, P., Kougias, P.G., Frison, A., Raga, R., Angelidaki, I., 2016. Improving methane production from digested manure biofibers by mechanical and thermal alkaline pretreatment. Bioresource Technology 216, 545–552.

IV Tsapekos, P., Kougias, P.G., Treu, L., Campanaro, S., Angelidaki. I., 2017. Process performance and comparative metagenomic analysis during co-digestion of manure and lignocellulosic biomass for biogas production. Applied Energy 185, 126–135.

V Tsapekos, P., Kougias, P.G., Larsen, U., Pedersen, J., Trénel, P., Angelidaki. I., Mechanical pretreatment at harvesting increases the bioenergy output from marginal land grasses. Submitted to Renewable Energy. September 06, 2016

VI Tsapekos, P., Kougias, P.G., Larsen, U., Pedersen, J., Trénel, P., Angelidaki. I., 2016. Improving the energy balance of grass-based anaerobic digestion through combined harvesting and pretreatment. Anaerobe. doi: org/10.1016/j.anaerobe.2016.12.00505

VII Tsapekos, P., Kougias, P.G., Vasileiou, S.A., Lyberatos, G., and Angelidaki. I., 2017. Effect of microaeration and inoculum type on the biodegradation of lignocellulosic substrate. Bioresource Technology 225, 246–253

VIII Tsapekos, P., Kougias, P.G., Vasileiou, S.A., Treu, L., Campanaro, S., Lyberatos, G., and Angelidaki. I., Bioaugmentation with hydrolytic microbes to improve the anaerobic biodegradability of lignocellulosic agricultural residues. Submitted to Water Research. December 02, 2016

In this online version of the thesis, **paper I-VIII** are not included but can be obtained from electronic article databases e.g. via www.orbit.dtu.dk or on request from.

DTU Environment
Technical University of Denmark
Miljoevej, Building 113
2800 Kgs. Lyngby
Denmark

info@env.dtu.dk.

The Department of Environmental Engineering (DTU Environment) conducts science based engineering research within six sections: Water Resources Engineering, Water Technology, Urban Water Systems, Residual Resource Engineering, Environmental Chemistry and Atmospheric Environment.

The department dates back to 1865, when Ludvig August Colding, the founder of the department, gave the first lecture on sanitary engineering as response to the cholera epidemics in Copenhagen in the late

Department of Environmental Engineering
Technical University of Denmark

DTU Environment
Bygningstorvet, building 115
2800 Kgs. Lyngby
Tlf. +45 4525 1600
Fax +45 4593 2850

www.env.dtu.dk