Thesis for the Degree of Doctor of Philosophy

Bioprocessing of Recalcitrant Substrates for Biogas Production

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Abstract

The application of anaerobic digestion (AD) as a sustainable waste management technology is growing worldwide, due to high energy prices as well as increasingly strict environmental regulations. The growth of the AD industry necessitates exploring new substrates for their utilisation in AD processes. The present work investigates the AD of two recalcitrant biomass: lignocelluloses and keratin-rich residues. The complex nature of these waste streams limits their biological degradation; therefore, suitable pre-processing is required prior to the AD process.

In the first part of the study, the effects of organic solvent pre-treatments on bioconversion of lignocelluloses (straw and forest residues) to biogas were evaluated. Pre-treatment with N-methylmorpholine-N-oxide (NMMO) resulted in minor changes in the composition of the substrates, while their digestibility significantly increased. Furthermore, due to the high cost of the NNMO, the effect of pre-treatment with the recycled solvent was also explored. Since it was found that the presence of small traces of NMMO in the system after the treatment has inhibitory effects on AD, pre-treatments of forest residues using other organic solvents, *i.e.* acetic acid, ethanol, and methanol, were investigated too. Although pre-treatments with acetic acid and ethanol led to the highest methane yields, the techno-economical evaluation of the process showed that pre-treatment with methanol was the most viable economically, primarily due to the lower cost of methanol, compared to that of the other solvents.

In the second part of the work, wool textile wastes were subjected to biogas production. Wool is mainly composed of keratin, an extremely strong and resistible structural protein. Thermal, enzymatic and combined treatments were, therefore, performed to enhance the methane yield. The soluble protein content of the pre-treated samples showed that combined thermal and enzymatic treatments had significantly positive effects on wool degradation, resulting in the highest methane yields, *i.e.* 10–20-fold higher methane production, compared to that obtained from the untreated samples.

In the last part of this thesis work, dry digestion of wheat straw and wool textile waste, as well as their co-digestion were studied. The total solid (TS) contents applied in the digesters were between 6–30% during the investigations. The volumetric methane productivity was significantly enhanced when the TS was increased from 6 to 13–21%. This can be a beneficial factor when considering the economic feasibility of large-scale dry AD processes.

Keywords: anaerobic digestion, biogas, lignocellulose, wool, keratin, pre-treatment, co-digestion, dry digestion, economic evaluation

LIST OF PUBLICATIONS

This thesis is based on the results presented in the following articles:

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II. **Kabir, M.M.,** Niklasson, C., Taherzadeh, M.J., Sárvári-Horváth, I. 2014. 'Biogas production from lignocelluloses by N-methylmorpholine-N-oxide (NMMO) pre-treatment: Effects of recovery and reuse of NMMO.' ***Bioresource Technology*, 161(0), 446-450.**

III. **Kabir, M.M**., Forgács, G. Sárvári Horváth, I. (2013). 'Enhanced methane production from wool textile residues by thermal and enzymatic pre-treatment.' **Process Biochemistry 48(4): 575-580.**

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Statement of Contribution

Paper I: Responsible for all the experimental work and data analyses, preparation and organisation of the manuscript, and its revision.

Paper II: Responsible for all the experimental work, data analyses, writing of the manuscript and its revision.

Paper III: Responsible for all of the experimental work, data analyses, writing of the manuscript and its revision.

Paper IV: Responsible for all of the experimental design, laboratory work, data analyses, writing of the manuscript and its revision.

Paper V: Responsible for all the experimental work, data analyses, parts of writing of the manuscript and its revision regarding the experimental data.

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NOMENCLATURE

AD	Anaerobic Digestion
AFEX	Ammonia Fibre Explosion
C/N	Carbon Nitrogen ratio
CBM	Carbohydrate Binding Module
CHP	Combined Heat and Power plant
CI	Crystallinity Index
COD	Chemical Oxygen Demand
CSTR	Continuously Stirred Tank Reactor
EBA	European Biogas Association
FTIR	Fourier Transform Infrared Spectroscopy
GHG	Greenhouse Gas
HRT	Hydraulic Retention Time
MSW	Municipal Solid Waste
NMMO	N-methylmorpholine-N-oxide
NPV	Net Present Value
OFMSW	Organic Fraction of Municipal Solid Waste
PBP	Payback Period
ROI	Return On Investment
TS	Total Solids
TW1	Textile Waste 1
TW2	Textile Waste 2
VFA	Volatile Fatty Acid
VS	Volatile Solids

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CHAPTER 1

INTRODUCTION

Waste generation is an inevitable part of human life and is increasing considerably as a result of rapid growth of population, urbanisation and industrialisation. In parallel with the waste generated, open dumping and unsophisticated land filling of solid waste has become a major concern, especially in developing countries, which can lead to significant environmental and health consequences, such as foul odours, climate change and epidemic diseases [1].

Additionally, the growing global energy demand and limited availability of fossil fuels, together with increasing energy prices, necessitate the use of renewable energies. According to the target set by the European Commission in 2011, 20% of the total energy demand and particularly 10% of the energy consumed within the transport sector must be produced from renewable resources by year 2020 [2]. Commercially renewable vehicle fuels available today include: ethanol, biogas, biodiesel and electricity produced from renewable energy sources.

Among the technologies producing these renewable fuels, biogas production via anaerobic digestion (AD) was the main focus in this thesis. Biogas is a versatile energy source, as it can be used for different purposes. The most common use of biogas, especially from small-scale plants in developing countries such as China and India, is to provide heat for cooking. It is assessed that 1 m^3 biogas with composition of 60% methane has a heating value of 21.5 MJ [3].

There is no doubt that the role of biogas in the European energy portfolio is progressively growing. The most dominant use of biogas in most countries, such as in Germany, the UK and Denmark, is its utilisation in combined heat and power (CHP) plants. However, in Sweden, the majority of the biogas produced is used as a source of transport fuel [4]. To use biogas for transportation purposes, or for natural gas grid injection, biogas needs to be upgraded and cleaned to achieve a methane content higher than 97% [5, 6]. Injecting the biomethane into the natural gas grid allows the methane storage for later use as transport fuel

or for heat and electricity generation at times of peak demand. Furthermore, the digestate residue remaining at the end of the process can be applied as a nutrient rich fertilizer in the agricultural sector [7].

Numerous types of feedstock can be used for the production of biogas, such as animal manure, slurries, crop residues, organic wastes from dairy production, food industries and agro-industries, wastewater sludge, organic fraction of municipal solid wastes, etc. One of the benefits of AD is its ability to treat both wet (moisture content higher than 60–70% such as sewage sludge, animal slurries) and dry biomass (with moisture content of less than 20%, including lignocellulosic biomass, agro-wastes, energy crops and textile wastes), applying two different technologies called wet and dry anaerobic digestion.

In the present study, the feasibility of two difficult to degrade waste streams, lignocellulosic biomass and keratin based residues, being used as feedstock for AD processes were explored. Due to their complex and rigid recalcitrant structure, efficient biogas production is hindered from these kinds of residues. Therefore, different pre-treatment strategies were applied and the subsequent effects on the biodegradability of these materials were investigated. Additionally, due to the naturally low moisture content of these residues, the application of dry AD technology was also examined.

1.1 Research journey

As a PhD student, the early stage of my research was started with comprehensive literature review on possible pre-treatment methods, which can be applied on the complex structure of biomass recalcitrance. Since a suitable pre-treatment method that claims the economic viability of the bioconversion of recalcitrant biomass for methane production has not yet been identified, the main objective of the present study was to explore appropriate pre-processing methods for effective conversion of difficult to degrade biomass to biogas. The first part of this study was focused on improving the yield of AD process from lignocellulosic biomass (*i.e.* forest residues, and barley straw) using a cellulosic solvent, N-methylmorpholine-N-oxide (NMMO) (**Papers I and II**) for the pre-treatment. Different process parameters and pre-treatment conditions were investigated. In terms of methane production, the results were promising; moreover, it was also revealed that this pre-treatment method could successfully disrupt the cellulose network without loss of biomass or unwanted carbohydrate degradation (**Paper I**). The most important economic factor related to this unique pre-treatment technique is the cost of the NMMO; therefore, the research was continued on examining the

effectiveness of this solvent on the final yield of methane after recycling and reuse (**Paper II**). The results of the pre-treatment with recycled NMMO showed that the effect of the recycled NMMO on lignocelluloses is solely dependent on the nature and the chemical composition of the substrate; thereby, NMMO can act differently when it is introduced to different substrates. Additionally, compositional and structural analysis of lignocelluloses before and after pre-treatment with fresh versus recycled NMMO was also determined (**Paper II**). After the NMMO project and the problems found related to it, attempt was made to find other possibly more effective solutions. This time, forest residues as the challenging biomass with high lignin fraction was chosen, and pre-treatment methods using low molecular weight alcohols and acids were selected (**Paper V**). The results were significant in terms of increasing the biogas yield. In addition, the problems associated with the previous pre-treatment technique such as the necessity of recycling the solvent for further use was not an issue. The next step was to perform a techno-economic study based on the results of the lab scale experiments, aiming to investigate the feasibility of a large scale industrial process including both the pre-treatment and the anaerobic digestion steps together with taking into the account the upgrading and utilisation of the produced biogas as well as the digestate residue (**Paper V**).

The second part of this work dealt with the bioconversion of wool textile waste, a keratin-rich waste fraction, to biogas. Keratin is a structural protein; therefore, the wool textile residues were pre-treated using suitable pre-treatments breaking down proteins, *i.e.* thermal and/or enzymatic pre-treatments, prior to biogas production (**Paper III**). The effects of the pre-treatments on two different wool textile residues were examined, and the effectiveness of the applied pre-treatment methods was evaluated by measuring the soluble protein and sCOD content in the produced hydrolysates, as well as the accumulated methane yields during the subsequent AD assays.

Lastly, dry anaerobic digestion of lignocellulose- and keratin-rich waste fractions and the co-digestion of these two were investigated (**Paper IV**). The idea behind this project was to explore the potential of dry AD system for the utilisation of these two residues, taking advantage of their naturally high solid contents. The aim of this study was to evaluate the differences in the performance of anaerobic fermentation of wheat straw and wool textile waste in conventional wet digestion system, compared to dry anaerobic digestion. To be able to improve the bioconversion of these highly complex biomasses, the effects of appropriate enzyme as well as nutrients supply were also studied. The enzymes or nutrients were added to the system at the start-up of the AD batch digestion assays, performed at different TS contents of between 6–30%, hence without the application of an additional pre-treatment step. The

compositional and structural analysis of these feedstocks before and after the degradation process was also studied (**Paper IV**).

CHAPTER 2

OVERVIEW OF ANAEROBIC DIGESTION SYSTEM AND ITS FEEDSTOCK

2.1 Global biogas driving force and market

Over the last few decades, the increasing energy demand worldwide led to the utilisation of massive quantities of fossil fuels (80% of todays energy demand) or increasingly relying on nuclear power which are extensively unsustainable and self-damaging [8]. For these reasons, sustainable energy sources are becoming much more of a necessity than a mere alternative.

The production of biogas through anaerobic digestion (AD) of organic waste is widely implemented today, as it offers an opportunity to deal with the reduction of large amounts of organic waste, while diminishing their environmental impact and thereby facilitating a sustainable development of energy supply [8, 9].

Biogas industry is already a well-known and reliable technology in Europe and Asia [10]. Compared to the combustion of fossil fuels, which accounted for 94% of the carbon dioxide emitted to the atmosphere in 2009, the utilisation of biogas from renewable sources in controlled conditions can significantly reduce the greenhouse gas (GHG) emissions [11].

The estimated trend on biogas market worldwide between 2012 and 2022 is presented in Figure 2.1. In 2012, the biogas production amounted to 17,200 kilo tonnes of oil equivalent (ktoe) per year; from this amount, 60% of the production is made by Europe (about 10,500 ktoe per year). The contribution from North America was limited to about 22%, followed by contributions made by the Asia-Pacific (11%), Latin America (6%) and the Middle East-Africa (about 1.0%). However, according to the National Renewable European Action Plans (NREAP), biogas production in Europe in 2020 is estimated to extend to approximately 28,000 ktoe per year [12, 13]. There is also a significant growth estimated for the Asia-Pacific area, while minor increases are predicted for Latin America and the Middle East.

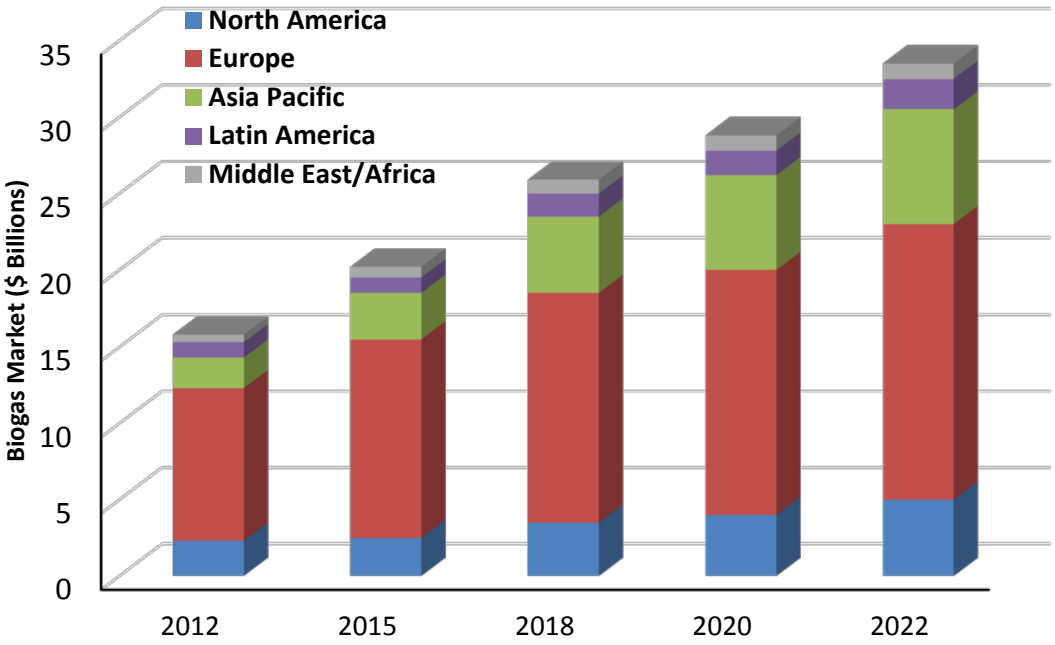

Figure 2.1. Biogas production at 2012 and the estimated trend until 2022 in different areas of the world (Adapted from [14]).

2.2. Biochemical reaction chain of the AD process

AD is a microbiological degradation process of organic compounds under anaerobic conditions. The main products of this process are biogas and digestate residue. Biogas mainly consists of methane and carbon dioxide, while the digestate residue is a nutrient-rich by-product containing the decomposed substrate, the microorganisms and other remaining products of the process.

The formation of biogas is a result of several interdependent, complex, sequential and parallel biological reactions, in which the products generated by one group of microorganisms serve as substrates for the next group [15]. Generally, the decomposition of the organic matter in AD process proceeds in four successive steps − hydrolysis, acidogenesis, acetogenesis and methanogenesis − as shown in Figure 2.2. However, the initial process flow as well as the degradation pathways depends on the nature of the organic substrate fed to the AD system. The microorganisms involved in this process are bacteria and archaea, which partially have a syntrophic relation to each other with different requirements on the environment [16].

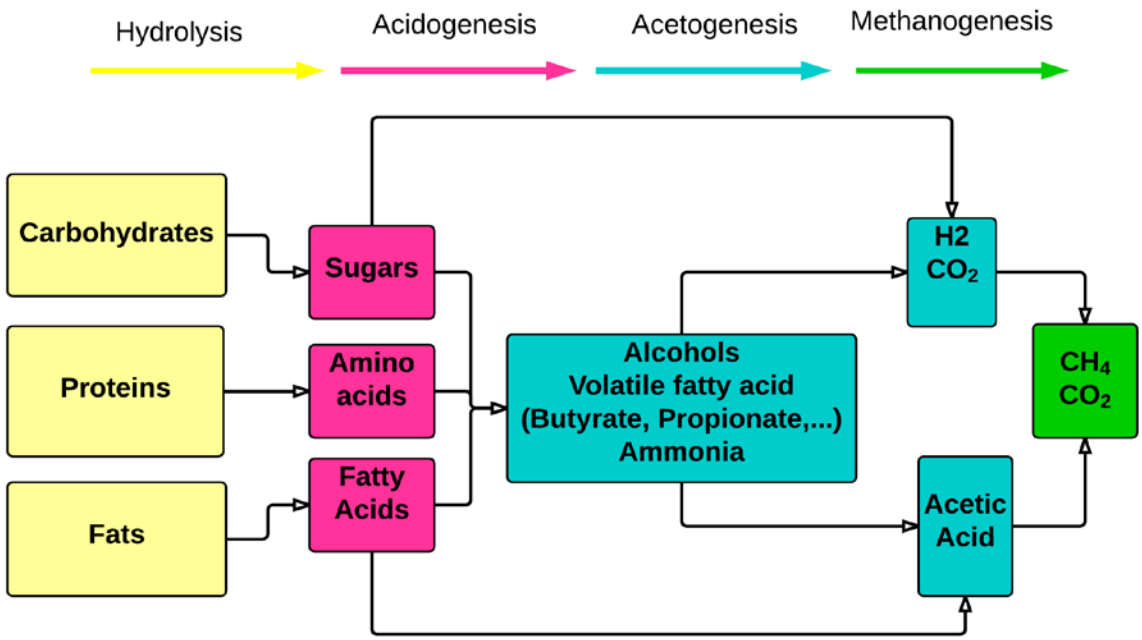

Figure 2.2. Series of biochemical reactions within the anaerobic digestion process (Adapted from [17]).

Hydrolysis

Hydrolysis is the first stage in the anaerobic digestion process, which includes the action of extra-cellular enzymes mediating the transformation of the particulate organic matter into solubilised monomers [18]. During hydrolysis, carbohydrates, proteins and lipids are disintegrated into simple molecules, such as monosaccharides, amino acids and fatty acids, respectively. The enzymes involved in this process are cellulases, proteases and lipases excreted by facultative anaerobes [19, 20]. The kinetics of the total decomposition process is determined by the slowest reaction within the chain. Therefore, if the digester is fed with difficult to degrade substrates, the hydrolysis acts as the rate-limiting step [21]. This is not due to deficiency of enzyme activity but to the limited availability of accessible surface area of the feedstock for the enzymes.

Acidogenesis

The monomers and the soluble oligomers formed in the hydrolytic phase are diffused into the bacterial cells and subsequently converted into other intermediate fermentation products. The products of acidogenesis are: volatile fatty acids (VFAs), such as valeric acid, butyric acid, propionic acid, acetic acid, formic acid, as well as hydrogen and alcohols. The acidogensis is

usually considered as the fastest conversion step in the AD chain [20]. This is mainly due to the higher microbial growth rates and higher product yields, compared to those involved in the methanogenesis step. Hence, if the digester is overloaded, the reactor might suffer from souring (sudden pH drop), due to the accumulation of VFAs. In this case, when the alkalinity is consumed by the acids, a pH drop will occur, which consequently will cause a severe inhibition for the methanogens [18].

Acetogenesis

The intermediate products of acidogenesis, which cannot be directly converted into methane by the methanogens (*i.e.* acetate and hydrogen), are further converted into methanogenic substrates in the acetogenic step by the action of the acetogens. In this step, VFAs and alcohols are oxidised into acetate and hydrogen, which might result in an increase of the hydrogen partial pressure. It is known that acetogenesis reactions are only thermodynamically feasible if the pressure of the hydrogen is low (*i.e.* lower than 10^{-5} bar). In the presence of a higher hydrogen pressure, the metabolism of the acetogenic bacteria will be inhibited. Therefore, a symbiotic relationship between acetogens (hydrogen producers) and hydrogenotrophic methanogens (hydrogen consumers) is of vital importance to regulate the hydrogen partial pressure in the digester [22, 23]. In a balanced anaerobic digester, with low partial pressure of hydrogen, the main degradation pathway results in formation of acetate, carbon dioxide and hydrogen. This degradation pathway is more favourable since it gives a higher energy yield for the microorganisms, and furthermore, the methanogenic microorganisms can directly consume these products.

Methanogenesis

The production of methane and carbon dioxide is carried out by methanogenic archaea using the intermediate products from the acidogenic and acetogenic reactions. Generally, the majority (two-thirds) of the formed methane originates through the acetoclastic pathway, where the two carbons of the acetate will be split: one will be oxidised to carbon dioxide and the other will be reduced to methane ($CH_3COOH \rightarrow CH_4 + CO_2$) [24, 25]. The remaining (one-third) part of the methane is produced from the conversion of H_2 and CO_2 through the hydrogenotrophic pathway [26, 27].

Methanogenesis is the slowest biochemical reaction within the anaerobic digestion process. Compared to bacteria, methanogenic archaea are known to be the most sensitive group of AD; hence, their activity is severely affected by operation conditions. Therefore, the

composition of feedstock, organic loading rate, temperature, ammonia and VFA concentration, as well as pH and alkalinity need to be controlled to maintain the balance within the degradation steps. Furthermore, methanogens have the longest generation time in the digester, *i.e.* between 5–15 days, compared to acid formers with doubled times of 1–1.5 days, which is why this step is usually considered as the rate-limiting stage for easily hydrolysed materials [28].

2.2.1 Parameters affecting biogas production

Anaerobic digestion is highly influenced by various factors including, feedstock characteristics, reactor design and operational and environmental conditions. Among the environmental conditions: pH, temperature and availability of nutrients and among operational parameters, the organic loading rate and the hydraulic and solids retention time, are the vital parameters. Therefore, as it was mentioned earlier, to maintain optimum conditions and maintain a high efficiency within the process, these parameters should be efficiently controlled [29].

2.3 Feedstock for AD

A wide range of feedstock can be fed to AD digesters. Historically, AD has been developed to treat liquid waste streams, such as industrial wastewater, sewage and sludge from biological or physical-chemical treatments of wastewater. However, the possibility of utilisation of organic solid wastes such as agricultural and municipal solid waste (MSW) started to draw attention during the 60s, because of their high organic matter content and high methane potential [30]. Some examples of substrates from agricultural, industrial and municipal sources are among others, manure, energy crops, algal biomass, food processing waste, slaughterhouse waste and organic fraction of municipal solid waste (OFMSW).

The properties of the substrate have a significant effect on the AD process, in terms of the quantity and the quality of the produced biogas, and ultimately the quality of the digestate residue [31]. The different components of organic wastes, such as lipids, proteins and carbohydrates, have different energy contents, thereby, producing varying amounts of gas with different methane contents [31]. Table 2.1 shows the methane yields of some usual substrates utilised in AD processes.

Based on the general chemical formulas for carbohydrates ($C_6H_{10}O_5$), proteins ($C_5H_7O_2N$) and lipids ($C_{57}H_{104}O_6$), the expected theoretical methane potential is 0.42, 0.50 and 1.01 Nm^3

CH_4/kg VS, respectively [32]. Furthermore, the CH_4:CO_2 ratio in the produced biogas from carbohydrates, proteins and fats are 50:50, 60:40 and 70:30, respectively [33].

Table 2.1. Potential methane yields from different feedstock (Adapted from [31]).

Substrate	**Methane yield (Nm^3/ton VS)**
Municipal solid waste	180–350
Food waste	400–800
Slaughterhouse waste	700
Sugar beets	300–800
Grass	200–400
Straw	100–320
Manure (cattle, pigs or chicken)	100–300

Since AD is a biological degradation process, the feedstock should contain all the nutritional requirements for the microorganisms working in the digester, in terms of energy source and other compounds that are vital for the activity of the microbial enzyme systems and for the optimum growth of the cells. The carbon/nitrogen (C/N) ratio of the substrate is therefore of great importance. A high C/N ratio results in a decrease in process efficiency, as the bacteria may suffer from nitrogen deficiency, while low C/N ratio can lead to ammonia inhibition.

The C/N ratio of the substrate can be adjusted by co-digestion of different substrate fractions or by the addition of an external carbon or nitrogen source. Therefore, the performance of AD system can be significantly enhanced using feedstock from different sources with “right” proportions of carbohydrates, fats and proteins [34]. Feeding the digester with diverse substrates taken from various sources amplifies the growth of a more diverse microbiological consortium, resulting in a more robust and stable process. Additionally, during co-digestion of different substrates, usually higher gas production is obtained than it is expected based on the gas production potential of the individual substrates [35, 36]. On the other hand, feeding the digester with a uniform composition substrate for a longer period will ultimately result in build-up of a consortium of microorganisms, which will cause difficulties in degradation of substrates from other sources.

2.3.1 Recalcitrance features of biomass

An effective conversion of organic substrates into biogas depends highly on their chemical and structural properties. Structural polymers, such as proteins occurring in nails, hair, wool and feathers, as well as cellulose presented in the cell wall of plants, are all equipped with either physical or chemical defence mechanisms against degradation. These sophisticated and integrated defence systems are naturally designed to protect the living organisms against mechanical stresses or microbial degradations, and they are known as biomass recalcitrance.

Considering the chemical composition of carbohydrate rich substrates such as lignocelluloses (carbohydrates content counts up to ≈ 50–80% of the dry weight) and/or keratin rich materials like feather and wool (protein content counts up to ≈90% of the dry weight), their theoretical methane potentials are expected to be high. In contrast, the rate of digestion is practically very low and inefficient in these cases. The reason for this low yield is not related to the inhibition caused by degradation products, but rather due to the low rate of hydrolysis. Therefore, it can be concluded that substrates with substantially lower yield, compared to that theoretically expected based on their chemical composition, can be considered as recalcitrant substrates. These kinds of feedstock are hardly hydrolysed by anaerobic microorganisms in their natural state; hence, they would need additional pre-processing prior to anaerobic digestion. For these reasons, this group of biomass is not commonly utilised in anaerobic digestion processes for energy production.

Structural proteins such as keratin in wool, hair, nail and feathers are not yet considered as a feedstock for large scale AD processes, and there is a scarce research on their hydrolysis efficiency so far. Therefore, new investigations are still needed on how the amino acid degradation is carried out in biogas production from keratin rich substrates. On the other hand, there are many research results available on bioconversion of lingocelluloses for bio-fuel production, mainly on pre-treatment and enzymatic saccharfication of the cellulose fraction prior to ethanol production. These studies are complemented with investigations focusing on the utilisation of lignocelluloses for biogas production. However, there are still many questions unanswered regarding the efficient degradation of these widely available biomass streams for biogas production.

In this thesis, forest residues and straw, among the lignocellulosic materials, and wool textile residues, as an example of keratin rich substrate, were chosen to be studied for biogas production.

2.3.2 Approaching the challenges of biomass recalcitrance

For all these complex polymers, the hydrolysis step is regarded as the rate-limiting step within the AD process [37, 38]. In order to improve the enzymatic hydrolysis, it is necessary to overcome the complex chemical and morphological network of these polymers. The pre-treatment of recalcitrant biomass is one of the possibilities to solve this obstacle, since a suitable pre-treatment will increase the accessible surface area of these macromolecules to provide a physical contact to the enzymes [38]. The available pre-treatment methods are usually classified into three main categories: chemical, physical and biological methods. These methods can be applied individually to improve the digestibility, or can be combined with each other, such as physicochemical pre-treatments, aiming to achieve more profound results. However, the suitability and success of different pre-treatments should be evaluated by comparing the yield and rates of the targeted products (the produced methane in the case of AD), with the costs and energy demand needed for the pre-treatment. It is also important to control the material balance for the organic materials over the pre-treatment, as well as the formation of inhibitory by-products, which could affect the subsequent fermentation process [39, 40].

Physical pre-treatment

Physical pre-treatments by means of size reduction are performed to increase the accessible surface area for the degrading enzymes and to reduce the degree of polymerisation of the biomass. However, a physical treatment is often not enough for an efficient enzymatic hydrolysis. Therefore, physical pre-treatments are generally applied prior to chemical or biological pre-treatments [41]. Grinding or milling, extrusion, irradiation, using microwaves and ultrasonication are among the common physical pre-treatment methods. The physical pre-treatments positively affect the efficiency of the process; however, they often negatively affect the process economy because of their high energy demand.

Biological pre-treatment

Biological pre-treatments are based on employing the natural ability of microorganisms or enzymes for degradation of biomass recalcitrance. Biological pre-treatments of lignocelluloses are usually aimed at delignification by the action of wood decay fungi. For degradation of feather or wool, keratinases produced by keratinolytic microorganisms are mostly applied. Biological pre-treatments are considered to be economically beneficial due to

their low energy and cost input; however, their main disadvantages compared to other pre-treatment technologies are the low rate of hydrolysis and long incubation times [42].

Chemical pre-treatment

Chemical pre-treatments involve chemical reactions modifying the structure of complex biomass. Chemical pre-treatments include treatments using alkali, acid, oxidising agents and organic solvents. Alkaline pre-treatments, using sodium hydroxide or calcium hydroxide, can be applied for the pre-treatment of a wide range of substrates, including both lignocelluloses and keratin-rich waste fractions. Alkaline pre-treatments on lignocelluloses are highly effective in lignin removal with removal of small percentage of acetyl groups from hemicelluloses and with minor cellulose solubilisation [43, 44]. The effectiveness of this process depends on the lignin content of the biomass. The alkaline pre-treatment on keratin aims at disrupting the peptide bonds, primary amide bonds and also the cysteine disulphide bridges within the complex keratin structure.

Acid pre-treatments applied on lignocelluloses enhance cellulose digestibility by solubilisation of the hemicellulose fraction. The acid pre-treatments can be performed with concentrated or diluted acids, and they have been investigated on a wide range of lignocellulosic feedstock. Even though the use of concentrated acids, such as sulphuric or nitric acids, allows efficient hydrolysis of hemicellulose and cellulose, the high operational and maintenance costs due to equipment corrosion problems and formation of inhibitory compounds make these treatments less favourable for biogas and bioethanol production [45].
Pre-treatments with oxidising agents using, for example, hydrogen peroxide (H_2O_2) or ozone, affect lignocellulosic biomass in similar manner as alkaline pre-treatments, since they can break down the lignin. However, in some cases the oxidant may cause cellulose and hemicellulose losses [46].

Organic solvent pre-treatments include solvents with low boiling points (such as methanol and ethanol), and high boiling points (ethylene glycol and glycerol), ethers, ketones and phenols [47]. Pre-treatment with ethanol is one of the most common methods for the fractionation of lignocelluloses. The disadvantage of this pre-treatment is the high volatility of the solvents. This pre-treatment must therefore be performed in extremely tight vessels. In this thesis, organic solvents such as N-methylmorpholine-N-oxide (NMMO), ethanol, methanol and acetic acids were used for the pre-treatment of forest residues and barley straw prior to anaerobic digestion (**Papers I, II** and **V**).

2.4 Types of anaerobic digesters

Anaerobic digester is an airtight tank, where the degradation of organic matter occurs and where the biogas is produced. There are many types of anaerobic digesters, operating around the world. Nevertheless, all the digesters can be classified and compared based on the biological and technical performance and characteristics:

- Reactor configuration based on the feedstock flow (*i.e.* batch vs continuous operation)
- Solid content of the feedstock (*i.e.* wet vs dry digestion)

2.4.1 Batch and continuous type reactors

In the batch-type digesters, the fresh feedstock is loaded at once at the start-up and removed when the digestion process is completed. The material stays in the digester throughout the entire digestion period; no new fresh substrate is added and no digestate residue is taken out during the process. Batch-type digesters are easy to build and are common for use in dry digestion. The biogas production is usually highest at the beginning of the digestion and decreases as the feedstock is degraded until the end of the process. A long lag phase at the beginning of the process might be observed, which is associated with the adaptation of the microbial community to the feedstock entered into the system [48].

In continuous digesters, the feed is charged and the digestate residue is discharged continuously or semi-continuously, providing a steady state to be reached in the reactor with a continuous biogas production. The continuously fed reactors are typically used when the feedstock is a liquid or for substrates with low viscosity, so that they can be pumped for continuous feeding. Otherwise, a semi-continuous process is used with a discrete amount of feed at regular intervals. Continuous reactors usually have higher operation and maintenance costs than batch reactors.

2.4.2 Wet and dry anaerobic digestion

Based on the TS content of the organic waste, the AD system can operate in wet digestion mode, *i.e.* when the average dry matter content (DM) is lower than 15% and in more dry digestion, *i.e.* when the DM content is higher than 15%, usually between 20–40%. Wet anaerobic digestion systems have been successfully applied to treat various ranges of low solid content wastes, such as sewage sludge and food industry effluents: nevertheless, the requirement of a relatively large digester volume, high capacity of wastewater treatment

facilities, and high heat and energy demands are some of the challenges associated with this technology. The most commonly used reactor configuration for wet anaerobic digestion is a continuously stirred tank reactor (CSTR) [10].

In dry digestion, due to the high solid content (25–40% TS) in the digester, the technical approach regarding the waste treatment is fundamentally different from those applied in the wet systems [49]. Because of the high viscosity of the feedstock used in dry digestion systems, the heat and nutrient transfer is not as efficient as it is in the wet AD process. Hence, mixing is of vital importance, as it guarantees sufficient contact between the microorganisms and the substrate throughout the digester, and it also prevents local overloading and acidifications [50, 51]. However, the conventional mechanical mixers that are commonly used in wet CSTR reactors are not suitable in dry digesters; instead, mixing is performed by the recirculation of the percolation liquid or by biogas injection back to the reactor [50]. A well-known example of dry digestion reactor is a so-called “garage type” model, which includes a series of digesters operating in batch mode. The reactors are usually made of concrete and the fresh substrate is mixed with the digestate residue and thereby inoculated before it is fed to the digester. Continuous recirculation of the percolation liquid is performed by spraying the liquid digestate over the digestion bed in the digester.

CHAPTER 3

LIGNOCELLULOSES AS SUBSTRATES FOR ANAEROBIC DIGESTION

3.1 Lignocellulosic materials and their availability

Lignocellulosic materials are abundant worldwide. Lignocelluloses have been estimated to account for up to approximately 50% of the biomass worldwide, with a production of about 200 billion tonnes per year [52, 53]. From an environmental perspective, lignocellulosic residuals are more favourable for the production of advanced biofuels, as they do not compete with land usage for food and feed production. According to the Scientific Committee of the European Environment Agency (EEA), only using additional plant growth or residues as a source of energy is contributing to carbon sequestration [54].

In this thesis, two different lignocellulosic waste fractions were studied as feedstock for AD processes and are discussed further in this chapter.

3.1.1 Forest and wood processing residues

Forest residuals refer to the remaining branches, leaves, tops, needles, bark, roots and damaged or unwanted stems after tree logging. Approximately 60% of the biomass from the harvested tree is left in the forest after logging. Furthermore, log squaring results in an additional 30–50% biomass waste, and sawing the wood logs generates an additional 8–10% biomass waste after logging [55]. Forest residues in Sweden is estimated to have an energy potential of between 49 to 59 TWh/year [56, 57]. Today, these residues are often recovered in the form of wood chips and used in production of particleboard, fibreboard or used as fuel [55]. However, a high energy potential (20 TWh/year) lies in the tops, branches, roots/stumps, which are currently not utilised [56]. This high abundance of forest residues makes them attractive, yet challenging feedstock for the AD process.

3.1.2 Agricultural residues

The agricultural residues mainly consist of different grains and straws, such as rice, wheat, barley and rye straw. Recently, the Biomass Futures project was initiated, which is a large European research project to estimate the bioenergy potential of biomass. According to its prediction, the energy potential of straw produced in EU will be 49.3 Mtoe from estimated 127 million tonnes in 2020 [58]. This calculation includes straw residues of barley, wheat, rye, oats and other cereals. Figure 3.1 presents the estimated biomass availability in 2020; as evident, the main potential is from wheat straw.

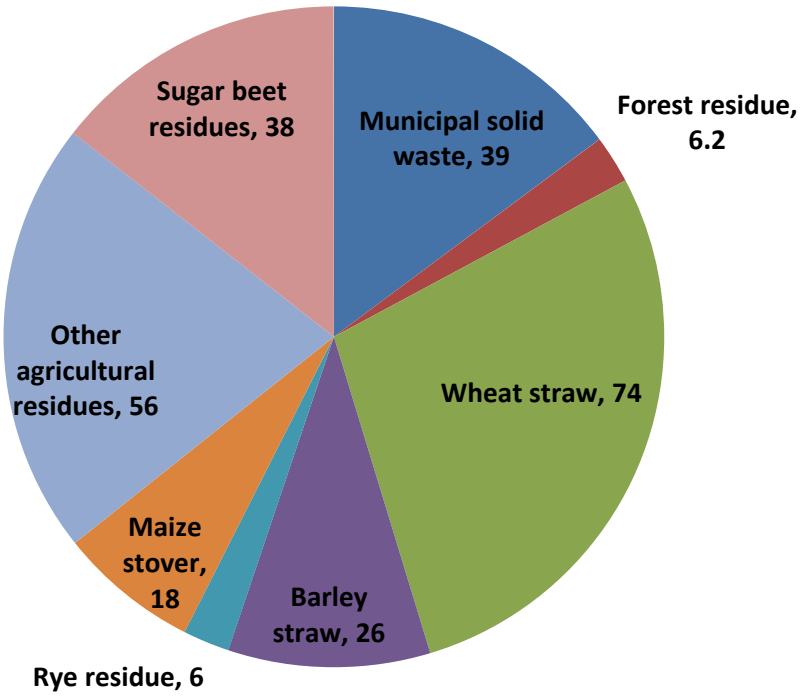

Figure 3.1. Availability of biomass residues in 2020, in million tonnes (Adapted from [59]).

3.2 Structure and composition of lignocellulosic materials

Lignocelluloses are naturally designed composites that play a vital role in survival of plants. As the plant cell grows, multilayer cell walls are formed around its plasma membrane, which serve as protection layers for the plant cell against mechanical stresses as well as chemical and microbial degradation [60]. The cell wall structure in most of the plants includes a primary and a secondary cell wall. The primary cell wall is created during the cell growth and

provides mechanical strength but also flexibility to the plant. When the plant growth is stopped, a secondary wall is constructed inside the primary wall, which is a thicker and stronger layer. The secondary cell wall is often divided into three sub layers (S1, S2 and S3). Furthermore, there is an empty chamber called lumen found in the cell wall structure of dead cells, surrounded by secondary and primary walls. Lumen was a place for cell organelles when the cell was alive [61] (Figure 3.2). The main components of the cell wall are: cellulose, hemicellulose, lignin, and small amounts of proteins, pectin and other minerals.

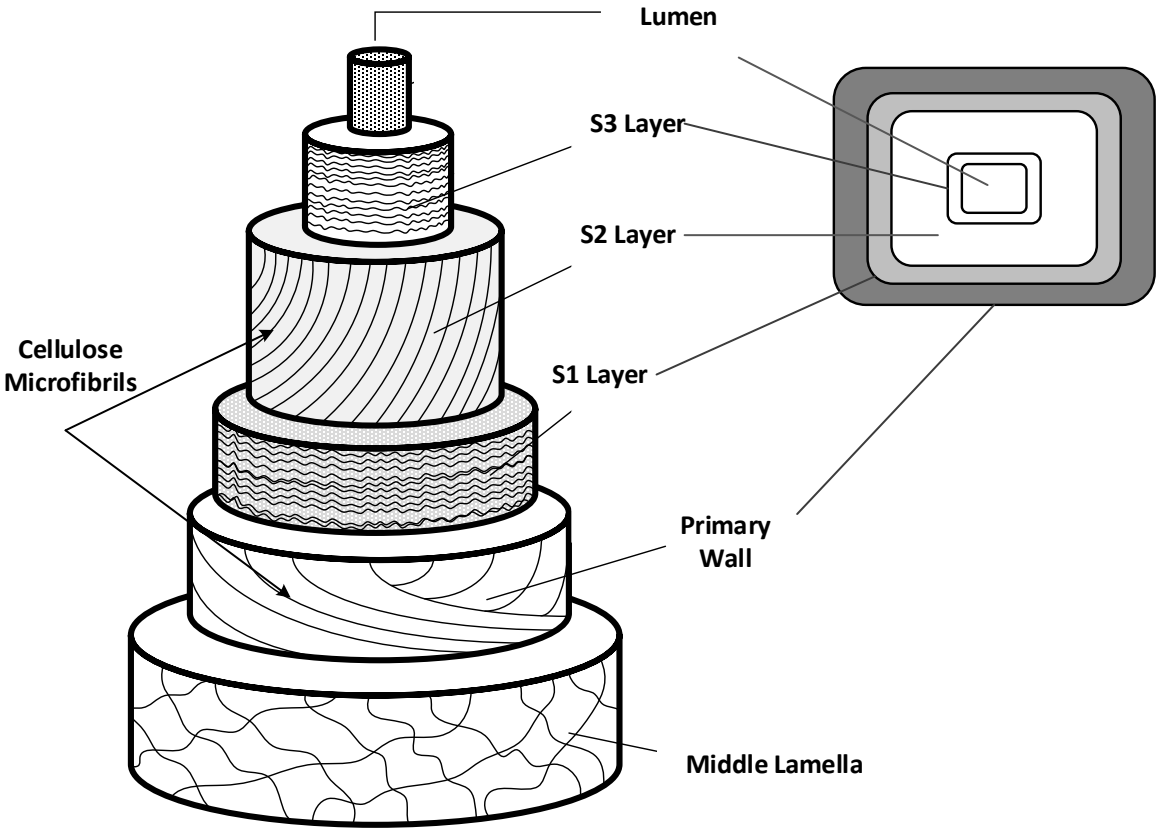

Figure 3.2. Three-dimensional sketch of the plant cell wall (Adapted from [62]).

Cellulose

Cellulose $(C_6H_{10}O_5)_n$ is the main component of lignocellulosic biomass that constitutes 20–40% of the plant cell wall. Cellulose is a linear polymer of D-glucose with β-1,4-glycosidic bonds. In the cellulose chain, cellobiose, which is a dimer of two glucose units, is a repeating unit of cellulose where two neighbouring glucose molecules are twisted 180° relative to each other. The hydroxyl groups in each glucose molecule can form both intra- and inter- molecular hydrogen bonds that are responsible for stabilisation and crystallinity of

cellulose [63, 64]. There are three hydroxyl groups present in each repeating unit of cellulose, as shown in Figure 3.3. Therefore, the inner and outer surfaces of cellulose are covered by OH-groups, which are able to make hydrogen bonds between the cellulose chains [65]. The inter-chain hydrogen bonding between the hydroxyl groups and the oxygen of the neighbouring ring molecules strengthens the linkage and forms the linear configuration of the cellulose chain (shown in Figure 3.3) [64, 66].

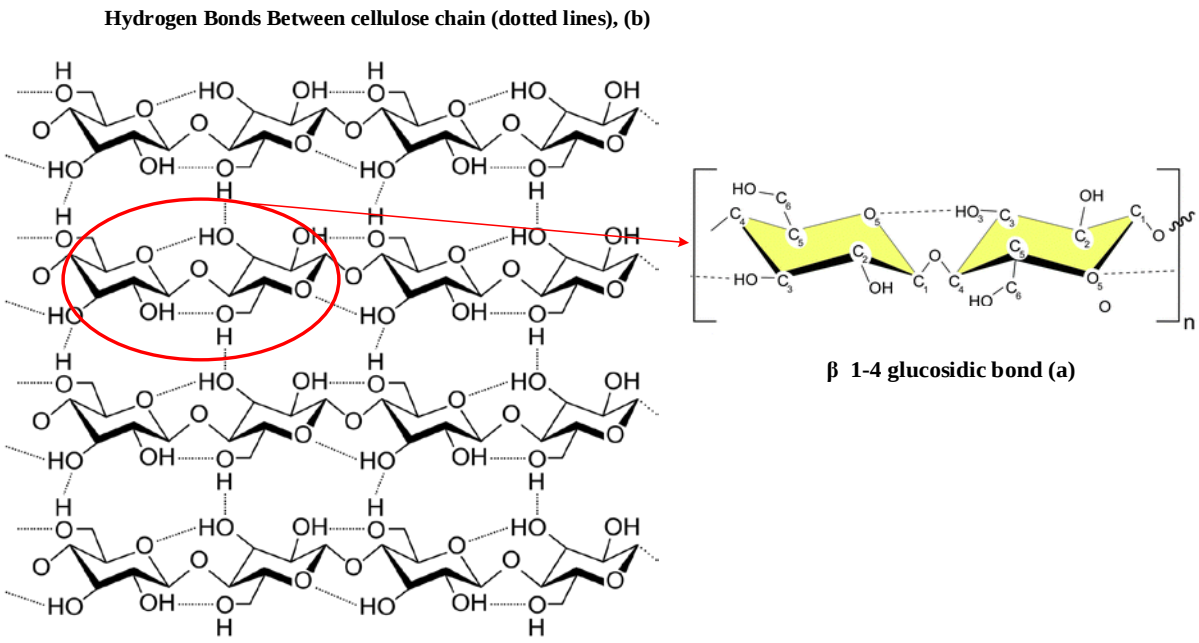

Figure 3.3. Molecular arrangement of a single cellulose unit, showing the β-1,4-glucosidic bond (a) and intra-chain hydrogen bonding between cellulose chains (dotted line) (b). (Adapted from [66]).

Intermolecular hydrogen bonds along with van der Waals forces provide the aggregation of the cellulose chains and cellulose fibrils. The aggregation of the cellulose chains, through different parts of the hydrogen bonds between and within strands, can form different cellulose crystals, such as Cellulose I, II, III_I, III_{II}, IV_I and IV_{II}. Natural cellulose has a crystalline formation of Cellulose I, which has two coexisting allomorphs: Cellulose I_α and I_β. These two polymorphic forms of cellulose differ mainly regarding their hydrogen-bonded pattern. The biological origin of the cellulose has an influence on the proportions of coexisting Cellulose I_α and I_β. The cell wall of bacteria and algae mainly contains Cellulose I_α, whereas the cell wall of higher plants like trees contains a higher proportion of Cellulose I_β [67, 68]. The process of

swelling of cellulose occurs by the forming of H-bonds between the OH groups of water and the free OH-groups of cellulose, which are not linked to each other [69].

Hemicellulose

Hemicelluloses are heteropolymers of polysaccharides and polyuronides, and they can be found in almost all lignocellulosic materials, comprising approximately 20–35% of the dry weight. The polysaccharide part of hemicelluloses consists of different monosaccharides, a mixture of pentosans (xylose and arabinose) and hexosans (glucose, mannose and galactose) [70, 71]. The dominant monomeric sugar found in hemicelluloses of softwoods is mannose, which is highly acetylated and has galactose side groups. Whereas the dominant monosaccharide of hemicelluloses in hardwoods and agricultural residues is xylose, which is less acetylated and contains arabinose side groups [72]. The polyuronides of the hemicellulose contain hexuronic acids and methoxyl, acetyl and free carboxylic residues. Polyuronides are found to be more sensitive to chemical and biological attacks than polysaccharides [70, 73]. Compared to the crystalline structure of cellulose, which is highly recalcitrant to hydrolysis, hemicelluloses have a more amorphous arrangement with short chain polymers as side chains [74, 75]. Hence, hemicelluloses can be hydrolysed and degraded easier in AD processes [76].

Lignin

Lignin is the second most abundantly present natural polymer after cellulose [77]. This is a very complex polymer that contributes to the structural support of the plant and reinforces the strength of the crystalline cellulose in cell wall layers, thus, supports the plant to grow higher and higher [65]. It has a highly hydrophobic character that facilitates the transmission of water and nutrients within the plant. In addition, it acts as a barrier shield against pathogenic microbial attacks as well as chemical and enzymatic degradation [78]. It was found that lignin seems to limit cellulose hydrolysis by two different mechanisms, *i.e.* by forming a physical barrier that inhibits the access of enzymes and by binding to the cellulolytic enzymes thereby reducing their activity. The lignin content in plant species varies enormously. Lignin makes up about 25–40% of softwoods, while 18–25% of the hardwoods, and the content of lignin decreases in grasses and other agricultural residues to 10–20% of the dry weight [69, 73]. In addition to the amount of lignin present in different species, there are major differences in monomeric units and linkage types within lignin found in different plants.

Other components

Usually more than 80% of the dry weight of lignocellulosic biomass consists of cellulose, hemicellulose and lignin. The remaining part is referred to a group, known as extraneous materials. Extraneous materials can be extracted by means of polar and nonpolar solvents; however, based on their solubility in water, they can be classified into two categories: *i.e.* extractives or non-extractives [79]. The major composition of extractives are resins (fats, fatty acids, resin acids and phytosterols), terpenes (isoprene alcohols and ketones) and phenols (residue and by-products of lignin biosynthesis) [69, 79].

The non-extractives are the inorganic components, including alkali earth carbonates, oxalates as well as organic components, like starches, pectins and proteins. Proteins are among the most important fractions of non-extractives, amounting to 2–10% of the cell wall. In some types of grasses and particularly in rice straw, a significant level of non-extractive silica crystals has been identified [79].

The extraneous fraction of lignocellulosic biomass is not considered to play a major role in bioconversion of lignocelluloses for biofuel production, and there is a lack of studies related to this faction and its effect on pre-treatment processes [80].

3.3 Lignocellulose degradation

3.3.1 Microbial degradation of cellulose and hemicellulose (Cellulosome)

Microorganisms use different approaches for degradation of cellulose in aerobic and anaerobic conditions. In aerobic conditions, microbes produce cellulases in high concentrations that act synergistically to hydrolyse cellulose. The cellulose degradation by cellulases is based on the action of three types of enzymes, including: endoglucanases, exoglucanases and β-glucosidase [81, 82]. The function of endoglucanases is to randomly break the internal β-1,4-glucosidic bonds within the cellulose chain, creating polysaccharides of varying length and thus new chain ends, while exoglucanases are responsible for cleaving cellobiose units from the end of the cellulose polysaccharide chains. Finally, the β-glucosidase hydrolyses the cellobiose units into glucose [83, 84]. The rate-limiting factor in cellulose hydrolysis is due to the difficulty for endoglucanases to reach amorphous regions within the crystalline matrix of cellulose aiming to create new chain ends, where exo-glucanases can attack. A similar enzyme system is required for the hydrolysis of hemicelluloses; however, other enzymes, rather than cellulases, are required for their

complete degradation. For instance, hemicelluloses from hardwood, *i.e.* branched acetyl xylan can be degraded by several different enzymes, including: β-xylosidase, α-glucuronidase, a-Larabinofuranosidase and acetylesterase. On the other hand, for complete hydrolysis of galactoglucomannans constituting hemicelluloses in softwood, the following enzymes are required: endo 1-4 b-mannanase, b-mannosidase, β-glucosidase and α-galactosidase [77].

In anaerobic conditions, the microorganisms develop a more energy conserving mechanism by producing a highly efficient multi-functional enzyme complex, called cellulosome, for cellulose degradation. A schematic picture of the cellulosome is shown on Figure 3.4. It has a non-catalytic subunit, referred to as scaffoldin subunit, which enables various catalytic enzymes to attach to the cellulosome [85].

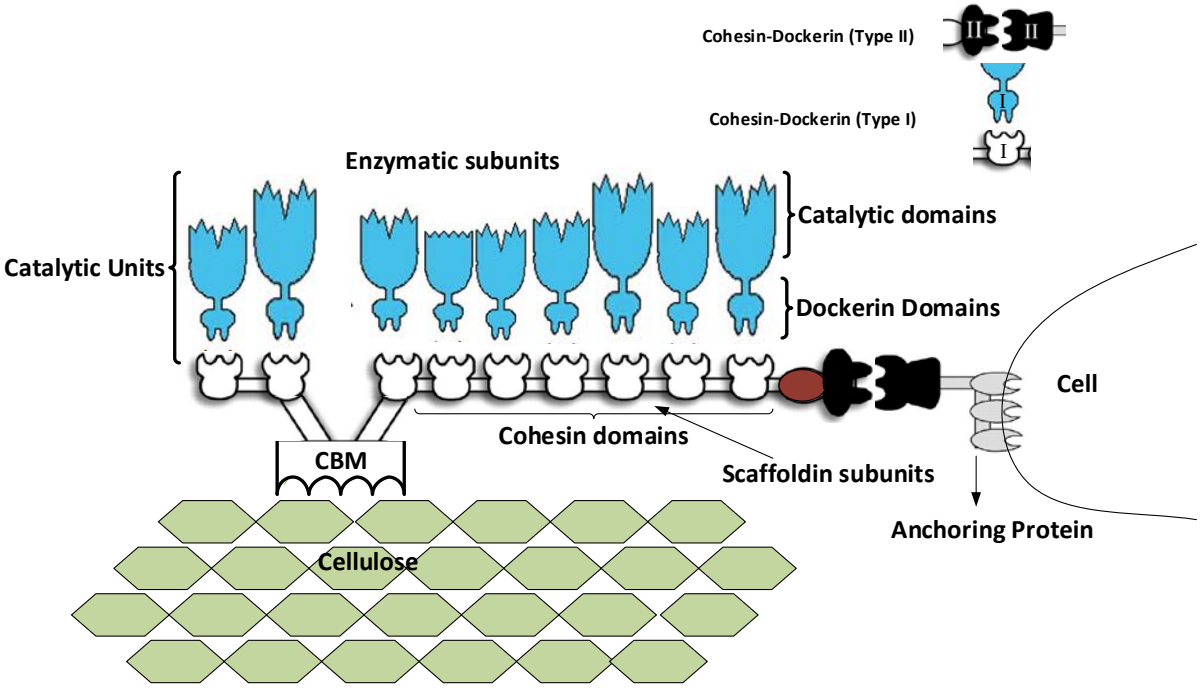

Figure 3.4. The complex structure of cellulosome (Adapted from [86]).

The enzymes are aligned on the scaffoldin via cohesin-dockerin (type I) interactions. The catalytic enzymes (hemicellulases, cellulases and pectinases) are linked to different dockerins (Figure 3.4). The scaffoldin also has a cellulose specific carbohydrate binding module (CBM) that is a linkage for the cellulosome to attach to the substrate (Figure 3.4) [82]. Anchoring proteins mediate the attachment of the scaffoldin subunit to the microorganism, providing a close proximity of the microbial cell to the polysaccharide. During the cellulosome-cellulose interaction, the CBM forms strong binding with the glucose chain on the hydrophobic face of the cellulose surface [87]. The CBM is able to bind to both highly crystalline cellulose and to

amorphous cellulose. However, the binding capacity to the amorphous cellulose is higher than that of the crystalline cellulose; therefore, CBM has a better accessibility to binding sites on amorphous cellulose [88].

3.3.2 Microbial degradation of lignin

Lignin is known to be the most recalcitrant constituent of the plant cell wall. As it was discussed earlier, the effect of lignin on biodegradability of cellulose and hemicelluloses is related to its role as a physical barrier, since the presence of lignin molecules will significantly decrease the available surface area for enzymatic penetration [89]. Therefore, the lignin content and the biodegradability of the substrate are inversely proportional.

The enzymes of white rot fungi are able to act on carbohydrates (cellulose and hemicellulose) and lignin in wood. Lignin degradation is mostly an aerobic process; thus, in anaerobic conditions, the lignin remains intact for a very long period of time [90]. Lignin degradation is a complex process and the enzymes involved possibly have synergistic effects on each other [91]. In lignin degradation, the extracellular oxidative enzymes of white rot fungi are initially involved. Ligninase and manganese peroxidase belong to the class of peroxidases, which oxidise their substrates by two sequential one-electron oxidation phases with intermediate cation radical formation. Ring-cleavage of aromatic rings is a key step of lignin mineralisation [77]. Environmental conditions, which favour the lignin biodegradation by white-rot fungi are: adequate nitrogen level, moisture, temperature, as well as the composition of the lignocellulosic substrate itself [77, 92].

The other lignin degrading enzymes are superoxide dismutase and glyoxal oxidase. These enzymes work together with peroxidases, and they never attack wood on their own. Superoxide dismutase reacts as a reducing or oxidising agent with other radicals produced by ligninolytic enzymes, contributing to further lignin transformation processes, such as aromatic ring cleavage [91] or demethoxylation [77, 93].

3.4 The importance of pre-treatment for degradation of lignocellulosic materials

The combined effects of lignin shields around the cellulose fibres, crystallinity of the cellulose and the lack of accessible surface area form a very recalcitrant structure inhibiting microbial and enzymatic degradation [94]. Therefore, pre-treatment is needed for the disruption of this naturally recalcitrance structure of plant cells aiming to improve their biological conversion in subsequent bioprocessing to produce bioethanol, biogas, lactic acid

or animal feed. The pre-treatment process will enhance the enzymatic or microbial degradation of lignocellulosic biomass by affecting the physicochemical properties of this substrate, such as molecular size, cellulose crystallinity, degree of acetylation of hemicellulose, surface accessibility, pore size distribution and biomass swelling capacity [46, 80].

3.4.1 Pre-treatment with a cellulose solvent, NMMO, and its challenges

N-methylmorpholine-N-oxide (NMMO) is a cyclic amine oxide, known as a cellulose solvent. This solvent has been industrially used for the production of regenerated cellulosic fibres with different commercial names, such as lyocell and newcell, since the 1990s [34, 95] However, research on using NMMO for pre-treatment of lignocellusic biomass was first conducted in 2009 [96]. Previously, it was found that the NMMO concentration had a key role in the extent of cellulose dissolution. Depending on the concentration of NMMO, there are different modes of action on cellulose fibres which have been identified [9, 97]:

1) Cellulose dissolution without major swelling (83–87% of NMMO)
2) Swelling and ballooning without dissolution (73–79% of NMMO)
3) Low homogenous swelling and no dissolution (below 65% of NMMO)

The results of **Papers I** and **II** in this thesis were obtained by the application of NMMO pre-treatment on forest residues and barley straw in two different modes of action, *i.e*. with NMMO concentrations of 75 and 85% (w/w %) at 120 and 90°C, for 3–30 hours. The pre-treated substrates were then subjected to subsequent anaerobic fermentation.

In **Paper I**, the effects of NMMO (75 and 85%) pre-treatment on different particle sizes (2, 4 and 8 mm) of forest residues at 120°C, and duration times of 3, 7 and 15h prior to AD were investigated. The results showed that the highest methane yields were obtained after applying the NMMO pre-treatment on 2 mm particle size. This is due to the fact that decreasing the particle size will increase the accessible surface area, which consequently results in better performance of enzymatic degradation [41, 98].

The study of the NMMO pre-treatment in two different modes, *i.e.* cellulose dissolution (85%) and swelling without dissolution (75%) (**Paper I**) showed that the dissolution mode is more effective in enhancing the final anaerobic digestion yields (Figure 3.5). The reason for this observation is the different mechanisms for changes in the structure of highly crystalline cellulose during the two different modes of NMMO pre-treatment. In cellulose swelling mode, the overall structure of the cellulose remains intact, and the physical changes occur only due to an increase in the sample volume caused by the uptake of NMMO [99]. On the

other hand, during the dissolution mode, the hydrogen bonds between the cellulose chain molecules might break and the van der Waals forces get weakened. During the cellulose regeneration process, when water is added as an anti-solvent, the NMMO can form hydrogen bonds with the water molecules setting the cellulose chain free. The cellulose chains will therefore be free to move and form a less crystalline arrangement, compared to their original native state. As a result, an inter-conversion of the cellulose polymorph from Cellulose I to Cellulose II will occur. Cellulose II is found to have less crystalline properties; thus, its accessibility for cellulolytic enzymes is higher [97]. It is worth mentioning that the process of cellulose transformation from form I into form II is irreversible; hence, Cellulose II will represent a more stable structure of cellulose, compared to Cellulose I.

When it comes to the effect of the duration time for the pre-treatment (3–15 h), it was shown that the 3-hour pre-treatment did not effectively increase the methane production of forest residues (**Paper I**). However, prolongation of the pre-treatment time to 7h could significantly improve the methane yield. Nevertheless, there were no significant differences between the methane yields obtained from 2mm forest residues pre-treated with 85% NMMO during 7 or 15 h, respectively (Figure 3.5). Since the long pre-treatment time might be a barrier for the economic viability of the process, selecting the shorter pre-treatment time with the same effect on digestibility would therefore be favourable.

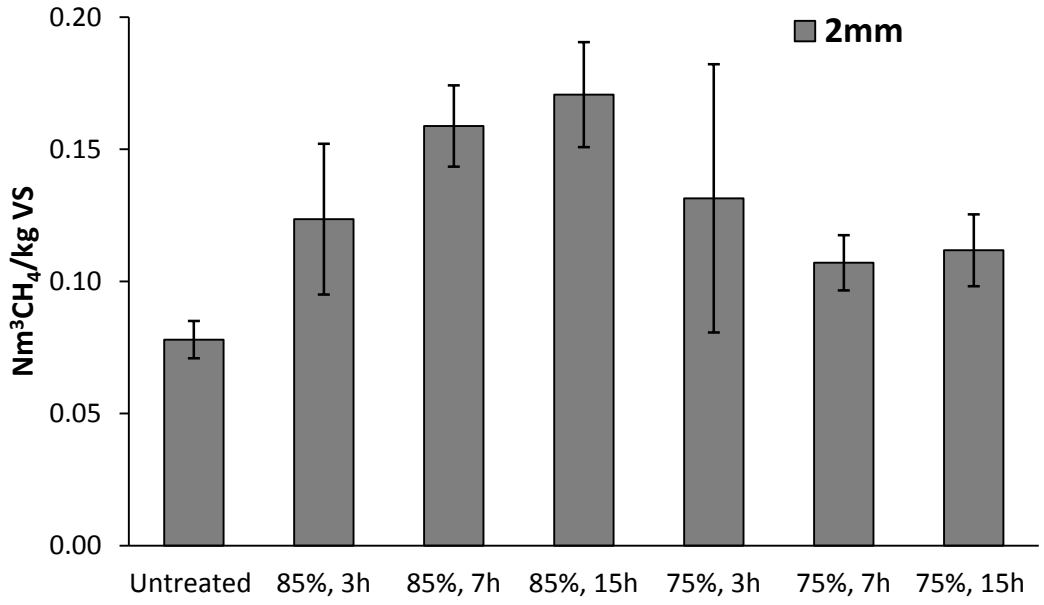

Figure 3.5. Accumulated methane yields obtained during 50 days of anaerobic digestion of untreated vs NMMO–pre-treated forest residues (2mm particle size), expressed as Nm^3CH_4/kg VS. Error bars represent ±1 standard deviations.

The energy consumption during the pre-treatment unquestionably has a key role in the economy of the whole process. For that reason, to consider more energy efficient process, the lower temperature was proposed. In **Paper II**, pre-treatment of barley straw and forest residues with 85% NMMO was therefore carried out at 90°C, taking advantage of available excess heat from power plants or district heating systems used for the pre-treatment. However, due to the lower severity of these pre-treatment conditions, compared to that applied in the previous study, longer duration times were also applied (3, 7, 15 and 30 h) (Figure 3.6 A&B) **(Paper II).**

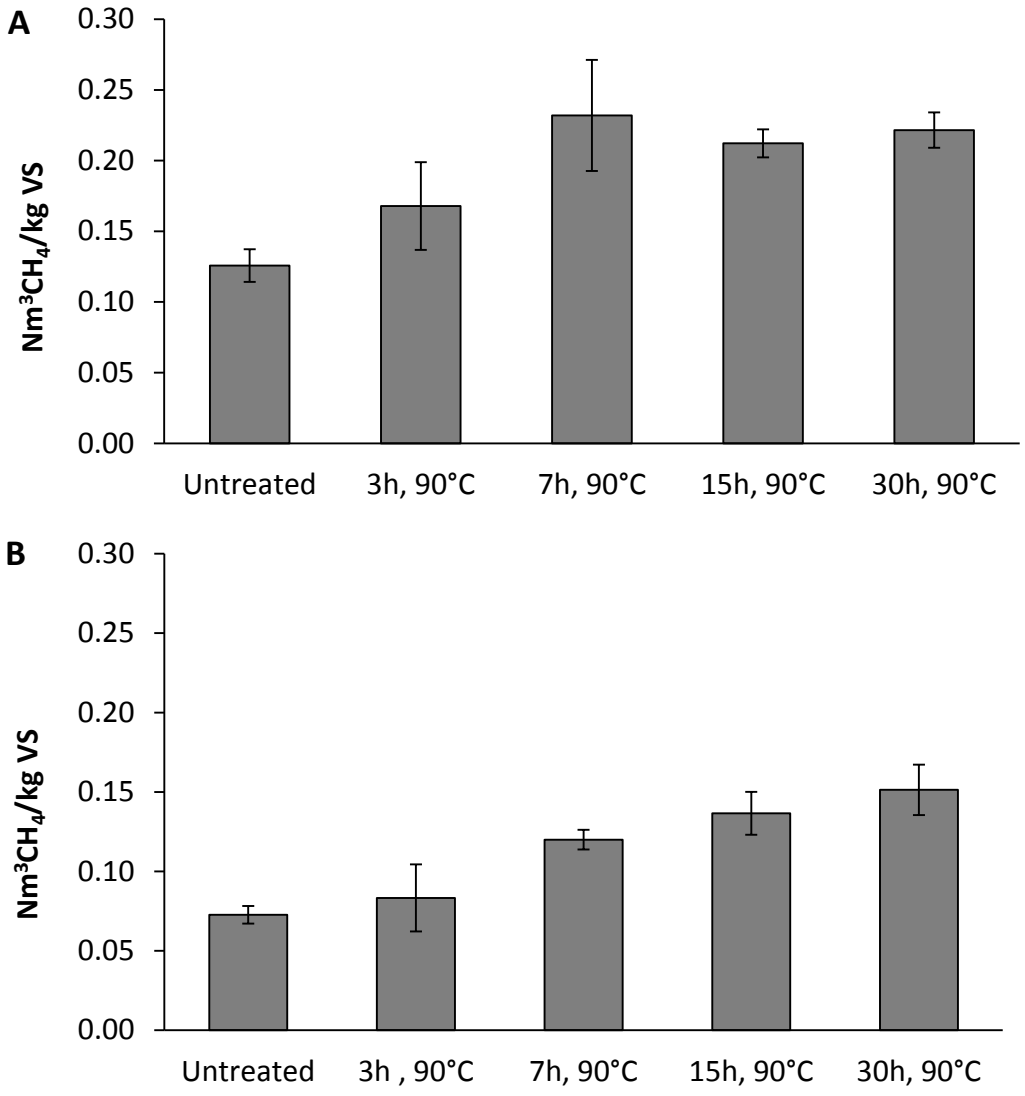

Figure 3.6. Accumulated methane yield (Nm^3CH_4/kg VS) obtained during AD assays of barley straw (A) and forest residues (B) untreated vs pretreated with NMMO at different pre-treatment conditions. Error bars represent ±1 standard deviations.

The results of the subsequent AD assays showed that the 3h-long pre-treatment at 90°C was not effective enough to degrade the complex lignocellulose structure, hence, enhance the enzyme accessibility (Figure 3.6 A&B). The highest methane yields from forest residues were obtained after 15 or 30h NMMO pre-treatment. The statistical analysis showed no significant differences in methane production after pre-treatment times of 15h and 30h (Figure 3.6B).

In case of NMMO–pretreated barley straw, the maximum methane potential was observed after 7h pre-treatment (Figure 3.6A). Increasing the pre-treatment duration longer than 7h did not significantly lead to further enhancement in the methane yield.

Regardless of the positive effect of the NMMO pre-treatment on anaerobic digestion of lignocelluloses, there are many factors that need to be considered to achieve economic viability for industrial-scale processes. Previously, Teghammar et al., [100] showed that the cost of the solvent reflected the core part of the material costs, when evaluating a process where forest residues after NMMO treatment was co-digested with the organic fraction of municipal solid waste (OMSW). Hence, an effective recovery of the solvent would play an important role in the economic viability of the process. The performance of the pre-treatment by employing recycled vs fresh NMMO on forest residues and barley straw was therefore also investigated (**Paper II**). The pre-treatments were performed with 85% NMMO for 30 h at 90°C, and the solvent was recovered and reused to examine the effectiveness of the pre-treatment after a course of five recovery cycles. The results showed that there was no significant difference between the methane production obtained from NMMO-pretreated barley straw with the fresh or with the recycled NMMO (Figure 2C in **Paper II**). However, interestingly in the case of the forest residues, the methane yield decreased by 45% and 55% after pre-treatment with recycled NMMO, which was recovered and reused three and five times, respectively, compared to the methane yield obtained using the fresh NMMO (Figure 3.7 and **Paper II**).

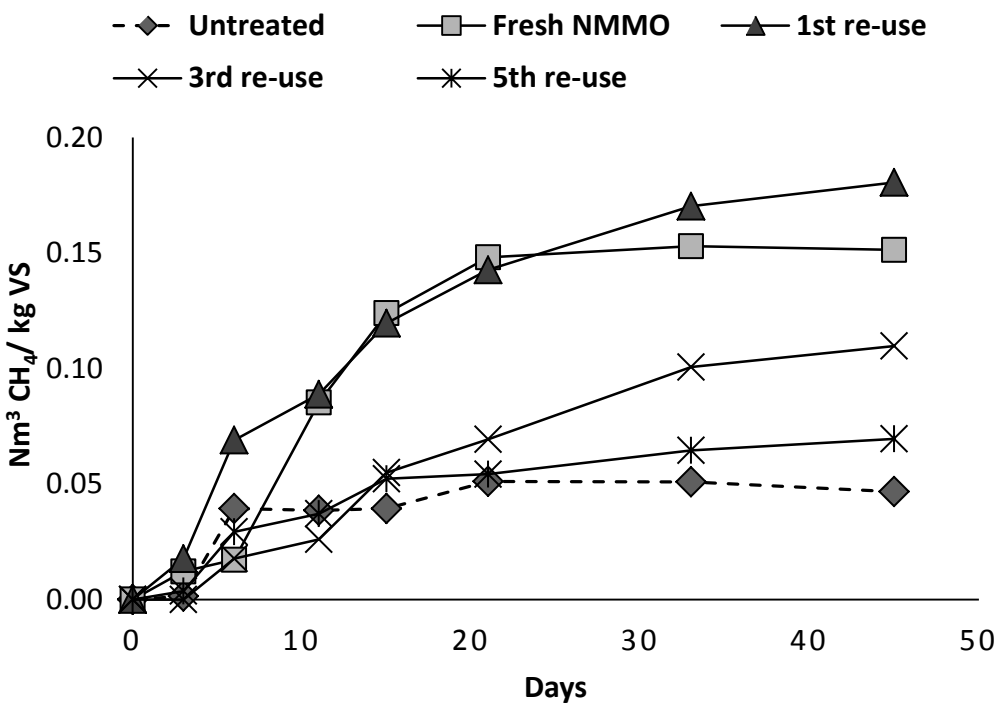

Figure 3.7. Accumulated methane production (Nm^3CH_4/kg VS) from anaerobic digestion of forest residues, untreated vs pre-treated with fresh or recycled NMMO.

A previous study [101] examined the effects of recycling of the NMMO on cotton and viscose fibres, consisting of almost pure cellulose. The results of that study showed no significant differences between using the fresh solvent, compared to that using the recovered one prior to biogas or bioethanol production. These observations may link to the composition and characteristic of the substrates studied. Forest residues mainly consist of a mixture of spruce and pine with high bark content. This mixture contains tannins, resin acids, volatile terpenes, phenolic acid and phenolic aldehyde, which are already known to be toxic in wastewater systems [102, 103]. Similar to the wastewater treatment process, these organic compounds could be liberated and accumulated in the NMMO monohydrate solution after being recycled a few times. Therefore, negative side reactions in the forest residues/NMMO/water mixture are more likely to occur, and the solvent will lose its dissolution power. Nevertheless, the mechanism of these side reactions is not quite clear yet, and more detailed investigations are needed in the future to find out the reasons behind the changes in the dissolution power of the solvent.

Inhibition effect of NMMO on the AD process

After NMMO pre-treatment of lignocelluloses and addition of anti-solvent for cellulose regeneration, a thorough filtration and washing needs to be applied. Thus, it is important to find out whether traces of remaining NMMO would have an effect within the subsequent AD process. Therefore, a detailed study was carried out to investigate the effects of different concentrations of NMMO (*i.e.* between 6.4×10^{-5}%–1%) on the anaerobic digestion of pure cellulose fibres (**Paper I**). It was found that NMMO, in concentrations higher than 6.4×10^{-5}% showed severe inhibition effects (Figure 3.8). Furthermore, a first-order kinetics model [104] was used to characterise the inhibition effects of NMMO on digestion of cellulose fibres. The results showed that not only the accumulated methane production but also the degradation rate declined with increasing NMMO concentrations present in the reactors (Table 3 in **Paper I**).

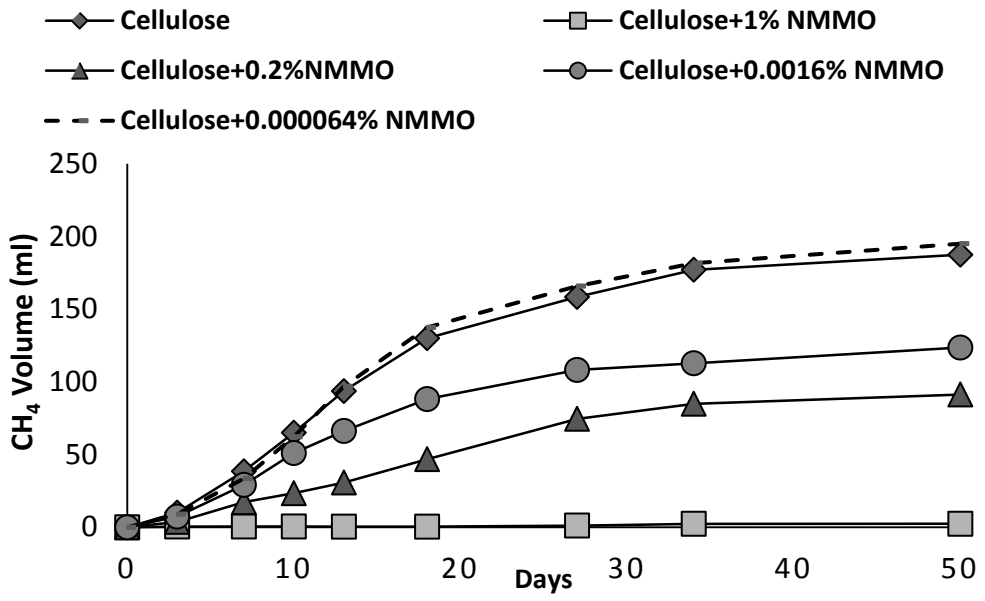

Figure 3.8. Accumulated methane production obtained from cellulose in the presence of different concentrations (6.4×10^{-5} %–1%) of NMMO.

3.4.2 Pre-treatments using other organic solvents

Pre-treatments of lignocelluloses with organic solvents have been used extensively for biomass fractionation or for improving the enzymatic hydrolysis. These pre-treatment methods partially hydrolyse the lignin fraction as well as break down the bonds between the lignin and carbohydrates, aiming to remove the major barrier for the enzymatic attack [105]. Among the organosolv pre-treatments, pre-treatment with ethanol is known to be one of the

most effective methods. According to Pan et al. [106], this process is effective even on softwood, which has a higher lignin content and higher content of methyglucronic acid in the hemicellulose fraction. In their study, hot aqueous ethanol was used for the pre-treatment of a hybrid poplar, resulting in an enhancement in the recovery of glucose by 85% after enzymatic hydrolysis [106]. Furthermore, a treatment with ethanol using sulfuric acid, as catalyst on lodgepole pine (softwood) showed 97% conversion of cellulose to glucose during the following enzymatic hydrolysis [107].

Pre-treatment of lignocelluloses with low molecular weight alcohols and organic acids aiming to improve biogas production is advantageous, since these organic solvents are not inhibitory for the methane producing microorganisms. Therefore, the efficiency of the pre-treatments with these organic solvents does not rely on using a high volume of process water to separate the solvent from the biomass prior to AD. Moreover, the recovery and recycling of these solvents can be done in a simple distillation process. This is a great advantage, compared to some other chemical pre-treatment methods, such as using alkaline, dilute acid or NMMO pre-treatments discussed above, where the chemical used for the pre-treatment can be a source of inhibition for the microorganisms even if only small traces remain in the pre-treated biomass.

Due to the challenges faced in the case of NMMO treatment presented in chapter 3.4.1, the effect of various other organosolv pre-treatments was also evaluated on biogas production from forest residues (**Paper V**). The organic solvents employed were 50% (V/V) ethanol, methanol or acid acetic. All pre-treatments, both with and without the presence of a catalyst, were carried out in an oil bath at 190°C for 60 min. Batch AD digestion tests in thermophilic conditions, *i.e.* at 55°C, were then carried out to determine the biomethane potential of the pre-treated vs the untreated substrates. The accumulated methane yields of pre-treated forest residues were between 0.23 and 0.34 Nm^3CH_4/kg VS, which indicates a significant improvement, compared to that of 0.05 Nm^3CH_4/kg VS, obtained from the untreated substrate. Moreover, among the organic solvents, pre-treatments with acetic acid and ethanol led to the highest methane yields, *i.e.* over 0.30 Nm^3CH_4/kg VS.

After the filtration step for separating solid biomass from the liquid fraction, approximately 96% solvent recovery was achieved. Hence, some amount of the solvent still remained in the solid fraction and accompanied the forest residues into the following digestion process. As all of these organic solvents (*i.e.* acid acetic, ethanol, methanol) are intermediate products in the AD pathways, each of them will rather contribute to than inhibit the methane production. The theoretical methane yield for acetic acid, ethanol and methanol is

0.37, 0.48 and 0.52 Nm^3CH_4/kg VS, respectively. Based on these theoretical yields, the amount of methane obtained from the solvents remaining in the substrates was calculated. Therefore, subtracting these calculated yields from the total methane productions obtained during the batch assays gives the methane yields from only the fraction of forest residues. Accordingly, methane productions of between 0.18–0.21 Nm^3CH_4/kg VS were determined from the pre-treated samples, corresponding to up to 4-folds improvements in methane yields, compared to that of the untreated substrate (Figure 1, in **Paper V**).

3.5 Characterisation of lignocelluloses before and after the pre-treatment

The aim of the pre-treatment is to modify the characteristics of lignocelluloses prior to a subsequent biological degradation. These modifications in the structure as well as in the composition of lignocelluloses, which in fact are also the main goals of the pre-treatments, include increasing accessible surface area, decreasing cellulose crystallinity, disrupting the protective layers of hemicellulose and lignin around the cellulose chains, and reducing the degree of acetylation of hemicellulose [41]. During the pre-treatment process, most of these changes occur simultaneously; hence, keeping track of only one single parameter is practically not possible. Therefore, an improvement obtained after the pre-treatment is a result of positively modified characteristics and not related to a single parameter.

In this thesis, some of these changes in lignocellulose characteristic, such as cellulose crystallinity and surface area accessibility were studied.

3.5.1 Cellulose crystallinity

The crystallinity of pure cellulose can be measured with Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD) and solid-state nuclear magnetic resonance (NMR) [80]. These methods can also be used for the determination of cellulose crystallinity in lignocelluloses. When FTIR is used, the lateral order index (LOI), also called crystallinity index (CI), at absorbance ratio of A1422/A898 cm^{-1} can be examined. LOI provides an indication of the transformation of cellulose from its natural state, *i.e.* Cellulose I to Cellulose II, with less crystalline structure (amorphous cellulose). As was mentioned earlier, the carbohydrate binding module (CBM) of the cellulosome complex can bind more easily to the amorphous form of cellulose than to crystalline cellulose [88]; therefore, a decrease in cellulose crystallinity can result in an improvement of anaerobic degradability, and subsequently a higher methane production can be achieved after an effective pre-treatment.

Pre-treatment of forest residues with fresh NMMO resulted in a decrease in the absorption band at 1422 cm^{-1}, while an increase in the absorption band at 898 cm^{-1}, representing that the amount of Cellulose II and/or amorphous cellulose was increased (**Paper II**). Moreover, the crystallinity index of the forest residues pre-treated with recovered NMMO after 1st, 3rd and 5th recovery have interestingly showed an increase in the crystallinity, indicating that the solvent had lost its dissolution power after being recycled several times (Figure 3.9)

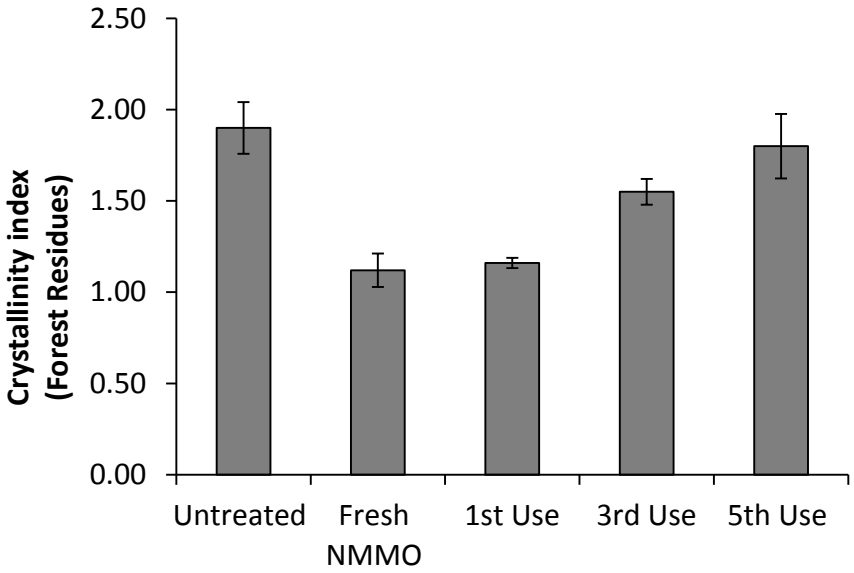

Figure 3.9. Crystallinity index of forest residues pre-treated with fresh or recycled NMMO. The error bars correspond to ±1standard deviations.

3.5.2 Simons' stain

Providing a sufficient accessible surface area is the core objective of all of the pre-treatment processes. If the enzymes do not have direct physical contact to the biomass, they may not penetrate to the cellulose chains. The cellulose degradation products formed by the action of the multi-functional enzyme complex (cellulosome) are subsequently channelled to the microorganisms through some produced fibrous corridors (see section 3.3.1). Therefore, the formation of pores is more important for the degradation of cellulose by anaerobic microorganisms than for the aerobic ones.

The accessibility of cellulose can be defined as milligrams of enzyme adsorbed per gram of biomass [108]. However, this method was not applied within this study, instead a method called the Simons' Stain technique was used. This method is a quantitative method developed earlier for the evaluation of porosity in pulp fibres [109]. However, Simons' Stain has also

been employed to estimate the pore distribution of lignocelluloses, as an indication of accessible surface area and its correlation to enzymatic hydrolysis since 2001. Later, the Simons' Stain method was modified and used for the prediction of improvements in digestibility as a result of different pre-treatments [110]. This staining method is based on the adsorption of two different dyes (blue and orange dye), with two different particle sizes and different cellulosic affinity. The diameter of the blue dye molecule is 1 nm, whereas the orange dye molecules have diameters of 5 to 36 nm [111]. Depending on the pore sizes on the surface of a material, different amounts of these two dyes can be adsorbed. Hence, the absorbed amounts of these two dyes will give an indication of the accessible surface area, and can hereby be measured [111]. Considering the average size of typical bacterial cellulase enzymes, which is between 4 to 16 nm [112], it can be concluded that the enzyme penetration into the substrate can occur at pores, where the orange dye can enter. Therefore, the total orange dye absorbed on lignocellulosic substrates can be a valid estimation of the active surface area of the biomass.

The Simons' Stain technique was applied to study the structural changes of barley straw and forest residues, after pre-treatments with fresh or recycled NMMO (**Paper II**). Interestingly, the results of the Simons' Stain analyses showed a tight correlation with the results of AD tests performed after the pre-treatments. Barley straw showed an increase in total amount of absorbed dyes after the pre-treatments, both in cases of fresh and recycled solvent, compared to that of the untreated sample (Figure 1C in **Paper II**). On the other hand, the pre-treatment with the recycled NMMO (3rd and 5th use) did not show similar effects as pre-treatment with the fresh NMMO regarding the changes in the pore size of the forest residues. The Simons' Stain analyses illustrated a reduction by 50% in the total amounts of the absorbed dye after recycling the solvent the 3rd and 5th time (Figure 1D **Paper II**). This is again an indication that the efficiency of NMMO decreases after a few times recovery and reuse, when forest residues are treated. These observations strengthen our findings, previously discussed in section 3.4.1, which also showed that the compositional difference between these two lignocellulosic materials plays an important role in the efficiency of the pre-treatment with reused NMMO.

CHAPTER 4

KERATIN WASTE AS A SUBSTRATE FOR ANAEROBIC DIGESTION PROCESS

4.1 Wool production

Wool is a natural, biodegradable, and sustainable fibre, which has highly valuable properties in textile industry. The inherent resistance of wool to flame and heat makes it one of the safest household textiles of all. Moreover, its ability to absorb and release moisture and its natural ability to breathe makes this garment comfortable and able to adapt to different climate conditions. These unique properties of wool fibre make it increasingly popular for global manufacturers and consumers. According to the Food and Agriculture Organization of the United Nations (FAO), the annual wool production is around 2.1 million tonnes [113, 114]. Australia is the largest wool producer with a production of one-fifth of that total amount, followed by China, New Zealand, Iran, Argentina and the UK, each producing more than 50,000 tonnes wool per year [113]. After the early processing of raw wool, which involves removing dirt and grease, it is then spun into yarn for further use in fabrics, knitted garments or hand-knitting.

In terms of production share for clothing textiles, wool has approximately 10% of the total textile market, together with similar market share for viscose and acrylic, followed by polyester 16%, and cotton fibres that account for more than 43% of all fibres [115]. However, the fibre by-products released from the textile industry, along with the disposal of a huge volume of textile clothing waste have become a major environmental concern for the textile and fashion industry. Over the last few centuries, efforts have been made for the developments of new commodities from biomass-derived materials, such as conversion of industrial wastes into value added products. Wool waste, as one of the most abundant non-

food protein sources, is a cheap and renewable feedstock, which is mostly discarded as waste from the textile production and also as part of regular consumer routine.

4.2 Wool structure

Wool is a fibrillar protein composed of keratin with mechanical strength and protective abilities against most of the stresses encountered by animals. Keratin is the main component of hair, nails, wool, horn and scales [116]. Two major groups of keratins are identified: α-keratins are commonly found in mammals and β-keratins are available in bird and reptiles.

Wool keratin belongs to α-keratins and is an unbranched polymer comprised of various amino acids, bounded together with peptide bonds. The sequence of amino acids is referred to as a primary structure of a protein. The secondary structure of proteins is locally formed three-dimensional structures defined by the pattern of hydrogen bonds, which formed between amine hydrogen (NH groups) and carbonyl oxygen atoms (C=O) presented in the backbone. The two major configurations of secondary structures are the α-helix and the β-pleated sheet (Figure 4.1).

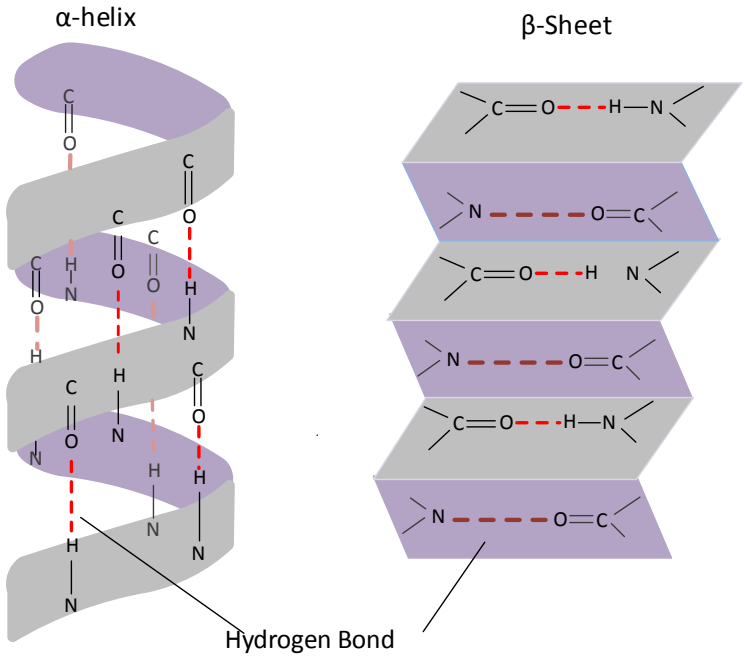

Figure 4.1. Secondary structure of protein, α-helix and β-pleated sheet [118].

The α-helix is formed when the polypeptide chains twist into a spiral shape. This stable and tightly coiled spiralling shape makes the α-helix structure very strong. The β-pleated sheet is formed when 2 (or more) segments of the protein structure come together. In other

words, the β-pleated sheet consists of several β-strands, which are kept together by hydrogen bonds. Depending on whether the strands align in the same or opposite directions (N- to C-terminals), β-pleated sheets are further subdivided into parallel or antiparallel sheets. In Figure 4.1, the α-helix and β-pleated sheet conformations are presented [117].

Keratin as the main component of wool protein is known to have a high content of cysteine. Cysteine is an α-amino acid with the chemical formula of $HO_2CCH(NH_2)CH_2SH$, which enables these molecules to form cystine cross linkages within and between protein chains, known as disulphide bonds. It is believed that over 95% of the cysteine residues in keratins are involved in disulphide bonds [119]. The peptide bonds between individual amino acids together with a range of non-covalent interactions (hydrogen bonding, van der Waals interactions and hydrophobic interactions) and covalent interactions (disulfide bonds) determine the highly resistant physical and chemical properties of wool protein. Among all these interactions and bonding within the protein molecule, the disulfide bonds stabilise the three-dimensional structure of wool proteins [120]. The disulfide bridges are responsible for cross-linking the protein chains, increasing the mechanical strength and stiffness and resistance to chemical degradation.

Elemental analysis of wool, as one of the most studied α-keratin fibres, shows that it consists of carbon, oxygen, nitrogen, hydrogen and sulphur (shown in Table 4.1). This composition is relatively typical for all proteins, excluding the sulphur content in wool, which is the main characteristic of α-keratins, due to the high concentration of “double cystine”, which has two sulphur atoms forming intra- and inter-disulphide crosslinks of protein chains [117].

Table 4.1. Elemental composition of wool fibre [117].

Element	**Weight %**
Carbon	50
Oxygen	22
Nitrogen	16
Hydrogen	7
Sulphur	5

The cysteine and sulphur content vary in different parts of the wool and generally in the keratin structure, leading to harder or softer materials [116]. For instance, the outer layer of wool fibre is rich in cysteine and highly cross-linked, whereas the inner part comprises

microfibrils of low-sulphur proteins surrounded by a matrix of high-sulphur proteins and glycine/tyrosine-rich proteins [121].

4.3 Wool degradation

Many studies have focused on the valorisation of keratin-based waste materials, made of low quality wools, fibre by-products from the wool textile industry as well as hairs, feathers, horns and nails from butchery industries. Incineration of these waste streams is not favourable due to the high rate of sulphur emissions [122]. Therefore, they are mostly disposed of at landfill sites. However, keratin, as a polymeric polyamide, is suitable for a high degree of chemical applications. It has great potential to be used as a bio-based material in production of sponges, films and matrices for agent retention [123, 124]. Keratins can be extracted by the cleavage of the disulphide bonds via reduction or oxidation treatments and then regenerated into various forms for biotechnological applications, alone or blended with other natural or synthetic polymers. The active sites of keratin include: amide, carboxyl, sulfoxide, sulphide and thiosulfate [125]. The hydrolysis of keratin from wool and feathers was exploited as an alternative way to deliver building blocks for the synthesis of novel polymers during the past few years [126]. Different chemical agents can be employed for the hydrolysis of keratin including alkaline media at elevated temperatures, which has been a common practice for strong hydrolysis of keratin. This treatment disrupts the peptide bonds, primary amide bonds, as well as the cystine disulphide bridges. Using this method, 68% to 80% recovery of pure keratin can be achieved, leading to the formation of intermediate filament proteins (IFPs) and constituent microfibrillar and matrix proteins. However, drawbacks of this technology are the high running costs and the destruction of certain amino acids. Partial hydrolysis under less harsh process conditions, such as steam explosion and treatment with superheated water have therefore been the focus of attention for many researchers lately [122, 125, 127].

4.3.1 Biological degradation of wool keratin

Another more environmentally friendly and economically viable process for the degradation of wool keratin is using keratinolytic microorganisms or enzymes. Keratinophilic fungi and keratinolytic bacteria are able to utilise keratin as a sole carbon, nitrogen and energy source. They are able to express a specific kind of protease called keratinases [128]. Keratinolytic bacteria and fungi mainly produce extracellular keratinases; however, some are able to liberate intracellular keratinases as well, which are deposited on the cell surface [129]. The

mechanism of keratin degradation by microorganisms is not yet fully understood, although a number of hypotheses have been proposed, particularly in relation to keratin degradation by fungi. One of the known hypotheses was suggested by Kunert [130] who explained that keratin degradation takes place in a two-stage process divided into sulfitolysis and proteolysis. During sulfitolysis, the disulphide bonds are disrupted leading to the production of sulphite [131]. The schematic reaction for splitting disulphide bridges during sulfitolysis is presented in the following reaction:

$$\textbf{cys – S–S – cys (cystine)} + H_2SO_3^- \rightleftharpoons \textbf{CysSH} + \textbf{cysS} - SO_3^- \textbf{ (S –sulfocysteine)}$$

During this step, denaturation of the keratin structure occurs, which provides the possibility for further attack of the enzyme (proteolysis). It is not yet known whether sulfitolysis and proteolysis occur at the same time or if sulfitolysis occurs first followed by proteolysis [132]. It is believed that an alkaline reaction environment would highly facilitate the sulfitolysis step [133].

The process of keratin decomposition by bacteria differs from the keratin degradation by fungi. Several studies on wool keratin degradation, as well as on decomposition of chicken feather [134] by bacteria, together with a study performed by Yamamura et al. [135] suppose that the keratin degradation occurs by the action of two types of extracellular enzymes, *i.e.* serine protease and disulphide reductase. Interestingly, neither of these extracellular enzymes showed keratinolytic activity individually; hence, the degradation of keratin relies on the cooperation of both of these enzymes. The proposed schematic mechanism of keratin degradation in this case is as follows:

$$\textbf{R–S–S–R} \xrightarrow{\text{Disulphide reductase}} \textbf{R–SH} \xrightarrow{\text{Protease}} \textbf{Amino acids/peptide}$$

The degradation process begins with the reduction of disulphide bonds by the action of disulphide reductase, followed by the decomposition of the denatured keratin by the action of serine proteases, which subsequently leads to a release of peptides and amino acids.

4.4 The importance of pre-treatment for keratin-rich residues

Previously, alkaline conditions in elevated temperature were applied for bioconversion of feathers (β-keratin protein) to biogas production. It is reported that this sulphur rich protein can be disintegrated and lose almost half of its sulphur content at elevated pH, through a

chemical reaction leading to the liberation of H_2S [136]. However, the presence of H_2S over a concentration of 200 mg/L is toxic for methanogens and inhibits the AD process [137]. In another study, a biological pre-treatment of feathers was carried out with a recombinant *Bacillus megaterium* strain, prior to anaerobic digestion [138]. A high amount of soluble protein was produced as a result of the pre-treatment, with the benefit that no endotoxins were generated in the cell wall. Although the biological method is considered to be an environmentally friendly process, a thermal pre-treatment was still necessary prior to the biological degradation; furthermore, a recombinant strain was applied in the process. The slow rate of reaction and restricted regulations on genetically modified organisms are among the disadvantages of this method.

4.4.1 Enzymatic pre-treatment of wool textile wastes

Proteases or proteolytic enzymes refer to a group of enzymes with the catalytic function of hydrolysing the peptide bonds of proteins. A wide range of applications have been recognised for these types of enzymes, such as textile processing, animal feed production, detergent formulation and within the leather manufacturing industries [139]. Proteases can be divided into serine proteases, cysteine proteases, aspartic proteases and metalloproteases, according to the functional groups found on their active sites. They can be further categorised into two groups, namely, endopeptidases or exoproteases, based on their cleavage abilities. The former cleaves non-terminal peptide bonds inside polypeptide chains, and the later attacks the peptide chain at the ends (either the N- or C-terminus of the protein) removing single amino acids or di- or tripeptides from the end of the peptide chain.

Keratinases are specific proteolytic enzymes, which are capable of hydrolysing insoluble keratins in feathers as well as in animal wool and hair. The keratinases are identified as belonging to endopeptidases, which are members of the serine protease family. For wool and feather keratin degradation, a higher operation temperature (thermophilic range) is required, and thermostable keratinase can be used for this purpose. At higher temperatures, a higher reaction rate, as a result of lower diffusional restrictions, can be obtained [140].

In **Paper III**, the enzymatic pre-treatment of two types of wool textile residues with different compositions were carried out. Textile waste type one, referred to as TW1, consisted of 70% wool (protein) and 30% polyamide, while type two (TW2) consisted of 70% wool (protein), 18% polyamide and 12% kermel. Kermel is a kind of polyamide-imide fibre, which is naturally non-flammable. This fibre is commonly used in manufacturing of heat and flame resistant protective clothing. During the experiments, wool textile waste was subjected to

hydrolysis by Savinase®, a thermostable protease for 0, 2 or 8 hours at 55°C. Treatment time of 0h was also investigated, referring to simultaneous enzymatic treatment, meaning that the enzyme was added to the digester directly at the start-up of the AD assay. Additionally, a thermal pre-treatment was also performed at 120°C for 10 min, both without and with a following enzymatic treatment. Soluble protein content as well as soluble chemical oxygen demand (sCOD) was investigated to measure the effects of the different pre-treatment conditions on the hydrolysis degree of keratin and the solubilisation of organic matters in the entire samples. All pre-treatments greatly increased the sCOD and the soluble protein content of both of these two textile samples, compared to those of the untreated wool residues. Statistical analyses of pre-treated TW1 and TW2 showed that the combined treatment, *i.e.* primary thermal pre-treatment followed by enzymatic pre-treatment, significantly increased the sCOD and soluble protein contents. However, according to the ANOVA analyses, there were no significant differences between the soluble protein concentrations and the sCOD contents of the samples that underwent combined treatments, where the enzymatic part was valid for 2 or 8 hours. Therefore, the combined pre-treated samples for 2h duration were chosen for biomethane potential tests under thermophilic conditions. Furthermore, as the enzyme is functional under thermophilic temperature range, the direct addition of enzyme (0h) to the digestion of thermally pre-treated samples was also studied. Figures 4.2A and B present the biomethane potential of untreated vs pre-treated wool textiles residues of TW1 and TW2, respectively.

The AD assays showed that the combined pre-treatments increased the methane yields from both TW1 and TW2; however, at different levels depending on the type of textile. The accumulated methane yields, as well as the initial degradation rates were higher for TW1 than for TW2. The methane yield from TW1 increased up to 20 times in comparison with the yield from the untreated samples, while the increase was only about a half, *i.e*. 10 times in the case of TW2 (Figure 4.2). A possible explanation for this is the presence of kermel fibres in TW2 that are known for their non-flammability, thermostability, thermal insulation and mechanical strength.

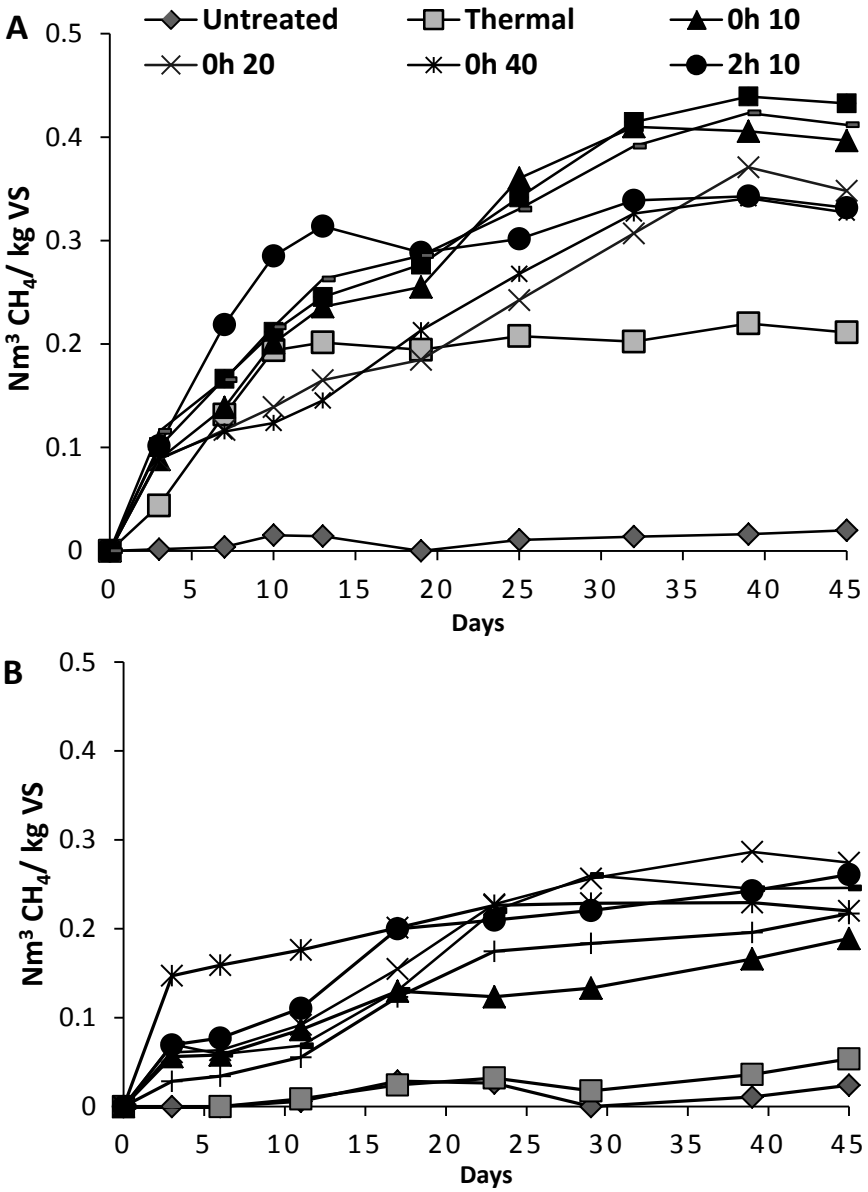

Figure 4.2 A & B, Accumulated methane production obtained during anaerobic digestion of untreated vs pre-treated wool textile waste (A; TW1 and B; TW2). 0h 10, 0h 20 and 0h 40 refer to direct addition of enzyme at the beginning of the batch digestion with enzyme loading of 10 µl/ml, 20 µl/ml or 40 µl/ml, respectively. 2h 10, 2h 20 and 2h 40 represent the thermo-enzymatically pre-treated wool textile, using similar enzyme loads at 2h duration.

CHAPTER 5

DRY ANAEROBIC DIGESTION OF LIGNOCELLULOSIC AND KERATIN WASTES

Dry anaerobic digestion, also called solid state AD, or high solids AD, has been regarded as an innovative waste management approach to treat organic matter with high solid content (>10% TS) [10]. Dry AD is beneficial due to the need for compact digester size with higher volumetric organic loading rate (and hence less space requirements), and lower energy requirements for heating [10]. Furthermore, solid state AD processes result in an easier and less expensive handling of the digestate residue, which can then be used as organic fertilizer [141]. However, one of the challenges with dry AD technology is the longer retention time needed due to the slower mass transfer than in wet AD processes.

Applications of both dry and wet AD have continued to increase during the last few years; however, the establishment of dry AD plants has been dominant since the beginning of the 90s. Table 5.1 shows the 5-year developments of wet vs dry AD plants. The increase in the installation of wet AD plants was observed between 2000–2005; however, since 2006, 71% of the newly installed AD plants use dry AD technology [142].

Table 5.1. 5- year development of wet and dry AD plants [142].

5-year development	1991–1995	1996–2000	2001–2005	2006–2010
Wet AD installed/5 year	37%	38%	59%	29%
Dry AD installed/5 year	63%	62%	41%	71%

Agricultural residues such as corn stover, wheat and rice straw are favourable feedstock for dry AD processes, due to their availability in large amounts, high potential biogas yields and high total solid contents. The high C/N ratio and low levels of trace elements, however, limit microbial growth and activity; therefore, these types of substrates can preferably be used

in co-digestion processes together with nitrogen-rich substrates to achieve a balanced nutrient composition. The biomethane potential of wheat straw, as a carbon rich substrate, and wool textile waste, as a protein rich waste stream was therefore investigated using four different TS concentrations (6, 13, 21 and 30%). The results of this study are discussed in the following sections.

5.1 Dry anaerobic digestion of wheat straw

In **Paper IV**, the AD of wheat straw was evaluated in wet, *i.e.* at 6% TS content; semi dry, *i.e.* at TS content of 13%; as well as in solid state AD conditions, *i.e.* at TS contents of 21 and 30%. The substrate and inoculum ratio was set to 2:1 based on the volatile solids contents. Furthermore, additions of cellulose degrading enzymes, as well as nutrients were also studied. The results of these investigations showed that the anaerobic digestion of wheat straw with or without the addition of enzymes was negatively affected, as the TS content increased to 30%. The highest volumetric methane productivity was achieved when TS of 13 and 21% were applied (Figure 5.1). Previous investigations showed that straw fibres have a tendency to form dense granules with microorganisms in less diluted environment, which ultimately increases the efficiency of the AD process [29]. However, according to the results of this study, the TS content applied must be adjusted to avoid overloading in the system (**Paper IV**).

5.2 Dry anaerobic digestion of wool textile

Anaerobic digestion of wool textile wastes was evaluated at similar TS contents of between 6–30% (**Paper IV**). The effect of addition of a protein degrading enzyme or nutrients was also studied. The methane yields obtained during the batch digestion assays without the addition of enzyme or nutrients were between 0.030 – 0.061 Nm^3CH_4/kg VS. These results correspond with only up to 8% of the expected theoretical yield based on the protein content of the wool textile waste. Interestingly, the varying TS contents in the reactors had no significant effect on the performance of AD in this case. However, the addition of a protein degrading enzyme resulted in an increase of the yield to 0.108 and to 0.131 Nm^3CH_4/kg VS, when TS of 6% and 13%, respectively, was used. This indicates that the simultaneous enzyme addition could enhance the methane yield, achieving, respectively, 30 and 38% of the theoretical yield from the wool protein. Nevertheless, the methane production decreased as the TS was increased to 21 and 30%. A possible explanation for this observation is the

accumulation of volatile fatty acids (VFAs) and ammonia due to the action of the enzyme added. In higher TS concentrations, the mass transfer barrier for diffusion of VFAs increases [143], which may lead to accumulation of VFAs leading to a deterioration in the system performance. On the other hand, the addition of nutrients did not have a significant effect on the accumulated methane yields from wool textile waste (**Paper IV**).

5.3 Co-digestion of lignocellulose and wool textile waste

Considering the importance of the C/N ratio for a balanced AD system, the co-digestion of wheat straw, as a carbon rich, and wool textile waste, as a nitrogen rich substrate may result in better performance. Therefore, in **Paper IV**, the co-digestion of these two waste streams, with mixture composition of 1:1 based on the VS contents, was also examined using different TS contents of 6–30%. The methane yields obtained from the co-digested mixtures were compared to the expected methane yields calculated from the methane production of the individual fractions at particular TS contents, respectively. The obtained methane yields from the mixtures were significantly higher, compared to the calculated levels, with an exception at TS of 13%, where the difference was not significant. This enhanced performance might have established due to the synergism developed in the digester between these two substrates. Interestingly, the highest synergistic effect was observed when this mixture was co-digested at 30% TS, with the addition of nutrients, corresponding to 58% increase in methane yield, compared to the calculated one. This might be due to the fact that digesters containing 30% TS can suffer more severely from nutritional imbalances, compared to those digesters operating at lower TS levels. Therefore, the supply of nutrients resulted in better performance. The volumetric methane productivity of the mixtures at different TS is shown in Figure 5.1. The highest values were achieved when TS of 21 and 30% were applied.

From the results presented in Figure 5.1, we can conclude that high methane productivities were achieved, both in the case of the digestion of individual substrates and during co-digestion of these two materials, in semi dry or dry AD conditions. This clearly shows the economic benefits of the dry AD system over the wet AD. Moreover, in common AD plants, operating at TS below 5%, a huge amount of process water is needed, together with further processing, cleaning and recirculation of this water. In contrast, dry AD process can offer a simpler and more economically efficient process with minimum amounts of water requirements.

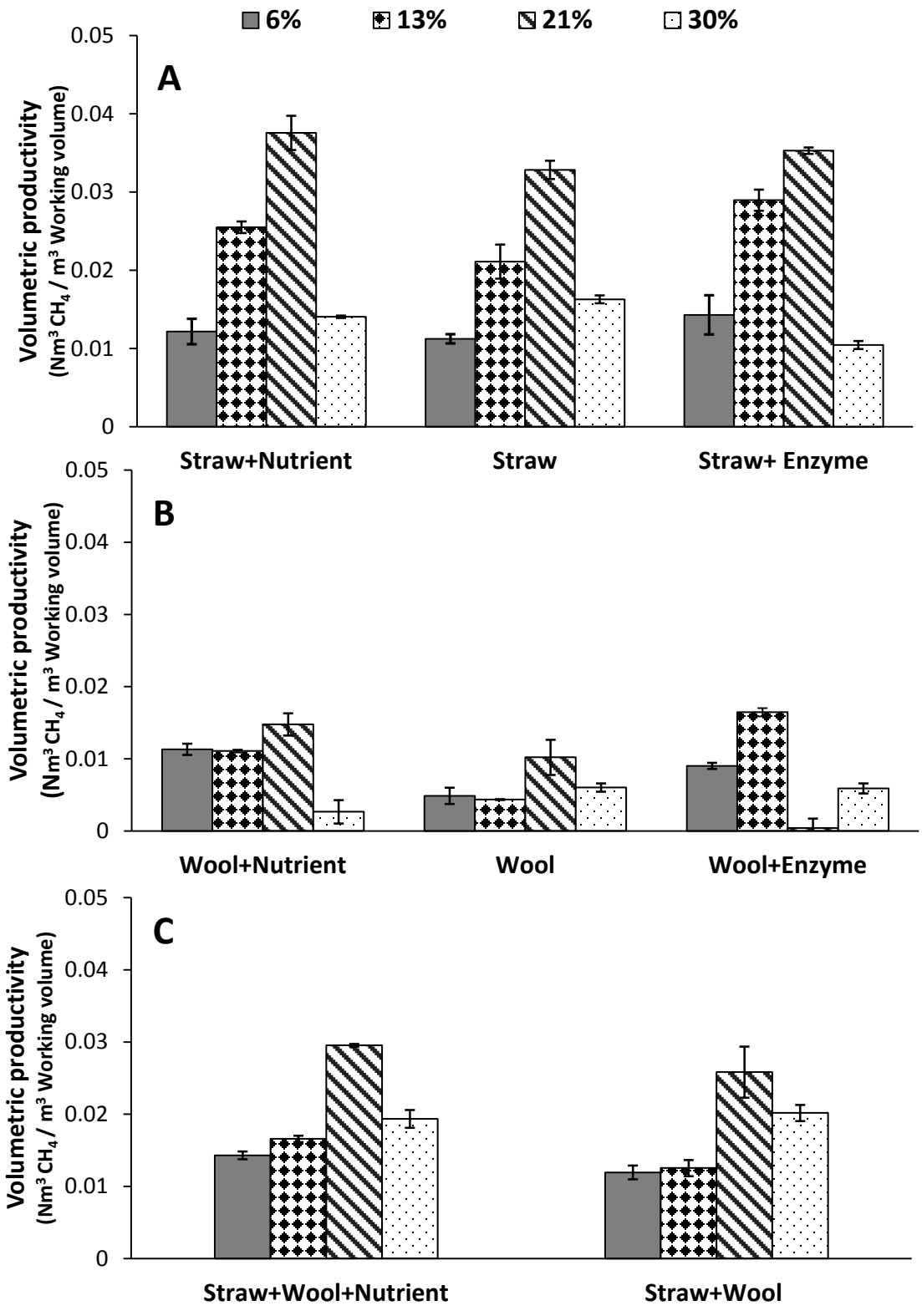

Figure 5.1. Volumetric methane productivity of wheat straw (A), wool textile waste (B) and during co-digestion of these two substrates (C), expressed as Nm^3CH_4/m^3 working volume and obtained at different TS contents used during the batch digestion assays. The error bars correspond to ±1 standard deviations.

5.4 Structural changes of the substrates during digestion

Structural analyses on cellulose of wheat straw and on the secondary structure of protein in wool textile waste were performed aiming to detect what happened with each of these feedstock during the degradation process.

Structural changes on wheat straw

Cellulose crystallinity (CI) in wheat straw was studied using FTIR at the absorbance ratio of A1422/A898 cm^{-1} (see detailed explanation in 3.5.1). CI provides an indication of the transformation of highly crystalline Cellulose I to Cellulose II (amorphous cellulose). The CI of cellulose in wheat straw, which had been digested for 50 days, increased, compared to that of the undigested wheat straw. For instance, during the dry-AD of straw, using 21% TS with addition of enzymes, its CI increased from 0.63 to 1.58. This is an indication that during the 50 day dry-AD process, the amorphous form of cellulose was primarily utilised, leaving the crystalline form of cellulose intact (**Paper IV**).

Structural changes on wool textile waste

The structural changes in the protein microstructure of wool textile were also studied by FTIR. The amide I (in the range between 1700–1600 cm^{-1}) bands, as the most predominant vibrational bands of the protein backbone, which are mainly associated with the stretching vibrations of peptide bonds, were investigated [144]. The most sensitive spectral region of the protein secondary structure is amide I; therefore, the deconvoluted amide I band was studied in detail to understand changes in the secondary structure of proteins in wool textile waste. The absorption regions of 1631–1621 cm^{-1} and 1694–1680 cm^{-1} represent secondary structures of β-sheet, and 1657–1651 cm^{-1} and 1679–1670 cm^{-1} display the secondary structures of α-helix and disordered regions, respectively. The original wool textile waste contained 29.3% α-helix, 44.4% β-sheet and 17% disordered regions (**Paper IV**). There was a slight decrease in the β-sheet region *i.e.* from 48% to 46%, and a more dramatic decrease, *i.e.* from 17% to 10% in the disordered regions after the wool was digested using 21% TS with enzyme addition (**Paper IV**). These results indicate that the disordered regions were probably the most affected regions during the digestion process.

CHAPTER 6

THE ECONOMICAL FEASIBILITY OF THE ANAEROBIC DIGESTION PROCESS

6.1 Economical parameters

One of the main driving forces for establishing an AD plant for the utilisation of lignocellulosic biomass is the potential economic feasibility. A techno-economic study is, therefore, a useful tool to evaluate the profitability and viability of the proposed process by using process simulation with economic calculations. The application of a techno-economic analysis gives valuable information about the optimisation of different process parameters or alternative process configurations. The capital cost is undoubtedly the main factor in the cost of biogas production. Depending on the share and type of substrates, the cost related to the feedstock (including transport and storage costs) is another major cost. Other factors such as heat and electricity consumption, maintenance costs, labour, wage, etc., all together called operating costs, which are determined by the properties of the feedstock, the applied technology, plant size and site conditions are also important.

6.1.1 Capital cost

The capital cost or fixed capital investment cost is affected by several factors including: plant size, location, type of pre-treatment unit and engineering. The composition and characteristics of the feedstock is also important, as it determines the essential units required for pre-processing prior to the AD process. Regarding the plant size, it is worth mentioning that the size of the plant does not linearly affect the capital cost of the plant, meaning that a larger plant size requires less investment per production unit [8].

6.1.2 Operating cost

The operation costs are the costs associated with price of the feedstock, energy, power consumption, maintenance, operating staff (salaries, insurances, etc.) and transportation. Among these, the cost for the type and amount of feedstock used and for the number of employees operating the plant are the direct costs. Whereas, insurances, taxes, salaries, etc. are among the indirect costs, as they are independent irrespective of whether the plant is in operation or not [145].

6.1.3 Type and cost of feedstock

The cost related to the feedstock, as one of the main factors, has a large impact on the economic viability of a biogas plant. The type of feedstock influences the digester configuration, in terms of design or operating conditions. It also determines the energy and mass balances as well as microbial physiology throughout the biological degradation process. The cost of the feedstock differs based on region/country where the AD plant is located. For instance, in England, the operator of an AD plant often charges a waste management gate fee for the waste taken care of by the plant. While in some other countries, like in Germany, the waste management sites compete for certain organic waste streams; therefore, the biogas plants must sometimes even pay for the waste, which they want to utilise [146]. In these countries, the demand for the waste streams used by other waste management sectors will also affect the cost of the feedstock. Biomass with no other potential use will obviously have a lower price than biomass that can be used for other purposes, such as in incineration for heat and electricity production.

6.1.4 Combined heat and power production

The majority of the biogas applications is its utilisation for heat or combined heat and power production (CHP). Most of the biogas plants are, therefore, coupled to electricity or CHP production. The CHP unit is often located rather close to the digester tank. Gas engines are normally used, and depending on the type and size, they can reach an electrical and thermal efficiency of 30–45% and 35–60%, respectively. The total efficiency of a CHP unit can reach to approximately 85%. In Sweden, 38% of the produced biogas is used for heat generation and 3% for electricity production [147]. The produced electricity can be sold to the public electricity net, while the produced heat is often used as heating within the AD process itself, but can also be sold as district heating.

To improve the quality of the raw biogas transferred to the boiler or to the CHP unit, substances such as hydrogen sulphide, nitrogen, water and particulates must be removed. Removing these substances from the gas prevents mechanical wear and corrosion of the equipment in which the gas is used [148]. The concentrations of these substances vary depending on the composition of the feedstock from which the gas was produced.

6.1.5 Upgrading to biomethane

Biogas produced via AD can be upgraded to be used as an alternative for natural gas or as a renewable vehicle fuel [149]. Upgraded biogas is usually denoted as biomethane. Biomethane is known as one of the cleanest vehicle fuels when it comes to the environment, climate and human health. Presently, the use of methane as a vehicle fuel is only widespread in Sweden, due to the Swedish financial and political support [4]. Since the biogas contains significantly more carbon dioxide than natural gas, further purification is necessary to be able to inject the produced methane to the gas grid. Typical techniques are chemical absorption, water scrubbing, pressure swing adsorption, chemical absorption and membrane separation [148]. Nevertheless, in different countries, different quality specifications are applied. The choice of the upgrading technique and its cost is therefore dependent on these regulations as well as on the composition and amount of the biogas produced. The most widespread technique, both globally and in Sweden, is the water scrubbing technique [150].

6.1.6 Digestate value or cost

Another cost saving source is the sale of the digestate residue, since due to its high nutrient content, especially nitrogen and phosphorus, it can be utilised as fertilizer on cropland. However, in case of lignocellulosic biomass used as feedstock, the digestate residue is usually rich in lignin, because of the limited decomposition of lignin in the AD process. The high lignin content in the dewatered digestate makes it a suitable energy-rich substrate for incineration. This gives an additional value to the overall AD process, since the utilisation of the digestate residue can contribute to a positive economical balance of the process.

6.2 Techno-economic evaluation of biogas production from forest residues

Forest residues are an abundant fraction of lignocellulosic waste streams in Sweden. Their utilisation in biogas processes has therefore been one of the main objectives in this thesis. The pre-treatment of forest residues with low molecular alcohols and organic acids (*i.e.* ethanol,

acetic acid and methanol) has shown to significantly improve the final methane yields during the subsequent anaerobic digestion (**Paper V**). Using the experimental data, a process simulation was performed to evaluate the techno-economic viability of the biogas production. The simulations were run separately for all three different pre-treatments, applying a plant capacity of 20,000 tonnes processed forest residues per year. The proposed process included operation units, as well as material storage tank, shredders, pre-treatment unit, solvent and water storage tanks, filtration unit, anaerobic digester, biogas upgrading unit and a unit for waste water handling (Figure 2 in **Paper V**). The anaerobic digester was operated at 55°C with HRT of 20 days.

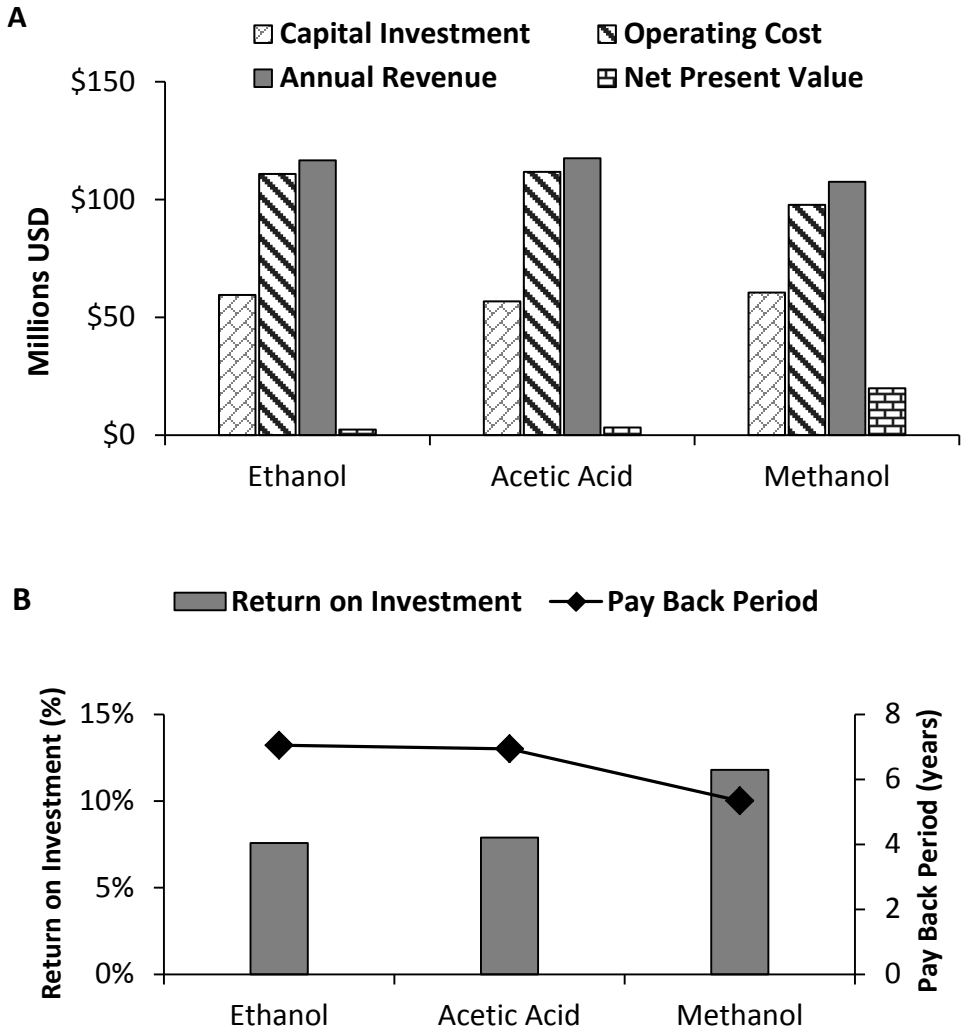

Figure 6.1. Comparison of the capital investments, operating costs, annual revenues and net present value (NPV) (A) and values for return on investment as well as the payback period (B) using different organic solvents for the pre-treatments.

For the evaluations, the lifetime of the biogas plant was assumed to be 15 years. The total energy consumption of the plant was 6.95, 7.0 or 6.85 GWh/year, when applying ethanol, acetic acid or methanol, respectively. The highest net methane production was obtained after the acetic acid pre-treatment amounting to 988 kg/h, whereas 954 kg/h and 890 kg/h methane was generated after pre-treatment with ethanol and methanol, respectively. Regardless of the highest methane yield achieved when applying the acetic acid pre-treatment, the techno-economic evaluation showed that this process alternative was less economically viable. When comparing the capital investments, operating costs, annual revenues and net present value (NPV) for the different pre-treatment methods investigated, it was found that the capital costs for the processes using the acetic acid, ethanol or methanol pre-treatments were 56.7, 59.4 or 60.5 million USD, respectively. However, after 15 years of operation, the NPV was highest, *i.e*. 19.9 million USD, in the case of methanol pre-treatment (Figure 6.1A). Consequently, the payback period (PBP) and return on investment (ROI) were also highest, when methanol was used for the pre-treatment (Figure 6.1B). This is due to the lower price of the methanol, compared to that of the other solvents used in this work.

CHAPTER 7

CONCLUDING REMARKS AND FUTURE DIRECTIONS

7.1 Concluding remarks

Biogas obtained via anaerobic digestion, one of the alternatives for renewable energy production, has been found to be an energy efficient process in terms of energy output/input ratio. The increasing demand for biogas production compels the exploration of new raw materials, preferably from waste streams, since in this way the process addresses both waste reduction and energy production. Lignocellulosic residues and keratin-rich waste streams are readily available and suitable feedstock for biogas production due to their high organic content. However, due to the recalcitrant nature of these materials, their bioconversion to methane is inefficient and very challenging. This thesis explores how to overcome the challenges related to anaerobic digestion of these waste streams.

The major findings of this thesis are summarised as follows:

- Pre-treatment with NMMO can effectively enhance the digestibility of lignocellulosic substrates, achieving nearly 90% of the theoretical methane yield during the subsequent AD process.
- The comparison between the performances when using fresh versus recycled NMMO for the pre-treatments revealed that the composition and structure of the substrate treated influences the dissolution power of the solvent after several courses of recycling. Therefore, the application of this pre-treatment method turned out to be more efficient on lignocellulosic materials with lower lignin content, like straw.
- It was found that even though the NMMO is presented only in small traces in the system, it could negatively affect the AD process. Therefore, a thorough filtration and separation step is recommended after the NNMO pre-treatment to avoid the inhibitory

effects. This also will lead to a more efficient NMMO recovery, which can further improve the economy of the process.

- Pre-treatments of forest residues with acetic acid, ethanol or methanol were successful in terms of increasing the biodegradability of the substrate. In addition, these organic solvents are intermediate products within the AD process; hence, they will not inhibit but rather promote the methane production. Less process water is, therefore, required to filter and separate these solvents from the biomass prior to AD, since small traces of these solvents possibly remaining in the biomass will also be converted into methane.
- Even though the highest methane production was obtained after the acetic acid pre-treatment, the techno-economic evaluation showed that among the treatments, pre-treatment with methanol was the most economically feasible due to the lower cost of this solvent.
- When investigating anaerobic digestion of wool textile waste, it was shown that the combined thermal and enzymatic pre-treatment led to the highest increase in soluble protein and soluble COD content, which subsequently enhanced the methane yield, achieving up to 20 times higher methane production, compared to that in untreated assays.
- Dry AD of lignocellulose- and keratin-rich waste showed higher methane yields and volumetric productivities; therefore, smaller reactor volume and thus less energy consumption for heating as well as cheaper maintenance of the digester would be needed. However, the TS content applied must be adjusted in a way to avoid overloading.

7.2 Future directions

The following investigations will be useful for further development of the AD process from highly recacitrance waste streams:

- Pre-treatment methods applied on the lignocelluloses or keratin-rich residues are mainly evaluated in batch digestion assays aiming to determine the methane potential. Further investigations are therefore needed to assess the long-term effects of the pre-treatment methods, including inhibition products and synergistic or antagonistic effects in a continuous digestion or co-digestion process where lignocelluloses and/or keratin-rich substrates are utilised.

- It will be interesting to examine the interaction of lignin and the extractive compounds in the NMMO-water solution to gain knowledge on the mechanism of the NMMO decomposition after recycling a few times, when lignocelluloses with high lignin content are treated.
- The degradation mechanisms and the effect of the extractive compounds of lignocellulosic biomass on the growth and activity of anaerobic microorganisms are still unclear. Further research in this area might therefore lead to further development in the field of lignocellulosic biogas production.
- The Life Cycle Assessment (LCA) of various pre-treatment processes used for processing recalcitrant biomass for biogas production will also be important to consider in the future.
- There is lack of knowledge on biodegradation of wool textile waste; further exploration of the pre-treatment optimisation and the mechanisms of these processes, among others, would be absolutely necessary for future applications.
- The techno-economic evaluation of solid state AD of lignocelluloses and keratin-rich residues can give a good insight for the economic viability of the process, and hence will approve or disapprove its advantages over the wet AD processes.

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Paper I

Effect of the *N*-Methylmorpholine-*N*-Oxide (NMMO) Pretreatment on Anaerobic Digestion of Forest Residues

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Pretreatment of forest residues using *N*-methylmorpholine-*N*-oxide (NMMO or NMO) prior to anaerobic digestion was investigated, where the effects of particle size, NMMO concentration, and pretreatment time were the primary focus. The pretreatments were carried out on forest residues; with different particle sizes of 2, 4 and 8 mm, at 120 °C for 3, 7, and 15 h in two different modes of NMMO-treatment: dissolution by 85% NMMO and swelling without dissolution using 75% NMMO solution in water. The pretreatment process led to minor changes in the composition of the forest residues. The best improvement in methane yield of the forest residues was achieved by pretreatment using 85% NMMO for 15 h at 120 °C. This treatment resulted in 0.17 Nm^3/kg VS methane yield, which corresponds to 83% of the expected theoretical yield of carbohydrates present in the material. Additionally, the accumulated methane yield and the rate of the methane production were highly affected by the amounts of remaining NMMO when it was not well separated during the washing and filtration steps after the treatment. The presence of concentrations even as low as 0.008% NMMO resulted in a decrease in the final methane yield by 45%, while the presence of 1% of this solvent in the digester completely terminated the anaerobic digestion process.

Keywords: Forest residues; NMMO; Anaerobic digestion; Inhibition; Degradation; Biogas; Lignocelluloses

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INTRODUCTION

Increased concern for the security of the oil supply and the negative impact of fossil fuels on the environment, particularly greenhouse gas emissions, has put pressure on societies to find renewable alternatives (Midilli *et al.* 2006). Bioenergy from renewable resources is a viable alternative to fossil fuels.

Among renewable energies, biogas has great potential as an alternative to fossil fuels. It can be utilized in the generation of power and heat, and it can also be upgraded to gaseous vehicle fuel (Börjesson and Mattiasson 2008; Klass 1998; Louwrier 1998; Saddler 1993). There are several studies that have been carried out on the conversion of wastes (*e.g.*, animal, industrial, household, and municipal) into biofuels by anaerobic biodegradation (Brown 2003; Cheng and Hu 2010; Elango *et al*. 2007; Forgács *et al*. 2012; Klass 1998). Large-scale biogas technologies utilizing a variety of wastes have already been developed in some countries in Europe, such as Germany, Sweden, and the Netherlands. However, to meet the increasing demand for bioenergy production, new raw

materials have to be considered (Petersson *et al*. 2007). One of the most abundant wastes available for biofuel production is lignocellulosic biomass.

Lignocellulosic biomass refers to plant biomass, which is mainly composed of cellulose, hemicellulose, and lignin (Hendriks and Zeeman 2009; Malherbe and Cloete 2002; Percival Zhang *et al*. 2009) and represents the majority of renewable sources of potentially fermentable carbohydrates on earth (Nakamura and Mtui 2003).

However, the anaerobic digestion of lignocellulosic materials is limited by the rate of their hydrolysis (Boone 1984; Noike *et al*. 1985). The main biodegradable polymers in these kinds of biomass, cellulose and hemicellulose, are protected by lignin, a relatively inert three-dimensional polyphenylpropane polymer (Grohmann *et al*. 1986; Sarkanen and Ludwig 1971). This complex structure of lignocellulosic materials therefore results in physical and chemical barriers to biofuel production unless the structure is subjected to a suitable pretreatment prior to anaerobic digestion.

N-methylmorpholine-*N*-oxide (NMMO) is a cellulose solvent that is used industrially for the spinning of cellulosic fibers (the Lyocell process). Recently, it has been shown that when NMMO is used for pretreatment, there is a great improvement in biofuel production from lignocellulosic materials. NMMO is known to change the highly crystalline structure of cellulose after its dissolution and regeneration (Cuissinat and Navard 2006).

A few studies have been carried out on the optimization of NMMO pretreatment conditions prior to bioethanol and biogas production. Shafiei *et al.* (2010) performed NMMO pretreatment on spruce and oak prior to bioethanol production. The pretreatment of oak and spruce at 130 °C with 85% NMMO resulted in almost total conversion of cellulose into ethanol and improved the ethanol yields up to 85.4 and 89%, respectively. Poornejad *et al.* (2013) investigated the effects of NMMO-pretreatment on rice straw for bioethanol production. The results of their study showed a significant improvement in the enzymatic hydrolysis of rice straw followed by fermentation into bioethanol. These results showed a promising effect of NMMO pretreatment on enzymatic hydrolysis. However, because bacterial hydrolysis has a different mechanism than cellulase enzymatic hydrolysis, it is not possible to conclude that the NMMO pretreatment can also be conducive to biogas processes.

Regarding biogas production, Jeihanipour *et al.* (2009) studied NMMO pretreatment of highly crystalline pure cellulose, which resulted in subsequent 100% conversion of cellulose into methane after 15 days of digestion. In another study, Teghammar *et al.* (2012) investigated biogas production from rice and triticale straws and spruce chips by NMMO pretreatment. The best conditions for the NMMO pretreatment in their work led to 87% of the theoretical methane yield.

In this work utilization of forest residues for biogas production was investigated due to its abundance in Sweden. In 2008, the tree branches and tops that were received from Swedish forests amounted to about 1.6 megatonnes total solids/year. This amount is expected to increase to 3.5 megatonnes total solids/year by 2018 (Thuresson 2010).

This paper addresses two main issues regarding NMMO pretreatment prior to biogas production: first, the optimization of the NMMO pretreatment was performed on an inhomogeneous waste stream of lignocellulosic biomass, *i.e.*, forest residues. The pretreatments were carried out in two modes of action, *i.e.* dissolution and swelling using 85% and 75% NMMO, respectively. Furthermore, the effect of particle size (2, 4, and 8 mm) and the treatment time (between 3 and 15 h) were also considered. Secondly, since, traces of remaining NMMO from the pretreatment may affect the anaerobic digestion

process, the presence of different concentrations of NMMO in anaerobic digestion process were studied. This is the first work on NMMO pretreatment of forest residues and the limitations caused by NMMO in anaerobic digestion systems.

EXPERIMENTAL

Materials

Native forest residue, an inhomogeneous mixture of spruce, pine bark, *etc*., was obtained from the forest outside Borås, Sweden. The material was dried at room temperature for a couple of days and then cut, milled, and screened to achieve three different fractions with particle sizes of 2, 4, and 8 mm.

Methods

Industrial-grade (50% w/w) NMMO solution (BASF, Ludwigshafen, Germany) was used in all pretreatment experiments. The concentration of NMMO was first increased to 75% and 85% (w/w) using a rotary evaporator (Laborata 20 eco, Heidolph, Germany) operating at an absolute pressure of 100 mbar and a maximum temperature of 130 °C. The NMMO solution was supplemented with 0.625 g/kg propylgallate to prevent oxidation of the NMMO during pretreatment (Bang *et al*. 1999; Kim *et al*. 2006).

For the pretreatments, 94 g of 85% or 75% NMMO solution were mixed with 6 g dry weight of forest residues with particle sizes of 2, 4, or 8 mm in 250-mL blue-cap bottles (Lennartsson *et al*. 2011). The bottles were then placed in an oil bath at 120 °C for 3, 7, and 15 h. The mixtures were stirred every 15 min with a glass rod (Shafiei *et al*. 2010), except for the 15-h pretreatment, where the mixtures were left overnight without mixing after 7 h. The pretreatment was stopped, and the cellulose was recovered by the addition of 150 mL of boiled distilled water followed by vacuum filtration and washing with hot (40 to 50 ºC) distilled water until a clear filtrate was achieved (Shafiei *et al*. 2010). The pretreated materials were stored at 4 °C until further investigations were conducted in anaerobic digestion assays. In addition, part of the materials was freeze-dried to prepare samples for further analyses.

Batch Anaerobic Digestion Assays

Batch digestion assays were carried out according to the method described by Hansen *et al*. (2004) using thermophilic inoculum obtained from a large-scale digester treating municipal solid waste at 55 °C (Borås Energy and Environment AB, Sweden). The total solids (TS), volatile solid (VS), and volatile fatty acids (VFA) content of the inoculum was 2.77 %, 1.68 %, and 1.90 %, respectively. The digesters used in the assays were serum glass bottles with 118 mL of total volume that were closed with butyl rubber seals and aluminum caps. Each flask contained 30 mL of inoculum and 0.25 g volatile solids (VS) of substrate to achieve a VS ratio of inoculums to substrate of 2:1. Furthermore, inoculums alone were used as blanks for the determination of the gas production of the inoculum itself. In addition, pure cellulose (Cellulose Fibrous Long, Sigma Aldrich, Germany) was used as a control substrate to check the quality of the inoculum. Moreover, the inhibition effect of NMMO was investigated by digestion of pure cellulose fibers in the presence of different concentrations (between 6.4×10^{-5} and 1%) of NMMO.

All experimental setups were performed in triplicate. Finally, the headspace of each bottle was flushed with a gas mixture of 80% nitrogen and 20% carbon dioxide to obtain anaerobic conditions. Gas samples were withdrawn regularly from the headspace of each bottle and analyzed by gas chromatography (GC) to obtain the accumulated methane production during the digestion period of 50 days. The amount of methane produced in the reactor headspace was then calculated using the data from the GC measurements as described by (Teghammar *et al*. 2010).

Analytical Methods

The total solids (TS) and volatile solids (VS) in the different samples were determined by first oven drying to a constant weight at 105 °C , followed by ignition at 575 °C in a furnace (Sluiter *et al*. 2008a). The cellulose, hemicellulose, and lignin contents of the pretreated or untreated lignocelluloses were determined according to NREL procedures (Sluiter *et al*. 2008b). In this method, a two-step acid hydrolysis with concentrated and diluted sulfuric acid was performed to liberate the sugars from the cellulose and the hemicellulose. The formed sugars were then quantified by HPLC. The acid-soluble lignin was measured using UV spectroscopy at 280 nm, and acid-insoluble lignin was determined after drying followed by ignition at 575 °C. All lignin and carbohydrate analyses were performed in duplicate.

The total carbohydrate (cellulose and hemicelluloses) were analyzed using HPLC (Waters 2695, Millipore, Milford, U.S.A.) equipped with a refractive index (RI) detector (Waters 2414, Millipore, Milford, U.S.A.) and an ion-exchange column (Aminex HPX-87P, Bio-Rad, U.S.A.) at 85 °C using ultra-pure water as the eluent with a flow rate of 0.6 mL/min.

The methane produced in anaerobic digestion was measured using a gas chromatograph (Auto System PerkinElmer, Inc., Waltham, MA) equipped with a packed column (PerkinElmer, 60x1, 800OD, 80/100, Mesh) and a thermal conductivity detector (PerkinElmer) with an injection temperature of 150 °C. The carrier gas used was nitrogen, with a flow rate of 23 mL/min at 60 °C. A 250-µL pressure-tight gas syringe (VICI, Precision Sampling Inc., LA) was used for the gas sampling. Excess gas was released through a needle after the gas analyses to avoid overpressure higher than 2 bar in the head space of the flasks. All methane volumes are presented at standard condition (temperature 273 K, and pressure 101,325 Pa).

Kinetic Model

A first-order kinetics model described previously by Jimenéz *et al.* (2004) was used to determine the inhibition effects of the presence of different concentrations of NMMO on the anaerobic digestion process,

$$G = G_m(1-e^{-K_0 t}), \quad (1)$$

where G is the accumulated methane volume (mL) after a time t (days), G_m is the maximum accumulated methane volume (mL) after an infinite digestion time, and K_0 is the observed specific rate constant of the overall process (days^{-1}). To calculate the value of the specific rate constant, Eq. (1) is transformed as follows:

$$Ln(\frac{G_m}{G_m - G}) = K_0 t \quad (2)$$

Statistical Analysis

All experiments in this study were carried out in triplicates. The significant differences between methane productions obtained by anaerobic batch digestion assays of untreated *vs* treated samples was verified by t-tests using a software package MINITAB® (V 15.0). All error bars and intervals reported represent 95% confidence intervals.

RESULTS AND DISCUSSION

Pretreatment of forest residues with particle sizes of 2, 4, and 8 mm, was performed using 75 and 85% w/w NMMO solution at 120 °C for 3, 7, and 15 h, and the effects of the pretreatment on the composition and the methane yield were investigated. This organic solvent has shown a high potential to enhance the digestibility of lignocellulose. However, so far little attention has been paid to possible inhibitory effects of this solvent in an anaerobic digestion system. Therefore, the effects of different concentrations of NMMO in the anaerobic digestion process were also explored in this study. The purpose of this investigation was to verify that the presence of the solvent after insufficient washing following the pretreatment step might inhibit the anaerobic digestion process.

Carbohydrate Composition of Untreated and NMMO-Treated Forest Residues

The results of the compositional analyses regarding the contents of total carbohydrates and total lignin were carried out only on the smallest particle size (2 mm) of the forest residues (Table 1). Other components, such as extractives and acetyl content, were not analyzed. The content of total carbohydrates in the untreated forest residues was 41.6 %. The content of total carbohydrates increased slightly as a result of the NMMO treatment, achieving values between 44.1 and 49.3 % (Table 1). The highest total carbohydrate content was obtained when the longest treatment time (15 h) and 85% NMMO was applied. While the content of total carbohydrates increased with increased treatment times, the total lignin content decreased. The total lignin content (acid soluble lignin and acid insoluble lignin) of untreated forest residues was 43.4 %, and this value was reduced after the treatment to between 37.4 and 39.2 % (Table 1). In general, the results of the compositional analyses show that the treatment did not seriously affect the composition of the substrate. These results are in accordance with previous findings of NMMO pretreatment of spruce, birch, and rice straw (Goshadrou *et al*. 2013; Poornejad *et al*. 2013; Teghammar *et al*. 2012).

Effects of NMMO-Pretreatment on Anaerobic Digestion

The results of accumulated methane yields obtained after 50 days of digestion are shown in Fig. 1. The methane potential of untreated assays of forest residues with particle sizes of 2, 4, and 8 mm were 0.07 ± 0.007, 0.03l ± 0.009, and 0.00 Nm^3 CH_4/kgVS, respectively. However, after the pretreatment, methane yields increased up to 10, 15, and 50 times for particle sizes of 2, 4, and 8 mm, respectively.

Table 1. Pretreatment Conditions, Lignin and Carbohydrate Content, Initial Methane Production Rates, and Accumulated Methane Yields of Untreated and Treated Forest Residues

NMMO Conc. (%)	Time (h)	Total Carbohydrates (%)	Total Lignin (wt %)	Initial methane production rates * (Nm^3CH_4/kg VS)	Accumulated methane yield (Nm^3CH_4/kg VS)
Untreated	-	41.6	43.4	0.005	0.07± 0.007
85%	15	49.3	38.1	0.012	0.17± 0.020
85%	7	45.3	39.6	0.009	0.15± 0.018
85%	3	45.2	40.6	0.003	0.12± 0.028
75%	15	46.2	37.4	0.004	0.11± 0.014
75%	7	46.0	38.4	0.003	0.11± 0.012
75%	3	44.1	39.2	0.000	0.13± 0.051

* Initial digestion rate determined as the methane production per day during the first 12 days of the digestion period

The best results of anaerobic digestion were obtained when forest residues with 2-mm particle size were treated with 85% NMMO for 15 h, resulting in a methane yield of 0.17 ± 0.020 Nm^3 CH_4/kgVS. This is an improvement by 152% compared with the yield of 0.07 ± 0.007 Nm^3 CH_4/kgVS measured from untreated forest residues (Fig. 1A). The decrease in NMMO concentration to 75% contributed to a lower methane yield of 0.13 ± 0.051 Nm^3 CH_4/kgVS after 3 h of treatment. In contrast, longer pretreatment time did not necessarily lead to a higher biogas yield (Fig. 1A). Treatment of forest residues with larger particle sizes of 4 and 8 mm resulted in methane yields of up to 0.10 and 0.05 Nm^3 CH_4/kgVS, respectively. This was to be expected because decreasing the particle size increases the surface area, which will in turn lead to better enzymatic degradation (Taherzadeh and Karimi 2008; Teghammar *et al*. 2012).

To verify the significance of differences between the methane yields obtained from untreated *vs.* treated samples, a statistical analysis using *t-test* was performed on data showing the best performance *i.e.* 2 mm particle size. The results showed that the enhancement in the accumulated methane production after the treatment was significant when pretreatment time of 15 h was applied in both dissolution (85% NMMO) and swelling (75% NMMO) mode (p-value 0.001 and 0.005, respectively) (Fig. 1A and Table 2). Whereas, applying pretreatment time of 7 h, showed significant effect only in the case of dissolution mode (85% NMMO), p-value 0.003 (Fig. 1A and Table 2). The 3-h pretreatment did not cause any significant increase in the accumulated methane production in any cases.

Table 2. Evaluation of significant differences between the accumulated methane yields of untreated *vs.* pretreated samples of 2mm particle size

NMMO Conc. (%)	Time (h)	P-Value Compared to Untreated Sample	Significant Difference Yes/No
85%	3	0.059	No
85%	7	0.003	Yes
85%	15	0.001	Yes
75%	3	0.240	No
75%	7	0.061	No
75%	15	0.005	Yes

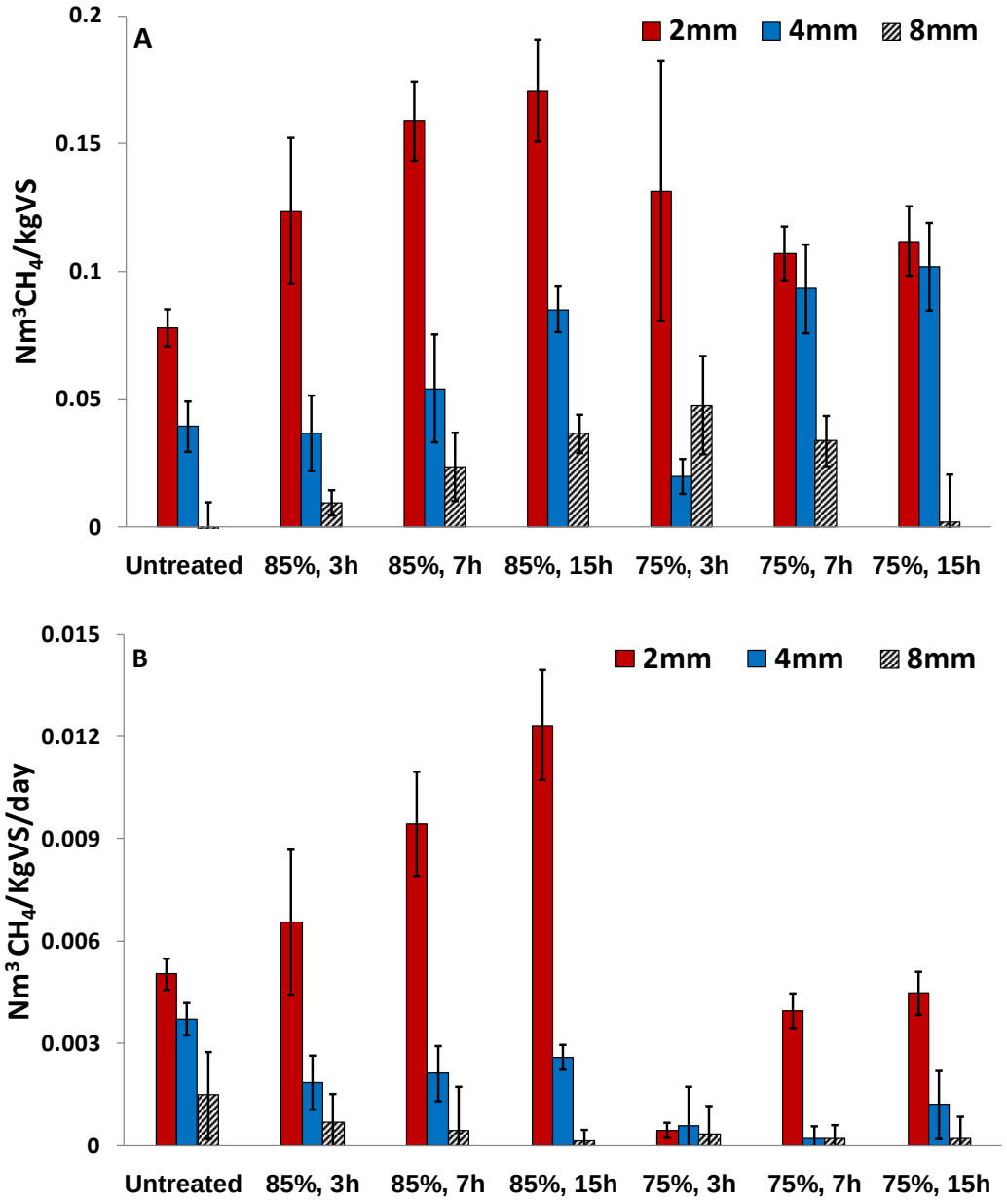

Fig. 1. Accumulated methane yield during 50 days of anaerobic digestion of untreated and NMMO-pretreated forest residues expressed as Nm^3 CH_4/kgVS (A) Initial digestion rate determined as the mean of the methane production per day during the first 12 days of the digestion period expressed as Nm^3 CH_4/kgVS/day (B).

These results indicate that changes in the structure of highly crystalline cellulose in cellulose dissolution and swelling without dissolution are completely different processes (Jeihanipour *et al*. 2009; Zhang *et al*. 2006). The dissolution mode of NMMO pretreatment was more successful because the hydrogen bonds and weak van der Waals forces between cellulose chain molecules break in this mode of action. Once these forces are broken during the dissolution, the chains are free to rearrange themselves. Subsequently, when NMMO is removed, the cellulose chains can create new bonds in a less crystalline state. However, in cellulose swelling mode, the gross structure of the

cellulose remains unchanged, even though significant physical changes resulting in an increase in the sample volume by uptake of the NMMO take place (Zhao *et al*. 2007).

The initial reaction rates were determined as the means of the methane production per day during the first 12 days of the incubation period and are presented in Fig. 1B. Again, the highest digestion rate of 0.012 Nm^3 CH_4/kgVS/day was achieved when forest residues with 2-mm particle size were treated with a higher concentration of NMMO (85% w/w) for 15 h.

For larger particle sizes (*i.e*., 4 and 8 mm), however, a long lag phase was observed (data not shown). This might be due to the low efficiency of the NMMO pretreatment on larger particle sizes for reducing the highly crystalline cellulose. Weimer *et al*. (1990) reported that the presence of highly crystalline cellulose in digestion may lead to much longer lag time compared to amorphous cellulose. Their explanation for this phenomenon was that the cellulolytic microorganism may attach more rapidly to and/or more readily recognize the amorphous cellulose than the crystalline cellulose (Weimer *et al*. 1990, 1991.

Additionally, comparisons between the initial reaction rates of the pretreated assays with particle sizes of 4 and 8 mm and untreated assays with similar particle sizes showed noticeably slower reaction rates (Fig. 1B). This might be due to the inhibitory effect of the remaining NMMO on the anaerobic digestion process. This finding is in accordance with previous work on oil palm empty fruit bunch (OPEFB), where it was found that the presence of commercial NMMO can significantly inhibit the process of digestion (Purwandari *et al*. 2013). In another study, the inhibitory effect of NMMO on Zygomycetes fungi was also observed during bioethanol production (Lennartsson *et al*. 2011). However, as shown in Fig. 1A, the accumulated methane production of the pretreated materials with larger particle sizes was higher compared to that of the untreated ones, which shows that the methanogen bacteria may adapt to the presence of small amounts of NMMO that is eventually present in the broth during the longer period of the digestion tests.

In general, pretreatment with NMMO is a beneficial method compared to many other pretreatments because the composition of the treated wood remains unchanged, including the hemicelluloses (Purwandari *et al*. 2013; Shafiei *et al*. 2010). Furthermore, it provides high flexibility in the choice of lignocellulosic feedstocks (Rosenau *et al*. 2001). However, the main drawbacks of NMMO pretreatment are longer pretreatment times and the need for a very efficient recovery and recycling of the treatment chemical after the treatment (Hall *et al*. 1999).

NMMO as an organic solvent possesses a highly polar nature that provides an excellent disruption of the extensive hydrogen-bonded network formed by carbohydrate polymers (Kuo and Lee 2009; Rosenau *et al*. 2001). The water added at the end of the treatment acts as an anti-solvent agent, leading to the regeneration of cellulose. During this dissolution regeneration process, the crystalline structure of cellulose I changes into cellulose II, making it more accessible to the degrading cellulolytic enzymes during the anaerobic digestion.

The results of this work shows that the interaction between the solvent and the forest residues seems to be more effective when decreasing the particle size and increasing the treatment time (Fig. 1). Additionally, increasing the concentration of the solvent (from 75% to 85%) showed considerable improvement in digestibility. This result is in agreement with Jeihanipour *et al.* (2009), who reported an efficient conversion of

cellulose I into cellulose II by treating cellulose fibers in 85% NMMO prior to enzymatic hydrolysis.

Inhibition Effects of NMMO on the following Anaerobic Digestion Process

Despite the positive effects of NMMO pretreatments, one of the drawbacks might be the presence of the solvent after insufficient washing, which might inhibit the subsequent anaerobic digestion process. Purwandari *et al.* (2013) examined the inhibitory effect of the NMMO in the batch mode of anaerobic digestion. For this purpose, 2.5 g/L commercial NMMO solution was added to the inoculum and digested at 55 °C. The results of their study showed that only 15% of the expected gas production from the inoculum was achieved in the presence of the NMMO at this concentration. For that reason, in this work, a more detailed analysis of the inhibitory effects has been carried out. Anaerobic digestion assays on pure cellulose with NMMO added at different concentrations (between 0 and 1%) were performed. All the reactors contained 8 g VS/L cellulose, and the results of the accumulated methane production during the 50-d incubation period are shown in Fig. 2A. The results indicate that NMMO concentrations as low as 0.0016% can reduce the accumulated methane yield by 34% (Fig. 2A and Table 3). No inhibition has been observed at concentrations below 0.000064%. However, the methane yield was decreased by almost 50% in reactors containing NMMO at concentrations between 0.0016 and 0.02%. Moreover, the highest concentration of NMMO (1%) resulted in negligible methane yield, indicating that the microorganisms involved in the digestion process were completely inhibited.

Previously, Jeihanipour *et al.* (2009), examined the effect of addition of 0.5% NMMO on enzymatic hydrolysis of cellulose, which reduced the hydrolysis rate by 12 %. In contrast in this work, 51% reduction in accumulated methane production from cellulose was obtained after addition of 0.2% NMMO in the anaerobic digestion system. This reveals a high adverse sensitivity of the methane-producing microorganism to this organic solvent. Additionally, it explains that the mechanism of the methane-producing microorganisms is rather different from the enzymatic hydrolysis.

The degradation pathway of NMMO begins with the reduction of NMMO to N-methylmorpholine (NMM), which is subsequently demethylated and transformed into morpholine and formaldehyde (Rosenau *et al*. 2001). NMMO was considered to be persistent until Meister and Wechsler (1998) showed that it could be metabolized by certain microbial species/environments as activated sludge, anaerobic degradation processes, and two yeast cultures (Fig. 3).

The adaptation of the microorganisms to NMMO and its metabolites is a sequential process. First, the microorganism must be adapted to NMMO to form NMM. The adaptation to NMM can take a number of days to reach a certain threshold concentration. Therefore, the NMM degradation cannot start until NMMO has been reduced to NMM. In the same way, morpholine degradation is only possible until the sludge is adapted to NMM. Morpholine is thus a much better biodegradable compound than NMMO or NMM (Schräder *et al*. 2000).

The reduction of NMMO to NMM was also observed under anaerobic conditions; however, the reaction stopped at NMM, and no further biodegradation was obtained, even with the presence of a co-substrate such as glucose, under the conditions tested (Knapp *et al*. 1996).

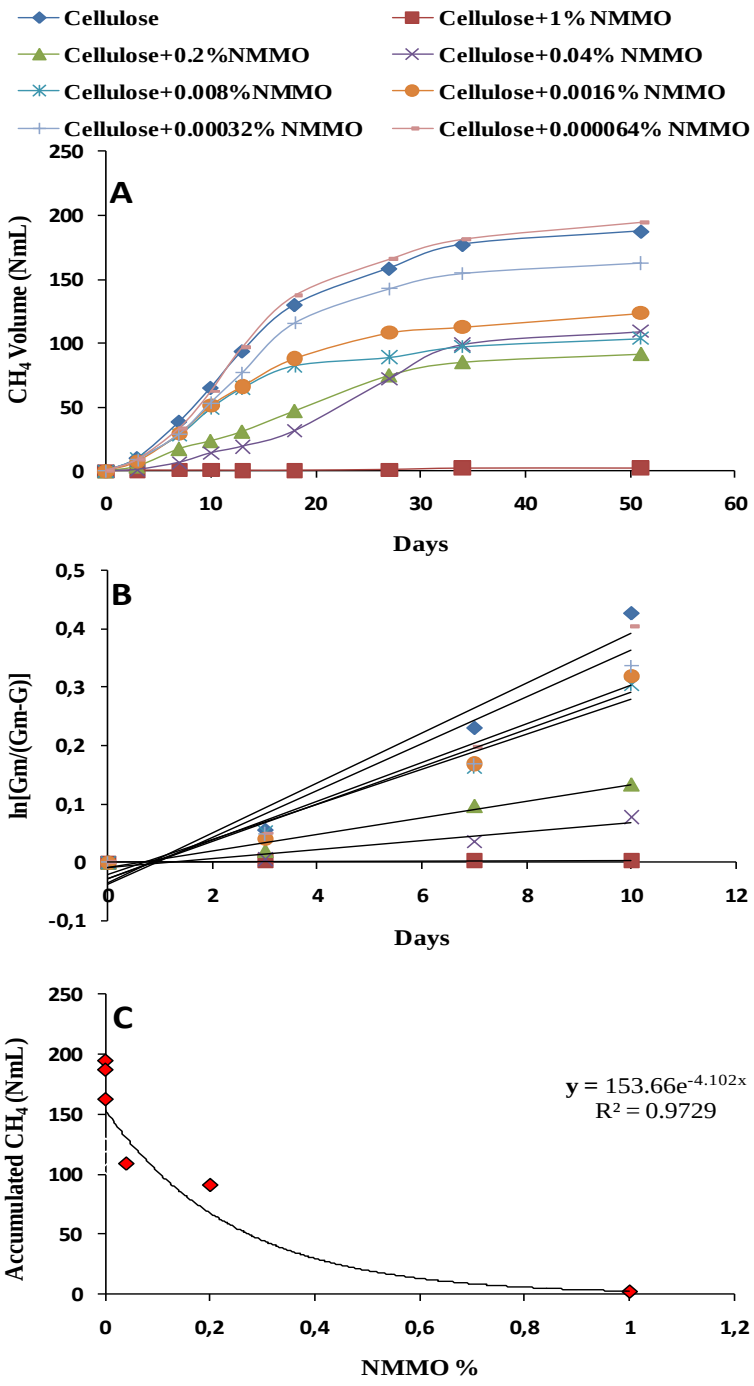

Fig. 2. Methane production obtained from cellulose with the addition of different concentrations (0.000064 to 1%) of NMMO. Accumulated produced volume CH4 (mL) during the incubation period of 50 days (A) Kinetic evaluation of the digestion process: values of ln[Gm/(Gm-G)] as a function of time (days) for pure cellulose and cellulose together with different concentrations (0.000064 to 1%) of NMMO (B) Correlation between accumulated methane yield (NmL) and NMMO concentrations (C)

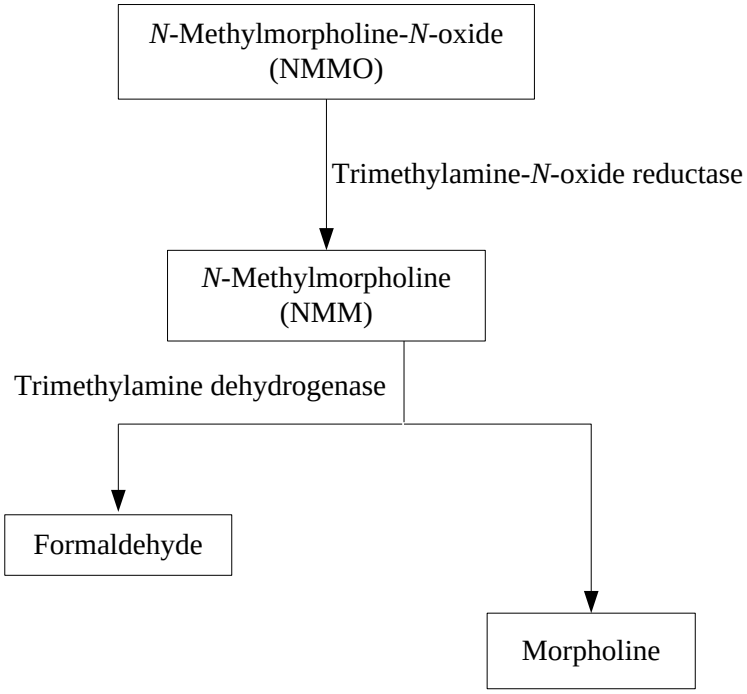

Fig. 3. Main degradation products of NMMO (Meier and Turnbull 2013)

To characterize the inhibition effects, a first-order kinetics model was used (Jiménez *et al*. 2004). Figure 2B provides information about the kinetics of the degradation within the first 10 days of digestion. The results show that not only accumulated methane production (Fig. 2A), but also the degradation rate declined with increasing NMMO concentrations in the reactors (Fig. 2B and Table 3). The methane production rate and NMMO concentration in the digester were correlated (R^2=0.973 in Fig. 2C). Moreover, the results presented in Table 3 show a direct correspondence between the NMMO concentrations and final methane yield in the systems.

Table 3. Accumulated Methane Production and Specific Rate Constant K_0 Obtained During 50 Days of Incubation of Cellulose with Different Concentrations of NMMO

Sample sets	Specific rate constant K_0 (day^{-1})	Accumulated methane production (NmL)	Final methane yield compared to pure cellulose (%)
Cellulose +1% NMMO	0.0003	2.42	1.30
Cellulose +0.2% NMMO	0.0142	91.30	48.71
Cellulose +0.04% NMMO	0.0077	109.15	58.23
Cellulose +0.008% NMMO	0.03	103.95	55.46
Cellulose +0.0016% NMMO	0.0319	123.63	66.00
Cellulose +0.00032% NMMO	0.0331	162.85	86.88
Cellulose +0.000064% NMMO	0.0399	194.99	104.05
Cellulose	0.0428	187.43	–

*The inhibition effects are expressed as percentage of methane yield of that obtained for the control, *i.e.,* pure cellulose fibers.

The values of K_0 obtained for cellulose with no addition of NMMO and in the presence of very low concentrations of NMMO (0.000064% and 0.00032%) was 0.04, 0.04, and 0.03 d^{-1}, respectively, which was considerably decreased in the presence of higher concentrations of NMMO (Table 3). Finally, the results of this study showed that NMMO could have a significant effect on anaerobic digestion. However, because no NMMO levels were measured throughout the digestion process, it is not possible to establish if NMMO was reduced to NMM and whether it was the NMM accumulation or the NMMO itself that was the factor resulting in the inhibition of the process. Further investigations are therefore recommended to study the degradability of NMMO and its metabolites in anaerobic systems.

CONCLUSIONS

1. The dissolution mode of NMMO treatment using 85% NMMO resulted in 83% of the theoretical yield, which is almost three-fold higher methane production compared to that observed from untreated forest residues.
2. The advantage of NMMO pretreatment is that it does not cause destruction of cellulose and hemicellulose, while the lignin content was decreased by approximately 7% when the longest pretreatment time (15 h) was applied.
3. The washing and filtering steps seem to be critical for the performance of the subsequent anaerobic digestion process, as NMMO remaining in concentrations higher than 0.002% considerably decreased the methane yield.

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Paper II

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Short Communication

Biogas production from lignocelluloses by *N*-methylmorpholine-*N*-oxide (NMMO) pretreatment: Effects of recovery and reuse of NMMO

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HIGHLIGHTS

- NMMO pretreatment of forest residues and straw for enhanced CH_4 yield was performed.
- The best pretreatment conditions resulted in 100% improvement in methane yield.
- Recovery of NMMO is critical for having an economically feasible process.
- Pretreatment efficiency with recycled NMMO depends on lignocelluloses' composition.
- The performance of recycled NMMO deteriorated when forest residues were pretreated.

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ABSTRACT

The effects of *N*-methylmorpholine-*N*-oxide (NMMO) pretreatment on barley straw and forest residues were investigated for biogas production. The pretreatments were performed at 90 °C with 85% NMMO for 3–30 h. The best pretreatment conditions resulted in 100% improvement in methane yield during the subsequent digestion compared to that of the untreated lignocelluloses. Methane yields of 0.23 and 0.15 Nm^3 CH_4/kg VS were obtained from barley straw and forest residues, respectively, corresponding to 88% and 83% of the theoretical yields. In addition, the effects of the pretreatment with recovered and reused NMMO was also studied over the course of five cycles. Pretreatment with recycled NMMO showed the same performance as the fresh NMMO on barley straw. However, pretreatment of forest residues with recycled NMMO resulted in 55% reduction in methane yield.

1. Introduction

Lignocelluloses are the major constituent in several waste streams such as; forestry, agriculture, and municipalities. The production of lignocellulosic materials is reported to be about 200 billion tons per year (Zhang, 2008). These abundant, carbon-rich substrates can be considered as an appropriate source for biogas production; however, the compact recalcitrant structure of lignocelluloses makes them difficult to degrade biologically. Therefore, a key step for producing biogas from lignocelluloses is the introduction of a suitable pretreatment prior to anaerobic digestion.

There are several pretreatment methods investigated on lignocellulosic biomass prior to anaerobic digestions. Among those, the pretreatments based on cellulose dissolution have several advantages using milder conditions than thermal pretreatments, and are effective for reducing the cellulose crystallinity (Taherzadeh and Karimi, 2008). *N*-methylmorpholine-*N*-oxide (NMMO) is a cellulose solvent, which is able to efficiently decrease the cellulose crystallinity due to the high polarity of its N—O bonds (Cuissinat and Navard, 2006). According to previous studies, the digestibility of spruce, birch, rice, triticale straw, and forest residues were increased by pretreatment with 85% or 75% NMMO at 120–130 °C, resulting in increased biogas yields (Goshadrou et al., 2013; Kabir et al., 2013; Teghammar et al., 2012b).

The current study deals with two main challenges regarding the NMMO pretreatment prior to biogas production. In the first part, the optimisation of the NMMO pretreatment in dissolution mode (85% W/W) on barley straw and forest residues was carried out at relatively low temperature (90 °C). Using a lower temperature is beneficial, since available excess heat from power plants or district heating systems can be used for the pretreatment. However, because of the lower severity of the treatment longer duration

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times, *i.e.*, 3, 7, 15 and 30 h were investigated. Additionally, due to the high cost of the solvent, recovery and reuse of the NMMO is a crucial factor for an economically viable process (Teghammar et al., 2014). However, so far no attention has been paid to examine the efficiency of the pretreatment on different lignocellulosic substrates, neither with low nor high lignin content, after the recovery and reuse of the NMMO. Therefore, in the second part of this study, the effects of the pretreatment to improve the biogas yield from barley straw and forest residues was examined after recycling the NMMO over the course of five cycles.

2. Methods

2.1. Materials

The lignocellulosic substrates used in this work were forest residues; mixture of spruce and pine with high bark content, obtained from the forest areas around Borås (Sweden), and barley straw received from a farmland outside Uppsala, Sweden. Prior to the treatment, these materials were milled to approximately 2 and 5 mm particle size, respectively, using a sieve shaker (Octagon 200, U.K.).

2.2. Pretreatment

A commercial grade 50% (w/w) NMMO solution (BASF, Ludwigshafen, Germany) was used in all experiments. This solution was concentrated to 85% (w/w) NMMO using a rotary evaporator (Laborata 20 eco, Heidolph, Germany), as described previously by Kabir et al. (2013). The pretreatment was carried out by mixing 6% (w/w) dry weight of substrates (forest residues and barley straw) with 85% NMMO solution in 5 L beakers. The reaction mixtures were then placed in an oil bath at 90 °C for 3, 7, 15, and 30 h and were stirred continuously throughout the pretreatment. The pretreatment was then stopped and the dissolved materials were recovered by the addition of an anti-solvent, which was boiled-distilled water, followed by vacuum filtration, and washing with hot distilled water. This washing/filtration step was repeated at least three times until no trace of NMMO was observed in the filtrate, *i.e.*, until a clear filtrate was achieved. The pretreated substrates were stored at 4 °C until being used in anaerobic digestion assays. In addition, some of the materials were freeze-dried to prepare samples for further compositional analyses.

2.3. Anaerobic batch digestion assays

The anaerobic batch digestion assays were performed for triplicate samples at 55 °C. The digesters were serum glass bottles with 118 mL total volume, closed with butyl rubber seals and aluminum caps (Hansen et al., 2004). The inoculum was obtained from a 3000-m^3 large municipal solid waste digester operating at thermophilic (55 °C) conditions (Borås Energi och Miljö AB, Borås, Sweden). Each flask contained 26 mL inoculum and 0.15 g VS (Volatile Solids) of untreated or treated substrate, and the final working volume was adjusted to 30 mL by the addition of deionised water. Furthermore, a mixture of deionised water and inoculum was used as a blank to determine the gas production of the inoculum.

The headspace of each bottle was flushed with a mixture of 80% nitrogen and 20% carbon dioxide to obtain anaerobic conditions. Gas samples were withdrawn regularly from the headspace of each bottle, and the accumulated methane production was determined using gas chromatography.

2.4. NMMO recovering and reuse

The performance of the pretreatment by utilising recycled NMMO instead of fresh NMMO was one of the objectives in this work. This is in fact one of the most critical parameters for an economically viable process. For this purpose, NMMO was recycled after 30 h pretreatment and reused over the course of five cycles.

The concentration of the NMMO/water solution obtained from filtration after the pretreatment was determined by titration with 0.1 M HCl and subsequently, the excess water was separated using a rotary evaporator (Laborata 20 eco, Heidolph, Germany) to re-concentrate the NMMO/water solution to 85%. NMMO is not volatile; therefore, water can be evaporated under vacuum conditions.

2.5. Analytical methods

Total Solids (TS) and Volatile Solids (VS) were determined as described by Sluiter et al. (2008a). The compositional analyses to determine the cellulose, hemicellulose, and lignin contents were performed according to the standard procedure presented by Sluiter et al. (2008b) as described previously in details by Kabir et al. (2013).

The methane produced in the anaerobic digestion was measured using a gas chromatograph (Auto System Perkin Elmer, Waltham, MA), equipped with a packed column (Perkin Elmer, 60 × 1, 800OD, 80/100, Mesh) and a thermal conductivity detector (Perkin Elmer) with an injection temperature of 150 °C. The carrier gas used was nitrogen, with a flow rate of 23 ml/min at 60 °C. A 250 μl pressure-tight gas syringe (VICI, Precision Sampling Inc., LA) was used for the gas sampling. Data analysis was performed as described by Hansen et al. (2004) and Teghammar et al. (2010).

In order to determine structural changes caused by the treatment, a modified version of the Simons' Staining procedure developed previously by Chandra et al. (2009) was used. The measurements were performed as described in details by Teghammar et al. (2012a).

The crystallinity of the forest residues pretreated with the fresh NMMO as well as with recycled NMMO versus that of the untreated materials was examined using Fourier Transform Infrared (FTIR) spectrometer (Impact 410, Nicolet Instrument Corp., Madison, WI), and the analyzing software used was Nicolet OMNIC 4.1.

Statistical analysis was performed using Tukey test to verify the effect of time on methane yields of pretreated assays, for a 95% confidence intervals with software package MINITAB® (V 17.0).

3. Results and discussion

3.1. Compositional and structural analyses before and after NMMO treatment

The results of the compositional analyses regarding the contents of the total carbohydrates and total lignin are presented in Table 1. The total carbohydrate content in the untreated barley straw and forest residues was 59.8% and 41.6%, respectively, and it increased slightly for both pretreated barley straw and forest residues, achieving values between 60.4% and 63.8% and between 41.8% and 44.6%, respectively. The highest total carbohydrate content for both of these substrates was obtained when the longest retention times (15 and 30 h) were applied to the treatment. The total lignin content of the untreated straw was 22%, and this value changed after the treatment to between 20.8% and 24.3%. For forest residues, the total lignin content for the untreated sample was 43.4%, which was reduced slightly to between 40.5% and 43.3% after the NMMO treatment (Table 1). In general, the results of the compositional analyses showed that the treatment does not

Table 1
Total carbohydrate and lignin contents as well as accumulated methane production together with applied pretreatment conditions for the untreated and NMMO-pretreated barley straw and forest residues.

NMMO Conc. (%)	Temperature (°C)	Time (h)	Total carbohydrates (w/w%)	Total lignin (w/w%)	Accumulated methane yield (Nm^3 CH_4/kg VS)
Pretreatment conditions					
Untreated St[a]	–	–	59.8	22.0	0.12 ± 0.011
85	90	3	60.4	20.8	0.16 ± 0.031
85	90	7	62.7	21.6	0.23 ± 0.004
85	90	15	63.8	24.3	0.21 ± 0.010
85	90	30	61.1	23.3	0.22 ± 0.012
Untreated Fr[b]	–	–	41.6	43.4	0.07 ± 0.005
85	90	3	41.8	42.7	0.08 ± 0.002
85	90	7	42.6	43.3	0.12 ± 0.006
85	90	15	44.4	40.5	0.13 ± 0.013
85	90	30	44.6	41.1	0.15 ± 0.015

[a] St stands for barley straw.
[b] Fr stands for forest residues.

significantly affect the composition of these materials. These results are in accordance with previous reports following the NMMO pretreatment of spruce, birch and rice straw (Goshadrou et al., 2013; Teghammar et al., 2012b). The observations on the compositional analyses also illustrated the advantage of the NMMO pretreatment on lignocellulosic biomass, since it does not lead to a loss of carbohydrates which can be a major problem in other pretreatment methods such as biological pretreatments, wet oxidation, steam explosion and alkaline pretreatment (Taherzadeh and Karimi, 2008). Furthermore, this composition analysis gives a comprehensive overview by comparing these two lignocellulosic substrates. The lignin content of the forest residues is almost twice as high as it is in the barley straw; on the other hand, the total carbohydrate content of the straw is higher. Since the lignin is particularly difficult to degrade and the presence of lignin reduces the availability of the other cell wall constituents for biological degradation, it is therefore expected that the straw will be degraded more easily in comparison to the forest residues during the following anaerobic digestion process.

The structural changes by the means of determining the inner and outer surface area of the untreated *vs*. treated substrates were investigated by using the Simons' Staining technique and the results are summarised in Fig. 1. Since the orange dye (OD) has a bigger molecular size of 5–36 nm compared to the size of a typical bacterial cellulosome of approximately 4–16 nm (Palmowski and Müller, 2003), the enzyme can therefore penetrate into a pore at all the places where the orange dye penetrates. Accordingly, the orange dye adsorption gives an indication of how accessible the substrate is for the degrading enzyme. Blue dye, however, has a lower affinity for cellulose with a smaller molecular diameter (1 nm). Therefore, it is preferably diffused into smaller pores.

The total adsorbed dye including both the orange and blue dye (mg/g), representing the number of overall pores, increased when the pretreatment times were prolonged (Fig. 1A and B). The overall dye adsorbed by the native barley straw and the forest residues was 86.7 and 40.6 mg/g, respectively, which increased as high as 133.0 mg/g for the treated barley straw and to 89.7 for the treated forest residues (Fig. 1A and B). Furthermore, the observed increase in the amount of adsorbed orange dye after the pretreatment indicates an increase in biodegradability, which in turn is expected to later result in an improved methane production.

3.2. Effects of the NMMO pretreatment on anaerobic digestion

The anaerobic batch digestion assays on the untreated and pretreated materials were performed for 45 days. The results in Fig. 2 indicate that the NMMO pretreatment could significantly increase the produced methane yield for both substrates. Furthermore, for barley straw, the degradation rate also increased (Fig. 2A and B).

The methane potential for the untreated assays of the barley straw and forest residues was 0.12 ± 0.011 and 0.07 ± 0.005 Nm^3 CH_4/kg VS, respectively. However, after the NMMO pretreatment, the methane yield showed approximately 100% increase for both the substrates. The highest methane production for the forest residues was obtained when the samples were subjected to the longest pretreatment time, *i.e*., 30 h, resulting in 0.15 ± 0.015 Nm^3 CH_4/kg VS, moreover, Tukey statistical test showed that there is a significant difference between the methane yield between 15 and 30 h, *P*-value <0.05 (data not shown). On the other hand, in the case of barley straw, increasing the pretreatment time longer than 7 h did not significantly result in further improvement in the methane yield (0.21 ± 0.010–0.23 ± 0.004 Nm^3 CH_4/kg VS, *P*-value >0.05; data not shown). Additionally, the results of the anaerobic digestion revealed that the 3 h-long pretreatment at 90 °C was not harsh enough to break down the complex lignocellulosic structure and enhance the enzyme accessibility to improve the methane production neither from the straw nor from the forest residues (Fig. 2A and B, Table 1).

3.3. Effects of the solvent recycling on anaerobic digestion process

One of the major advantages of this pretreatment is the possibility of recycling the NMMO, which potentially can make the process economically feasible. Thus, the performance of the process in using the recycled solvent instead of the fresh one was in particular focus in this study. The efficiency of the reused NMMO for the pretreatment of the two different lignocellulosic substrates was investigated. The results in Fig. 2C and D show that the recycled NMMO had similar effects as the fresh NMMO on barley straw regarding the methane yield, even after recycling five times. However, for the forest residues, the methane yield decreased by 45% and 55% after the treatment with the NMMO, which was recovered and reused three and five times, respectively, compared to the methane yield obtained using the fresh chemical. Further investigations on the crystallinity index of the NMMO-treated forest residues were performed by FTIR spectroscopy. The bands obtained at 1427 and 898 cm^{-1} are assigned to crystalline cellulose I and cellulose II (amorphous form), respectively. Hence the absorbance ratio of A_{1427}/A_{898}, also called crystallinity index, was used to determine the crystallinity of cellulose. The crystallinity index for untreated forest residues and those for samples pretreated with the fresh as well as with recycled NMMO for 1st, 3rd, and 5th time (data not shown) were investigated. The crystallinity index of the native forest residues was high (1.90); however, the pretreatment with the fresh NMMO resulted in a reduced absorption band at

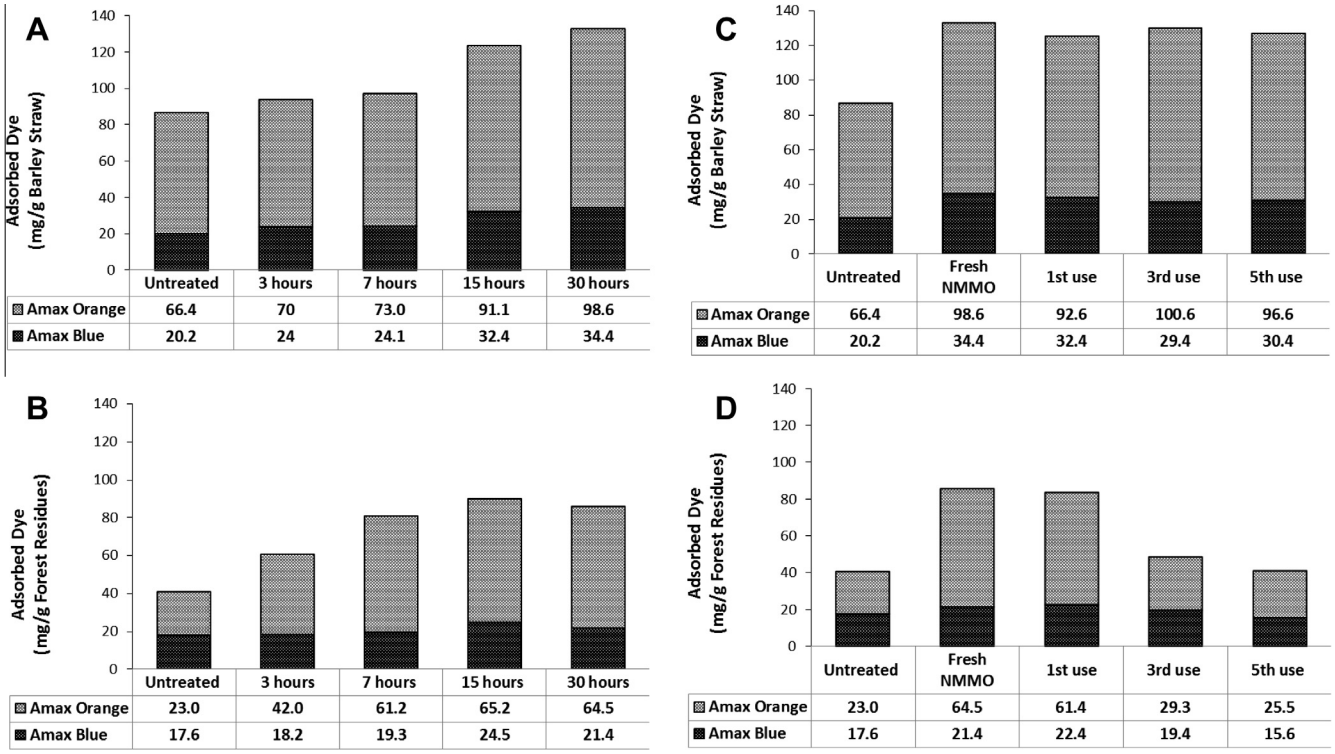

Fig. 1. Adsorbed dye, expressed as mg/g (barley straw, forest residues), from Simons' stain analysis on untreated and NMMO – pretreated barley straw (A) and forest residues (B) after treatments at 90 °C for 3, 7, 15, and 30 h, as well as untreated and NMMO – pretreated barley straw (C) and forest residues (D) after treatments with fresh and recycled NMMO at 90 °C for 30 h. Gray bars show the adsorption of the orange dye and black bars show the adsorption of the blue dye.

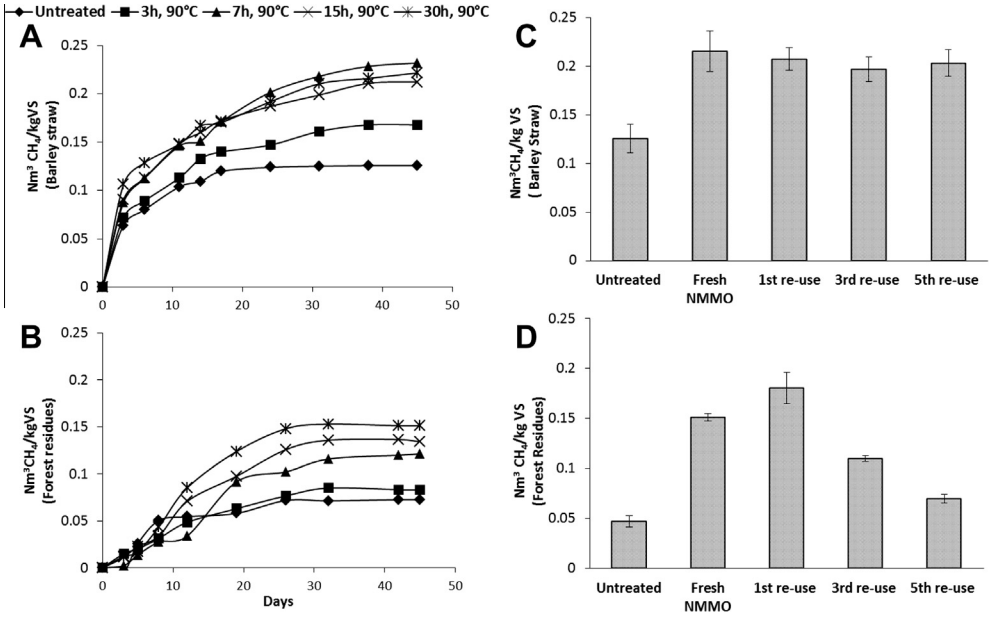

Fig. 2. Accumulated methane production (Nm^3 CH_4/kg VS) obtained during anaerobic batch digestion assays of untreated *vs.* pretreated barley straw (A) and forest residues (B) after different pretreatment conditions. Furthermore, yields of methane (Nm^3 CH_4/kg VS) obtained after 45 days of anaerobic digestion of untreated *vs.* pretreated samples of barley straw (C) and forest residues (D). The pretreatments were carried out at 90 °C for 30 h with fresh as well as with recycled NMMO. Error bars represent ±1 standard deviation of triplicates. The pretreatment conditions are described in the figure.

1427 cm^{-1}, and an increased band at 898 cm^{-1}, corresponding to crystallinity index of 1.12. This indicates a decrease in the crystalline cellulose I and a subsequent increase in the form of the crystalline cellulose II. The crystallinity index of the forest residues pretreated with the recycled solvent (recovery and reuse on the 3rd and 5th time) increased again to 1.81, indicating that the solvent had lost its dissolution power after being recycled the 3rd time.

Moreover, the Simons' Stain analyses were also performed on the barley straw and forest residues, after the pretreatment with the recycled NMMO (Fig. 1C and D). Interestingly, the findings of the Simons' Stain analyses showed a tight correlation between the results of the anaerobic digestion tests performed after the

pretreatment with the recycled NMMO (Fig. 1C and D and Fig. 2C and D). Barley straw showed an increase in the total amount of absorbed dyes after the pretreatments with both the fresh and the recycled solvent compared to that of the untreated sample (Fig. 1C). The total amounts of the absorbed dyes in the barley straw after the pretreatments with the fresh and recycled NMMO was between 124 and 133 mg/g barley straw. On the other hand, the pretreatment with the recycled NMMO (3rd and 5th use) did not show similar effects as the pretreatment with the fresh NMMO regarding the changes in the pore size of the forest residues (Fig. 1D). The pretreatment with the fresh and 1st recycled NMMO resulted in the total amount of absorbed dyes of 86 and 84 mg/g forest residues. However, the Simons' Stain analyses on the forest residues illustrated a descending trend (50% reduction) in the total amounts of the absorbed dye after recycling the solvent the 3rd and 5th time (Fig. 1D). This is an indication that the NMMO efficiency decreases after a few times recycling. These observations suggest that the compositional difference between these two lingocellulosic materials plays an important role for the efficiency of the pretreatment with reused NMMO.

A previous study (Jeihanipour et al., 2010) examined the effects of recycling of the NMMO on cotton and viscose fibers, consisting of pure cellulose. The results of their study showed no significant differences between using the fresh solvent compared to reused NMMO prior to both biogas and bioethanol production. Similarly, in this work the efficiency of the pretreatment of barley straw with the recycled chemical was reserved and similar methane yields were obtained in the batch digestion assays as they were after the treatment with fresh NMMO. In contrast, a 55% reduction in accumulated methane production from the forest residues was observed when the solvent was recycled after five times.

These observations show that the efficiency of NMMO treatment, especially after recycling, is highly dependent on the composition of the treated substrate. Forest residues, comparing to the other substrates (cotton and barley straw), contain remarkably higher amount of lignin and bark which might be the reason for solvent inactivation after recycling it a few times. According to Rosenau et al. (2001), the performance of NMMO treatment during the industrial cellulose fiber making process (Lyocell) is greatly affected by the side reactions occurring in the cellulose/NMMO/water system. Formation of by-products, degradation of cellulose and decomposition of the solvent itself into *N*-methylmorpholine and morpholine can all finally lead to a decrease in process performance. Based on the results of the current study we can assume that negative side reactions and formation of by-products are more likely occur in the case of forest residues/NMMO/water mixture, resulting in faster degradation of the solvent, which will thereby lose its efficiency. Previous studies showed that compounds released from the wood and bark such as tannins, resin acids, phenolic acid and phenolic aldehydes cause severe toxicity in wastewater treatment and anaerobic digestion systems (Field et al., 1988; Sierra-Alvarez et al., 1994). Similar to the wastewater process, these organic compounds could be released and accumulated in the NMMO monohydrate solution after being recycled a few times. However, this is only a hypothesis hence the identification of side reactions with the mentioned impurities and their actions in lignocellulose/NMMO/water system needs further investigations. These findings would be crucial for the application and commercialisation of this pretreatment process for lignocellulosic materials.

4. Conclusions

The digestibility and accessible surface area of the barley straw and forest residues were successfully increased as a result of the NMMO pretreatment. The longest pretreatment time (30 h) led to 88% to 83% of the theoretical methane yield from the carbohydrate fraction of the barley straw and forest residues, respectively. However, the efficiency of the pretreatment after recycling the NMMO is highly dependent on the composition of the lignocelluloses. The performance of the pretreatment with the recycled NMMO deteriorated when forest residues with high lignin and bark content were pretreated.

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Enhanced methane production from wool textile residues by thermal and enzymatic pretreatment

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ABSTRACT

Methane production from two types of wool textile wastes (TW1 and TW2) was investigated. To improve the digestibility of these textiles, different pretreatments were applied, and comprised thermal treatment (at 120 °C for 10 min), enzymatic hydrolysis (using an alkaline endopeptidase at different levels of enzymatic loading, at 55 °C for 0, 2, and 8 h), and a combination of these two treatments. Soluble protein concentration and sCOD (soluble chemical oxygen demand) were measured to evaluate the effectivity of the different pretreatment conditions to degrade wool keratin. The sCOD as well as the soluble protein content had increased in both textile samples in comparison to untreated samples, as a response to the different pretreatments indicating breakdown of the wool keratin structure.

The combined treatments and the thermal treatments were further evaluated by anaerobic batch digestion assays at 55 °C. Combined thermal and enzymatic treatment of TW1 and TW2 resulted in methane productions of 0.43 N m^3/kg VS and 0.27 N m^3/kg VS, *i.e.*, 20 and 10 times higher yields, respectively, than that gained from untreated samples. The application of thermal treatment by itself was less effective and resulted in increasing the methane production by 10-fold for TW1 and showing no significant improvement for TW2.

1. Introduction

Global fiber production for textiles and other applications has shown a constant increase in the past few decades. In 2009, the fiber production exceeded 70.5 million tons, where wool held more than 1.1 million tons of the market [1–3]. The fibers can be reused a few times, but finally end up in waste stations. Consequently, the disposal of this huge volume of fiber waste has evolved into a major concern for the textile industry today.

Wool is mainly composed of protein (≈97%) and lipids (≈ 1%)[4]. Due to its high protein content, it can be utilized as an alternative renewable biomass for the production of value added products, *via* chemical, physicochemical, and microbial processes. One of the options for utilizing wool-based textiles is biogas production. Biogas is a renewable energy product that can be a substitute for fossil fuels. The nutrient-rich digestate residue of a biogas process can be utilized as fertilizer [5,6]. However, anaerobic digestion of wool waste is indeed a challenging process. Wool protein belongs to a family of fibrous structural proteins known as keratin, which due to the presence of disulfide bridges and other intermolecular interactions, are extremely resistant to physical and enzymatic attacks [7]. This particular property of keratin constitutes an obstacle in anaerobic digestion in terms of achieving sufficient microbial degradation, which results in a low methane yield [8,9]. Pretreatment of keratin-based materials prior to anaerobic digestion has however been shown to be a promising strategy, enhancing methane production. Several researchers have reported increased biogas yield of keratin-based materials after enzymatic, chemical, or biological treatments [10,11]. These pretreatments increased methane production, yielding up to 0.40 N m^3/kg VS, corresponding to 80% of the theoretical yield from proteins [8,9]. The studies were carried out on another keratin-rich waste, namely feather. No research on biogas production from wool-based textile waste is as yet reported in the literature.

The present study explored two different wool textile wastes for utilization in biogas production. To investigate feasibility of enhancing biodegradability, three options of pretreatment were tested, namely thermally at high temperature, and enzymatically, by using a protein degrading enzyme. The third pretreatment option comprised a combination of these two treatments, applied consecutively. The treatment parameters (enzyme load, pretreatment time, and the combination of thermal and enzymatic pretreatment) were statistically evaluated, using soluble chemical oxygen demand (sCOD) as response variable. The effects of the different pretreatments were further investigated by means of

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anaerobic batch digestion assays, run under thermophilic conditions.

2. Materials and methods

2.1. Preparation of wool samples

The wool textile waste samples were obtained from Woolpower AB, Östersund, Sweden. Two types of samples were investigated. Type one (TW1) consisted of 70% wool (protein) and 30% polyamide, while type two (TW2) consisted of 70% wool (protein), 18% polyamide and 12% kermel. Besides, polyamide fiber (Nylon 6), provided from the School of Textiles, University of Borås, was also tested to determine methane production from this pure fraction. All samples were grinded into approximately 2 mm particle size using a miller (Retsch GmbH SM 100 comfort miller, Germany), and stored at 5 °C until investigation.

2.2. Pretreatment conditions

The treatments were carried out in 118 ml serum bottles. In each bottle, 0.32 g of milled textile waste, corresponding to 0.3 g VS (Volatile Solids), was mixed with 10 ml potassium-phosphate buffer (pH 8.0). The thermal pretreatment was accomplished in an autoclave at 120 °C for 10 min. The enzymatic hydrolysis was performed in a shaker bath at 55 °C for 0, 2, or 8 h, using an alkaline endopeptidase (Savinase® 16 L, Type EX, activity 16 Kilo Novo Protease Unit per gram of sample (KNPU/g) Novozymes, Denmark) at an enzyme loading of 10 μl/ml, 20 μl/ml, or 40 μl/ml, corresponding to 5.9 KNPU/g $VS_{textile}$, 11.8 KNPU/g $VS_{textile}$ or 23.6 KNPU/g $VS_{textile}$. The enzymatic treatment time 0 h refers to the enzyme being added to the digester directly when starting up the anaerobic digestion process, *i.e.*, no separate enzymatic hydrolysis was applied. The pretreatment combination was conducted by thermal pretreatment (120 °C for 10 min) being followed by enzymatic hydrolysis, using different enzyme loads as described above.

For the polyamide fiber (Nylon 6), only the combined treatment, using the highest enzyme load of 23.6 KNPU/g $VS_{textile}$ for 2 h was carried out.

2.3. Anaerobic batch digestion experiments

Anaerobic batch digestion experiments were performed under thermophilic conditions (55 °C), in accordance with a method developed by Hansen et al. [12]. The bacterial inoculum was obtained from a large-scale biogas plant (Borås Energi Och Miljö AB, Sweden), operating under thermophilic (55 °C) conditions.

To each serum bottle, containing pretreated or untreated wool samples, 40 ml inoculum was added. Blanks containing no textile were prepared, constituting either 10 ml potassium phosphate buffer and 40 ml inoculum, or 10 ml buffer with enzyme and 40 ml inoculum being added, in order to determine methane production by inoculum alone, and by enzyme alone. All digestion set-ups were performed in triplicates.

The prepared bottles were sealed and purged with a gas mixture comprising 80% N_2 and 20% CO_2, to achieve anaerobic conditions. The bottles were stored in an incubator (MMM-group, Einrichtungen GmbH, Venticell) at 55 °C, and were shaken once a day during the experimental period of 46 days. Gas samples of 0.25 ml were taken regularly from the reactor headspace, using a pressure-tight syringe, and the gas composition was determined directly by gas chromatography (GC). After each analysis, excess gas was released through a needle (Sterican® Ø 0.4 × 20 mm B. Braun, Germany) to avoid overpressure in the headspace, and after the release, the gas composition in the headspace was measured again. These measurements were conducted 2–3 times during the first two weeks of the experimental period, and then once a week during the remaining weeks.

2.4. Analyses

Methane was analyzed by using a gas chromatograph (Auto System, Perkin Elmer, USA), equipped with a packed column (Perkin Elmer, 6′ × 1.8″ OD, 80/100, Mesh, USA) and a thermal conductivity detector (Perkin Elmer, USA), and with the inject temperature set at 150 °C. Nitrogen was used as carrier gas, operating at 70 °C with a flow rate of 40 ml/min. A 250 μl pressure-tight gas syringe (VICI, Precision Sampling Inc., USA) was used for the gas sampling. At each measurement occasion, gas samples were taken and measured under overpressure conditions, and after releasing the overpressure, under atmospheric pressure conditions. Assuming ideal gas mixtures and using the ideal gas law, the methane content in the reactor headspace can be calculated by using the data from the GC measurements, without measuring the actual pressure in the bottles [13]. A gas of known composition was used as standard at each measurement occasion. All gas volumes are expressed under standard conditions (1 atm., 0 °C) [14].

Samples taken for monitoring the keratin degradation were centrifuged at 20 °C and 12,000 rpm in order to determine the sCOD and protein concentration in the supernatant. A HACH apparatus, equipped with a UV–VIS Spectrophotometer (HACH, Germany), was used to determine soluble chemical oxygen demand (sCOD). Samples were first digested at 150 °C for 2 h in a COD reactor, using Digestion Solution sCOD vials (with an operating range of 0–15,000 mg/l COD), after which absorbance was measured at 620 μm.

Table 1
Experimental design with different factors varied at different levels.

Factor	Type	Levels	Values
Additional thermal pretreatment	Fixed	2	0; 1
Enzyme concentration (g/g VS)	Fixed	3	5.9; 11.8; 23.6
Enzymatic hydrolysis duration (h)	Fixed	2	2; 8

The protein content in the hydrolyzates was measured by the Lowry method [15]. Subsequently, the absorbance was measured at 750 nm with UV–VIS Spectrophotometer (HACH, Germany).

3. Statistical analysis

The pretreatments of TW1 and TW2 were statistically evaluated, using the sCOD concentrations as response variables, with 3 different factors (additional thermal pretreatment, enzyme concentration, and enzymatic hydrolysis duration) at two and three factor levels, respectively (Table 1). The software package MINITAB® was used for the calculations of factor effects, pooled deviations, and standard errors. ANOVA (General Linear Model) was applied to test for significant effects, using a significance level of 5% [16].

4. Results

4.1. Effect of pretreatment conditions on solubility

The effects of the different pretreatment conditions, *i.e.*, thermal treatment, enzymatic hydrolysis, and combined thermal-enzymatic pretreatment, applied under different conditions on two different wool textile wastes are presented in Table 2. Soluble protein content was measured in the treated samples to gain information about the hydrolysis degree of keratin present in the textile waste, while sCOD was investigated to measure the effects of the different pretreatment conditions on the solubilization of organic materials in the entire samples. All pretreatments profoundly increased the sCOD and the soluble protein content of the two textile samples, in comparison with samples not undergoing pretreatment. The pretreatment conditions were, however, more effective on TW1 than on TW2 (Table 2). After thermal treatment, the sCOD value was 1120 mg/l in TW1, while being almost zero in TW2. However, the combined treatment dramatically increased the sCOD values in both TW1 and TW2 to 13,820–27,920 mg/l and 350–9100 mg/l, respectively. Applying enzymatic treatment alone, was not as effective in terms of increasing the sCOD in any of the textiles, sCOD values reaching 2920–18,940 mg/l in TW1 and 180–7600 mg/l in TW2. Increasing the enzyme load from 11.8 to 23.6 KNPU/g $VS_{textile}$ enhanced the sCOD released by 5-fold and 4-fold during 8 h pretreatment of TW1 and TW2, respectively. In TW1, 8–95% of the keratin content was solubilized after the pretreatments. The combined pretreatment was more effective than the enzymatic treatment, achieving a protein solubilization degree of 77–95%, corresponding to protein concentrations of 16.2–20.1 mg/ml. The enzymatic treatment resulted in solubilization degrees of 8–36%, *i.e.*, protein concentrations of 1.8–7.5 mg/ml. In comparison, the untreated TW1 showed a very low solubilization degree, almost zero (data not shown in Table 2).

The protein solubilization of untreated and thermally treated samples of TW2 was negligible (Table 2). Enzymatic treatment of this textile increased the protein solubilization degree to 6–36%, corresponding to protein concentrations of 1.2–7.5 mg/ml. By applying the combined thermal and enzymatic treatment in TW2, higher soluble protein concentrations were however obtained also in this textile, resulting in protein values of 2.2–10.6 mg/ml, corresponding to a solubilization degree of approximately 10–50%.

Table 2
The concentrations of sCOD and soluble protein obtained in the hydrolysates of TW1 and TW2 after thermal, enzymatic and combined (thermal followed by enzymatic) pretreatments with different conditions.

TW1				TW2			
Pretreatment				Pretreatment			
	Enzyme (KNPU/g VS)	Time (h)	sCOD (mg/l)	Protein (Lowry) (mg/ml)	Enzyme (KNPU/g VS)	Time (h)	sCOD (mg/l)	Protein (Lowry) (mg/ml)
Without thermal treatment					Without thermal treatment			
	5.9	2	4420	1.78	5.9	2	180	1.19
	11.8	2	5840	3.42	11.8	2	1260	4.08
	23.6	2	2920	5.77	23.6	2	4450	7.48
	5.9	8	5940	5.27	5.9	8	1660	2.57
	11.8	8	4880	5.07	11.8	8	1480	3.48
	23.6	8	18,940	7.48	23.6	8	7600	7.08
Thermal treatment (120 °C, 10 min)					Thermal treatment (120 °C, 10 min)			
	5.9	2	15,100	19.13	5.9	2	350	2.19
	11.8	2	16,000	18.53	11.8	2	3900	5.18
	23.6	2	25,880	20.14	23.6	2	9100	10.64
	5.9	8	13,820	16.20	5.9	8	3420	3.20
	11.8	8	15,520	16.40	11.8	8	4160	5.13
	23.6	8	27,920	18.09	23.6	8	7400	10.30
	0	0	1120	0	0	0	0	0

The most effective pretreatment of TW2, resulting in the highest protein solubilization, was the combined treatment with 2-h duration, where thermal treatment was followed by addition of enzyme at a concentration of 23.6 KNPU/g $VS_{textile}$. Under these conditions, approximately half of the protein content of the sample was solubilized, corresponding to 10.6 mg/ml soluble protein concentration. However, comparing the two textile samples after identical pretreatments disclosed that the protein solubilization in TW2 only reached 25%, *i.e.*, approximately half of what was achieved in TW1 (Table 2).

4.2. Statistical evaluation

The enzyme load, and the application of an additional thermal treatment prior to the enzymatic treatment, significantly increased the solubilization of both TW1 and TW2 (Tables 3 and 4; *p*-values being <0.0005, <0.0005 for TW1, and 0.014, <0.0005 for TW2). However, increased treatment duration of enzymatic hydrolysis did not significantly affect the sCOD. The assays with enzymatic hydrolysis lasting 8 h did not result in significantly higher sCOD values in comparison with assays where the hydrolysis lasted 2 h ($p = 0.062$, 0.140). Comparing the factor effect on TW1 and TW2 revealed that additional thermal treatment increased the sCOD by 12,068 mg/l in TW1, but only by 1950 mg/l in TW2. Increasing the enzyme concentration from 5.8 KNPU/g $VS_{textile}$ to 11.9 KNPU/g $VS_{textile}$ and further to 23.6 KNPU/g $VS_{textile}$, improved the sCOD by 5185 and 6768 mg/l in TW1, and by 2093 and 4688 mg/l in TW2. The ANOVA analyses of the sCOD values of TW1 and TW2 disclosed an interaction between enzyme concentration and hydrolysis duration in TW1 ($p = 0.027$, see Table 3), confirming that adequate enzyme concentration in conjunction with longer hydrolysis duration positively affects the sCOD concentration in this textile. In contrast, no significant synergistic effect on the sCOD concentration was found in TW2 ($p = 0.473$, see Table 4).

4.3. Anaerobic biodegradability of treated wool textile wastes

Based on the results of the statistical evaluations (see above), only thermal and the combined pretreatment, using different enzyme loads and 2 h duration, were chosen for assessing anaerobic digestion performance of the TW1 and TW2 samples. Since the enzyme is functional under thermophilic conditions, the results of thermally pretreated samples with instant addition of enzyme (0 h) were also evaluated to establish the effectivity of simultaneous enzymatic hydrolysis and anaerobic digestion, using assays of untreated samples, run together with treated samples, as control.

Fig. 1 shows accumulated methane production after 13 days of incubation and after the whole experimental period (46 days), from pretreated and untreated wool textile samples. After 46 days of incubation, pretreated samples (TW1, TW2) showed a higher accumulated methane production than samples receiving no pretreatment, methane yield being negligible in these samples. Furthermore, also the initial methane production rates were improved after pretreatments, which indicate that degradation rates had increased as well.

In general, all textiles pretreated with the combined treatment had higher methane yields in comparison with textiles undergoing only thermal treatment or no pretreatment at all. Moreover, the combined treatment, using different enzyme concentrations and hydrolysis duration (0 h, 2 h), had a stronger effect on TW1 than on TW2, resulted in higher methane yields from TW1 (0.32–0.43 N m^3/kg VS) than from TW2 (0.18–0.27 N m^3/kg VS). Thermal pretreatments alone resulted in methane productions of 0.21 ± 0.005 N m^3/kg VS from TW1 and of 0.05 ± 0.013 N m^3/kg VS from TW2.

The highest accumulated methane production from TW1 (0.43 ± 0.026 N m^3/kg VS) was observed after 2 h combined treatment with an enzyme load of 11.8 KNPU/g $VS_{textile}$. The maximum yield from TW2 (0.27 ± 0.001 N m^3/kg VS) was achieved after simultaneous enzymatic hydrolysis and digestion with an enzyme load of 11.8 KNPU/g $VS_{textile}$ (0 h, *i.e.*, right at the start of the digestion process).

Accumulated methane production obtained after 13 days of digestion provides information about the initial methane production rates (Fig. 1). The initial methane production of assays subjected to the combined pretreatment ranged between 0.14 ± 0.012 and 0.31 ± 0.050 N m^3/kg VS from TW1, and between 0.05 ± 0.001 and 0.11 ± 0.002 N m^3/kg VS from TW2. After 13 days of thermal pretreatment, the accumulated methane yield almost reached the final methane production value (46 days, 0.20 ± 0.011 N m^3/kg VS; see Fig. 1a), while after 13 days under identical pretreatment conditions, the methane yield in the assay of TW2 was negligible (Fig. 1b).

4.4. Anaerobic biodegradability of polyamide (Nylon 6)

According to the results achieved from the biomethane potential tests of TW1 and TW2, the best treatment conditions were selected to be applied on polyamide to determine the biomethane

Table 3
General Linear Model ANOVA table on pretreatment of TW1, with sCOD as the response variable, calculated by MINITAB® software package.

TW1	DF	Seq SS	Adj SS	Adj MS	*F*	*p*
Thermal treatment	1	866161350	866161350	866161350	99.52	<0.0005
Enzyme conc.	2	399709033	399709033	199854517	22.96	<0.0005
Time	1	36064017	36064017	36064017	4.14	0.062
Thermal treatment *Enzyme conc.	2	46278900	46278900	23139450	2.66	0.092
Thermal treatment *Time	1	42188017	42188017	42188017	4.85	0.053
Enzyme conc. *Time	2	95619633	95619633	47809817	5.49	0.027
Error	14	121846633	121846633	8703331		
Total	23	1607867583				

$S = 2950.14$. $R^2 = 92.42\%$, $R(\text{adj})^2 = 87.55\%$.
DF: degree of Freedom, Seq SS: sequential sum of squares, Adj SS: adjusted sum of squares, Adj MS: adjusted mean squares, *F*: *F*-distribution, *p*: probability.

potential of polyamide (Nylon 6). The combined treatment, comprising initial thermal treatment and subsequent enzymatic treatment, using 23.6 KNPU/g VS_{Nylon} enzyme load and 2 h treatment duration resulted in a sCOD release of 7000 mg/l from the polyamide fibers, and their methane yield reached 0.27 ± 0.031 N m^3/kg VS_{added}. No anaerobic degradation occurred in untreated polyamide fibers, as proven by methane production being non-detectable.

5. Discussion

The main objective of the present work was to investigate whether wool textile waste might be utilized in an anaerobic digestion process. The compact structure of wool keratin presents a problem in terms of achieving an adequate digestion process. Finding suitable strategies for improving digestibility is thus of great significance in order to facilitate the use of wool in methane production. The attempts in this study to increase digestibility of wool textile samples by using various pretreatments resulted in improved methane potential of wool textile waste. The potential differed, however, between the two types of wool textile, and was depending on certain factors included in the different pretreatments.

The soluble protein and the sCOD content of the treated samples revealed that the combined treatment was the most effective one of those tested, resulting in the highest values of sCOD and soluble protein. The digestion assays showed that the combined pretreatments, although increasing the methane yields from both TW1 and TW2, resulted in different levels obtained from the textile types. The yield increase was up to 20 times in TW1, *i.e.*, double the increase (10 times) in TW2, both seen in comparison with the yield from untreated samples.

The theoretical methane yield of proteins is 0.50 N m^3/kg VS, it is calculated by the Buswell's formula using an average chemical formula for proteins ($C_5H_7NO_2$) [17]. Based on the protein content of TW1 and TW2 (70% protein), the expected theoretical methane yield of both substrates was calculated to be 0.34 N m^3/kg VS. However, the highest methane production of TW1 (obtained after the combined treatment) was 0.43 N m^3/kg VS, thus exceeding the theoretical yield. This indicates that degradation of the polyamide fibers present in this textile type contributed to the acquired yield. This finding is in accordance to previous work on polyamide degradation, reporting that polyamide can be degraded in soil [18,19], and in an activated sludge process [20]. However, these reported degradation processes were carried out under aerobic conditions, while the polyamide fibers in this study were subjected to anaerobic degradation. The theoretical methane yield of polyamide can also be calculated to 0.74 N m^3/kg VS using its chemical composition of $(C_6H_{11}NO)_n$ [17]. Since the examined textile wastes were composed of 70% wool and 30 or 18% polyimide, the theoretical yield of TW1 and TW2 were calculated to 0.57 and 0.48 N m^3/kg VS, respectively.

The highest methane yield obtained from TW1 in the present study, 0.43 N m^3/kg VS, counting up to 75% of the theoretical yield. Moreover, according to the results of batch digestion of only polyamid fibers, the methane yield of pretreated polyamide was 0.27 N m^3/kg $VS_{added.}$ Using this data and assuming that all of the proteins were degraded we can calculate the expected methane yield as follows:

$$0.70 \times 0.50\,(\text{N m}^3/\text{kg VS}) + 0.30 \times 0.27\,(\text{N m}^3/\text{kg } VS)$$
$$= 0.43\ \text{N m}^3/\text{kg VS}$$

This suggests that the combined thermal-enzymatic treatment, applying an enzyme load of 11.8 KNPU/g $VS_{textile}$ for 2 h, succeeded in utilizing the whole amount of protein in the textile waste.

Although thermal treatment alone resulted in a tenfold increase of the methane yield from TW1 (compared with control) to a value of 0.21 N m^3/kg VS_{added}, this yield reached only 60% of the theoretical yield from the protein fraction. According to the literature, thermal pretreatment at this conditions are suitable to open up the disulfide bonds presented in the keratin structure through β-elimination reaction [21], which led to an enhanced degradation and a higher methane yield in the subsequent anaerobic digestion process.

Table 4
General linear model ANOVA table on pretreatment of TW2, with sCOD as the response variable, calculated by MINITAB® software package.

TW2	DF	Seq SS	Adj SS	Adj MS	*F*	*p*
Thermal treatment	1	22815000	22815000	22815000	7.97	0.014
Enzyme conc.	2	144707033	144707033	72353517	25.29	<0.0005
Time	1	6998400	6998400	6998400	2.45	0.140
Thermal treatment *Enzyme conc.	2	3099900	3099900	1549950	0.54	0.593
Thermal treatment *Time	1	1728067	1728067	1728067	0.60	0.450
Enzyme conc. *Time	2	4519300	4519300	2259650	0.79	0.473
Error	14	40059233	40059233	2861374		
Total	23	223926933				

$S = 1691.56$, $R^2 = 82.11\%$, $R(\text{adj})^2 = 70.61\%$.
DF: degree of freedom, Seq SS: sequential sum of squares, Adj SS: adjusted sum of squares, Adj MS: adjusted mean squares, *F*: *F*-distribution, *p*: probability.

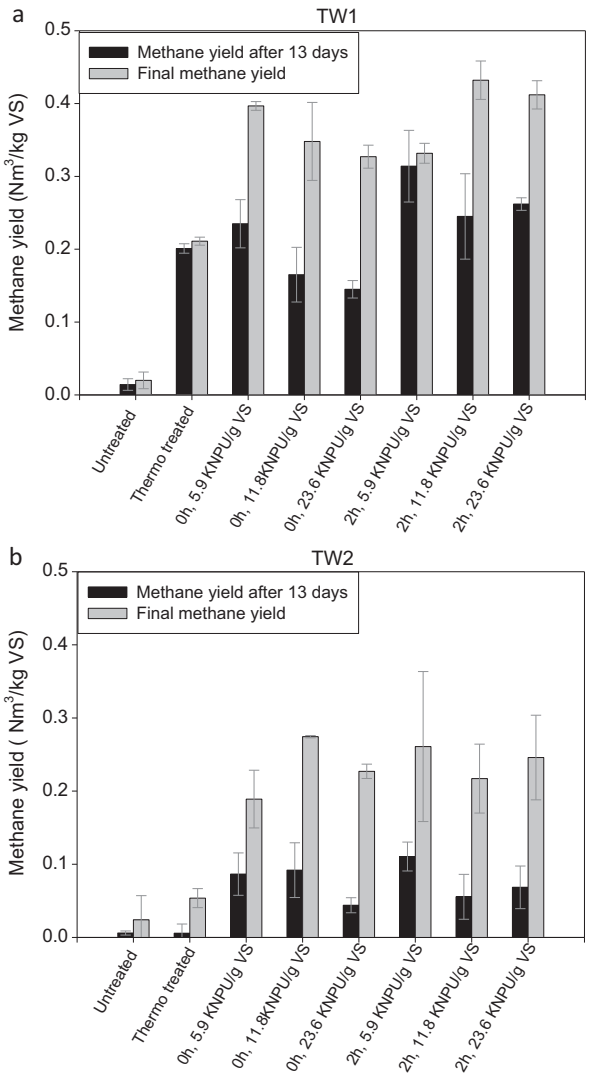

Fig. 1. Accumulated methane production (N m^3/kg VS) from treated and untreated samples of TW1 (a) and TW2 (b), obtained after 13 days (black bars) and final methane yield (grey bars). Error bars represent ±1 standard deviation of triplicate assays.

Other research also reported that thermal treatment alone on keratin-rich materials prior to anaerobic digestion improved the methane yield [8,9]; thermal pretreatment of chicken feather at 120 °C for 5–10 min, improved methane production by 25% (about 45% of the theoretical yield) in comparison with untreated material.

The increase in enzyme load (11.8 and 23.6 KNPU/g VS) caused a slower initial digestion rate, which is illustrated during the first 13 days of incubation (Fig. 1). Enzyme added at higher concentration caused higher hydrolysis rate of the substrate, leading to a temporary overload of degradable materials in the anaerobic digestion process. This might be the reason of transitional imbalance observed in the anaerobic food chain present in the system, while different groups of microorganisms converting organic matter to methane. [22,23]. Another reason could be due to the presence of antimicrobial components, (*i.e.*, sodium-azid or thimerosal) in enzyme solution which could also inhibit microbial growth at the higher enzyme concentrations [24]. However, this negative effect did not appear in the final methane yields (46 days of incubation); concluding this effect was temporary.

Final methane production and initial digestion rate being higher in samples of TW1 than of TW2 conveys that the pretreatment chosen for improving the biodegradability was more effective on TW1 than on TW2. This might be explained by 12% of TW2 consisting of kermel, a recalcitrant polyamide-imide fiber, in all likelihood inhibiting complete degradation.

This is supported by the sCOD content, measured after thermal treatment, being 6 times higher for TW1 (12,014 mg/l) than for TW2 (1905 mg/l). Kermel-containing fibers are known for their non-flammability, thermostability, thermal insulation, and mechanical strength. They are also resistant to chemicals at ambient temperature, particularly acids and dilute alkalis, even if they are immersed for a fairly long time period. Due to these properties, kermel can retain the integrity of textiles, and is commonly used in the manufacture of heat and flame resistant protective clothing [25].

6. Conclusions

The present study proved that after a suitable pretreatment, converting wool textile waste into biogas *via* anaerobic digestion is feasible. The highest methane yield achieved, 0.43 N m^3/kg VS methane, was 20 times higher after combined thermal and enzymatic pretreatment of the TW1 textile type than in controls receiving no pretreatment, thus equaling the theoretical yield, calculated on the basis of the composition of this waste textile.

The total global wool production amounts to 1.1 million tons/year [26]. According to the present results utilizing the generated waste would yield approximately 473 million m^3 of methane, replacing 532 million liters of gasoline, corresponding to 15% the yearly gasoline consumption in Sweden [27].

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Paper IV

Dry anaerobic digestion of lignocellulose and protein wastes

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Abstract

Utilisation of wheat straw and wool textile waste in dry anaerobic digestion (AD) process was investigated. Dry-AD of the individual substrates as well as co-digestion of those were evaluated using different total solid (TS) contents of between 6 to 30%. Additionally, the effects of the addition of nutrients and cellulose or protein degrading enzymes on the performance of the AD process were also investigated. Dry-AD of wheat straw resulted in methane yields of 0.081 – 0.200 Nm^3CH_4/kgVS with the lowest and highest value at 30 and 21% TS, respectively. The addition of cellulolytic enzymes could significantly increase the yield in the reactor containing 13% TS (0.231 Nm^3CH_4/kg VS). Likewise, degradation of wool textile waste was enhanced significantly at TS of 13% with addition of protein degrading enzyme (0.131 Nm^3CH_4/kg VS). Furthermore, the co-digestion of these two substrates showed higher methane yields, compared to those calculated from the methane potential of individual fractions at all TS contents investigated due to synergetic effects and better nutritional balance.

Keywords: anaerobic digestion, lignocellulose, keratin, wool, wheat straw, dry-AD, TS, enzyme

1. Introduction

Anaerobic digestion (AD) has received tremendous attention recently as a consequence of a growing demand for renewable energies together with increasing fuel prices. This technology has been used in processing of municipal and industrial sludge for almost 100 years (1). The product of AD, the biogas, is a versatile energy source, which can be utilised for the generation of heat and/or electricity, either separated or combined; in addition, it can be upgraded and used as vehicle fuel (2). Anaerobic digesters are classified as wet-AD and solid-state/dry-AD, based on the total solids (TS) content of feedstock in the digester. Generally, TS contents in wet-AD digesters is lower than 10%, whereas in dry fermentation the TS content is usually higher than 10% (3, 4). During the past few years, over 63% of the new AD plant installations in Europe were dry-AD plants (3).

The increasing interest of dry-AD plants can generally be explained by the advantages of dry-AD processes over wet-AD processes. Due to the increase in TS content in the digester, it requires a smaller reactor volume, which reduces the material cost and the energy required for heating. Moreover, the digestate residue can easily be subjected to composting and then used as soil conditioner or fertilizer as no or minimal dewatering is needed. Furthermore, problems related to wet-AD, such as floating of scum layer can be avoided (5, 6).

Lignocellulosic biomass and textile wastes could be ideal substrates for dry-AD, due to their high abundance and their low moisture content. The global production of fibres has shown a constant increase in the past few decades. According to the Food and Agriculture Organization of the United Nations (FAO), the annual wool production is around 2.1 million tonnes per year (7). These fibres, after recycling and reuse for textile and other applications, would finally end up in waste stations. Consequently, the disposal of this large volume of fibre waste has evolved into a major concern for the textile industry today.

Wool is mainly composed of a recalcitrant protein, keratin, (≈ 97%) and lipids (≈ 1%) (8). Due to its high protein content, it can be used as an alternative renewable biomass source for the production of value added products, via chemical, physicochemical, and microbial processes. One novel approach is the application of biological degradation of wool-based textiles for biogas production.

Among the large amounts of lignocellulosic biomass produced within the EU-27, the potential for straw is estimated to about 127 million tonnes in 2020. This is equal to 49.3 Mtoe (million tonnes oil equivalent) (9). This high potential availability of straw and its high carbon content makes it a suitable substrate for biofuel production.

The main aim of this study was to explore the performance of AD of wheat straw and wool textile waste alone as well as in co-digestion using wet, semi dry and dry-AD processes. The TS concentrations were therefore varied between 6 – 30% during the investigations. The effect of addition of cellulose or protein degrading enzymes, as well as the effect of addition of nutrients on the performance of the AD process was also determined. Furthermore, chemical compositions and structural analyses were performed on both of these substrates aiming to determine what happens with each of these feedstock during the degradation process. To the authors' knowledge, there is no publication found in the literature to study the dry-digestion process of these two co-substrates.

2. Materials and methods

2.1. **Preparation of wool and wheat straw substrates**

Wool textile waste, consisting of 70% wool (protein) and 30% polyamide, was obtained from Woolpower AB, Östersund, Sweden. This textile waste was then grinded into approximately 2 mm particle size using a miller (Retsch GmbH SM 100 comfort miller, Germany) and stored until investigations. Wheat straw used in this study was supplied by Lantmännen Agroetanol

(Norrköping, Sweden). The composition of wheat straw was determined based on the dry matter of the wheat straw: 35% cellulose, 22% hemicellulose, 18% insoluble lignin and 16% extractives. The straw was milled to particle size between 2 and 5 mm, using a sieve shaker (Octagon 200, UK) and stored prior to analyses and anaerobic digestion.

2.2. **Anaerobic digestion assays**

The methane potential of different substrate combinations at different conditions were determined using batch anaerobic digestion assays. The milled wheat straw or wool textile waste as well as a mixture of these two were mixed with a predetermined amount of inoculum and deionized water to achieve a substrate to inoculum ratio (S/I) of 2:1 (based on the volatile solids content), and initial TS contents of 6%, 13%, 21% and 30%. The inoculum was obtained from a farm-scale digester, Vårgårda-Herrljunga Biogas AB (Sweden), operating at mesophilic temperature (37°C). The anaerobic digestion of wheat straw or wool textile waste was investigated even with the addition of enzymes, Cellic® CTec3 enzyme (Novozomes, Denmark) or an alkaline endopeptidase (Savinase® 16 L, Type EX), respectively. The cellulolytic enzyme load was 10 FPU (Filter Paper Unit)/gram straw and the endopeptidase load was 10 KNPU (Kilo Novo Protease Unit)/g textile. These enzymes were added to the digesters directly when starting up the anaerobic digestion process.

Furthermore, wool or straw was also digested with addition of nutrients. The final nutrient concentrations for the basal medium (1g/L substrate, containing inorganic macronutrients) were (in mg/L): NH_4Cl (76.4), KH_2PO_4 (5.18), $MgSO_4 \cdot 7H_2O$ (0.27), $CaCl_2 \cdot 2H_2O$, (10.00), and trace nutrients, 1 ml/L (10). Finally, the co-digestion of wheat straw and wool textile waste (ratio 1:1 based on the VS content) was investigated using similar TS concentrations as in the mono-digestion assays.

After the set-ups, anaerobic conditions were obtained by purging the reactors with nitrogen gas for about 2 min, and the reactors were then incubated in a convection oven at mesophilic conditions (37 ± 1°C). Inoculum without adding any substrate was evaluated as a blank to determine the methane production of the inoculum itself. The accumulated methane production was measured by taking gas samples regularly from the headspace during a 50-day long examination period.

2.3. **Analytical methods**

The total solids (TS) and volatile solids (VS) of the investigated substrates were determined according to Sluiter et al. (2005) (11). Total nitrogen content of wool textile waste before and after digestion was determined by Kjeldahl digestion, using a 2020 Kjeltec Digestor and a 2400 Kjeltec Analyser unit (FOSS analytical A/S Hilleröd, Denmark). The extractive content of wheat straw was measured according to Sluiter et al. (12). Furthermore, the structural carbohydrate's content were determined using a two-step hydrolysis method described by Sluiter et al. (13). The sugars were quantified by high performance liquid chromatography (HPLC) (Waters 2695, Millipore and Milford, USA) equipped with a refractive index (RI) detector (Waters 2414, Millipore and Milford, USA), using a Pb-based ion exchange column (Aminex HPX-87P, Bio-Rad, USA). The eluent was pure water with a flow rate of 0.6 mL/min at 85°C.

Fourier transform infrared spectroscopy (FTIR), (Impact 410, Nicolet Instrument Corp., Madison, WI) was used to measure the cellulosic crystallinity. The spectra were achieved with an average of 64 scans and a resolution of 4 cm^{-1} in the range from 600 to 4000 cm^{-1} and controlled by Nicolet OMNIC 4.1 analysing software. The crystallinity was analysed considering the absorbance bands at 1422 and 898 cm^{-1}, assigned to cellulose I and cellulose II (amorphous cellulose), respectively (14). The absorbance ratio of A_{1422}/A_{898} was used to

measure the crystallinity index of wheat straw before and after digestion, and of the digested straw in the presence of the enzyme.

The protein secondary structure in wool textile waste was also investigated by FTIR spectrometer. The most sensitive spectral region to the protein secondary structural components is the amide I band, which appears in absorption bands of 1700 – 1600 cm^{-1} due to the C=O stretch vibrations of the peptide linkage. To analyse the amide I band component, the second derivative spectra was curve fitted and used to identify the composite absorptions attributed to α-Helix, β-Sheet and disordered microstructural components, respectively, before and after 50 days of anaerobic digestion (15). The percentage of the α-Helix, β-Sheet and disordered microstructures were determined by adding the sum of absorptions for each and stating their sums as a fraction of total Amide I band area (16).

The methane production was measured using a gas chromatograph (GC) (Auto System, Perkin Elmer, USA) equipped with a packed column (Perkin Elmer, 6' x 1.8" OD, 80/100, Mesh, USA) and a thermal conductivity detector (Perkin Elmer) with inject temperature of 150°C. Nitrogen was used as carrier gas at 75ºC with a flow rate of 20 mL/min. A 250µL pressure-tight syringe (VICI, Precision Sampling Inc., USA) was used for gas sampling. The results are presented as Nm^3 CH_4/kg VS and were calculated as the volume of methane gas produced per kg of VS loaded into each reactor at start-up and corrected by subtracting the methane volume obtained from the blank reactor running with just the inoculum. The volumetric productivity of methane expressed as $V_{methane}/V_{working\ volume}$, meaning that m^3 of methane gas produced ($V_{methane}$) per m^3 of working volume of the reactor ($V_{working\ volume}$), was also determined.

All AD assays as well as the compositional and structural analyses were run in triplicates. Statistical analysis was performed using software package MINITAB®. All error bars and intervals reported represent one standard deviation. ANOVA general linear model was

applied to evaluate the methane production and the effect of the varying TS content in the digesters with a significance threshold (p-value) of 0.05. The effects of the addition of nutrients and enzymes on the anaerobic digestion process were evaluated via two sample comparative t-test using MINITAB®.

3. Results and discussion

3.1. **Anaerobic digestion of wheat straw and wool textile waste**

Anaerobic digestion of wheat straw and wool textile waste was investigated using four different TS contents (6, 13, 21 and 30%) in batch operation mode. The accumulated methane production was determined during a 50-day long digestion period. The S/I ratio (based on VS content) was set to 2 in each digester, while the TS concentrations varied between 6 – 30%. The obtained accumulated methane yields and volumetric productivities are presented in Figure 1 and Table 1.

3.1.1. Anaerobic digestion of wheat straw

In anaerobic digestion of wheat straw (without addition of enzyme and nutrients), the methane yields obtained were between 0.081 – 0.231 Nm^3CH_4/kg VS (Table 1, Figure 1). The highest methane yields were determined when TS concentrations of 13 and 21% was used, with methane productions of 0.170 and 0.200 Nm^3CH_4/kg VS, respectively. In comparison, the methane productions were significantly reduced when the straw was digested using TS of 6% (0.135 Nm^3CH_4/kg VS) or TS of 30% (0.081 Nm^3CH_4/kg VS), as these were confirmed by the statistical analyses. However, according to the statistical analyses, the methane productions from dry-AD using 13 or 21% TS did not differ significantly (p–value greater than 0.05).

The effect of addition of nutrients in anaerobic digestion of wheat straw was also examined, and the obtained methane yields were 0.141, 0.204, 0.225 and 0.070 Nm^3CH_4/kg VS, in

reactors containing 6, 13, 21 and 30% TS, respectively. The statistical analyses of the results showed that there was no significant difference between the methane yields obtained without and with the addition of nutrients.

The enzymes that are responsible for degradation of biomass in the AD system are already presented in the digester as they are excreted by the microorganisms. However, to improve the breakdown process, excess enzyme loading was applied. The enzymes used were directly added to the vessels at the start-up of the digestion process. Anaerobic digestion of wheat straw with excess cellulolytic enzyme supply resulted in methane yields of 0.171, 0.213, 0.211 and 0.052 Nm^3CH_4/kg VS, for TS contents of 6, 13, 21 and 30%, respectively.

Comparing the methane potential reported previously for wheat straw and obtained in batch wise wet-AD studies (ranging between 0.189 – 0.200 Nm^3CH_4/kg VS) at mesophilic conditions (18) to the results of the current work, it can be concluded that almost similar methane yields (0.170 – 0.200 Nm^3CH_4/kg VS) were achieved even in the dry digestion mode, with the advantage of 2 times more organic loading in the system. Moreover, the simultaneous addition of cellulose degrading enzymes at the start up of the digestion process could significantly enhance the methane production at TS of 13%, leading to 0.231 Nm^3CH_4/kg VS methane production. However, the statistical analyses showed that the addition of enzyme caused no significant differences (p-value greater than 0.05) at all the other TS contents applied. A possible explanation for that might be the presence of a lignin shield surrounding the cellulose fraction of the straw. As reported earlier (17), the binding of lignin to the cellulase enzymes is irreversible, and it can lead to the deactivation of the enzymes, thus reducing their effectiveness.

The volumetric methane productivity (shown in Table 1) was also evaluated. The determination of the volumetric methane productivity is an important factor when considering the economy of AD processes. The volumetric productivities determined during this study

showed that the highest methane production from straw could be achieved when the TS content was 13% (i.e. 0.021 – 0.030 m^3CH_4/ m^3working volume) or 21%, (i.e. 0.033 – 0.037 m^3CH_4/ m^3working volume), respectively (Table 1).

Generally, the higher methane yields obtained at TS of 13 and 21% might be related to the nature of the straw, which can act as a structural biofilm carrier. In AD systems, when biofilm carriers are present in the reactor, syntrophic interactions between a large variety of microorganisms can occur, which subsequently leads to higher methane yields. This is in agreement with the findings obtained in previous studies where additional biofilm carriers were applied using inert or naturally degradable materials, like straw, to which the microbial population could be attached (19, 20). Due to the cell's natural tendency to form dense granules, additional biofilm carriers would enhance process stability, which will lead to increased methane yields (20). Previously, Andersson and Björnsson (21) also found that addition of straw in a two-stage anaerobic digestion system resulted in an improvement in digestion performance with enhanced methane production. Therefore, we can expect that anaerobic digestion of straw in a dry system can lead to increased cell tendencies being attached to the surface of straw making the degradation more efficient, as it was shown from the results of this study, containing high gas production per unit volume in digesters operating at 2 or 3 times higher TS content of straw, compared to that of wet AD systems. During these conditions, high efficient degraders can be developed and/or retained in the system due to an increased accessible surface area. Hence, the cost effectiveness of the dry-AD process increases (22). On the other hand, the biomethane potential of straw in reactors with the highest TS concentration of 30% was not as high in this study. This can be a consequence of excessive shortage of moisture in the reactor, which finally deteriorates the digestion process. This result is in accordance with previous studies that showed a dramatic decrease in biogas production with TS contents of 30 to 50% (22). Moreover, according to Wujcik and Jewell

1980 (23), high TS contents of 30 to 40% cause inhibition of AD due to accumulation of VFAs.

3.1.2. Anaerobic digestion of wool textile waste

The anaerobic digestion of wool textile waste was investigated as well at similar TS concentrations (i.e. 6 – 30%). Furthermore, the effect of simultaneous addition of a protein degrading enzyme and the addition of nutrients were also studied. The results are summarised in Figure 1 and in Table 1.

The methane yields obtained without addition of enzyme and nutrients were low, between 0.030 – 0.061 Nm^3CH_4/kg VS at TS contents of 6 – 30% (Figure 1). Interestingly, no significant differences in methane yields were observed as the TS increased from 6 to 30% (p-values greater than 0.05). Similarly, addition of nutrients did not cause a significant improvement in AD digestion of wool textile waste. However, the addition of the protein degrading enzyme could significantly enhance the methane production at TS content of 6 and 13%, resulting in 0.108 and 0.131 Nm^3CH_4/kg VS, respectively. Previously, in wet-AD system and at thermophilic conditions, only 5% of the expected theoretical yield from the protein fraction of this textile waste could be achieved (i.e. 0.020 m^3CH_4/kg VS) (24). On the other hand, in this study, the degradation of wool textile waste in dry-AD mode resulted in 3 and 6.5 times higher methane yields, without and with the addition of a protein degrading enzyme, respectively. The highest protein conversion of wool textile waste to biomethane was obtained using enzyme supplement at TS of 13% (0.131 m^3CH_4/kg VS), corresponding to 38% of the expected theoretical yield from this substrate. However, a descending trend in the methane yield of 0.040 and 0.032 m^3CH_4/kg VS was observed when the addition of enzyme was provided at TS of 21% and 30%, respectively. The reason for this low gas production at higher TS concentrations might be the increase in the mass transfer barrier for the diffusion of VFAs (25), which leads to the accumulation of VFAs in the system. Besides, as the addition

of enzyme accelerates the degradation rate of proteins (26), the accumulation of ammonium nitrogen might be another reason behind the low methane yield (26). Furthermore, there was a 20-day long lag period observed in digestion of wool textile waste, in all TS concentrations investigated (Figure 1). However, the process was recovered, and the methane production started up in reactors containing 6 – 21% TS. On the other hand, the digestion was failed when the TS concentration was increased to 30%. These results are in accordance with those obtained previously in another work, when chicken feather (β-keratin protein) was used as a feedstock in a semi-continuous anaerobic co-digestion process. After an initial period of 20 days, the ammonium-nitrogen concentration increased to 4,200 mg/L in the reactors where enzyme was added together with feather, resulting in a decrease of the methane production (26). However, the system had been stabilised afterwards together with a slow decrease in ammonium-nitrogen concentration.

Table 1 presents the volumetric methane productivity determined when wool textile waste was digested at different TS levels. The results show that the volumetric productivity was the highest when TS of 13% was used. However, the volumetric productivity decreased when the TS was further increased to 21 and 30%.

3.1.3. Co-digestion of wheat straw and wool textile

The co-digestion of wool textile wastes and wheat straw (in ratio of 1:1 based on VS) was also investigated using similar TS contents with or without addition of nutrients. The expected methane yields for the co-digested mixture can be calculated from the methane potentials obtained for the individual fractions (Table 1). All the co-digestion assays showed higher accumulated methane yields than the calculated expected levels, with an exception of when TS of 13% was used. According to Álvarez and Lindén 2008 (27) and Pagés Díaz et al., 2011 (28), the accumulated methane yield in a co-digestion process can be higher than the calculated expected value, due to synergic effects developed in the digester. Interestingly, the

methane production of co-digested wheat straw and wool textile waste was the highest, showing an increase of 58%, compared to the expected value, when 30% TS was applied together with addition of nutrients. Furthermore, similar TS condition without nutrient supply led to an increase of 39%, compared to the expected methane yield. Since, higher TS concentration or higher substrate/inoculum ratio results in a slowdown in the microbial growth (29, 30), it is expected that digesters containing 30% TS, more likely suffer from nutritional imbalances, compared to those digesters that are running with lower TS content. Therefore, co-digestion of the carbon rich straw with the nitrogen rich wool textile waste provided a nutrient efficient feedstock with a more balanced C/N ratio even though applying 30% TS; consequently, this resulted in a noticeable improvement in methane production. Furthermore, this improvement was even higher when additional nutrients were added to the digestion system. The results of this study are in agreement with the study of Kayhanian and Rich (1995) (31), which suggested that mixing of two or three organic wastes could provide a nutrient sufficient feedstock for a dry-AD process.

3.2. **Degradation of substrates and compositional changes**

Compositional analyses on wheat straw and wool textile waste were performed, before and after a 50-day long digestion period at TS of 13% and 21%. When investigating the straw samples, changes in the composition (cellulose, hemicellulose and extractives) was defined as the corresponding mass reductions between the beginning and end of the 50-day digestion (Figure 2.) The compositional analysis of native wheat straw showed 35.2% cellulose and 22.2% hemicellulose content (Figures 2A and 1B). The cellulose content of the straw residues obtained after dry-AD digestion at 13% TS with and without addition of enzymes were 10.0% and 11.2%, respectively. The highest levels of cellulose degradation of 69 and 71% were observed when cellulolytic enzymes were added to the digestion process using TS of 13 and 21%, respectively. A similar trend was observed in the hemicellulose removal as well,

showing a reduction in hemicelluloses by 69 and 66%, when the digestion process was performed at TS contents of 13 and 21%, respectively, both with the addition of enzymes. On the other hand, the hemicellulose reduction in dry digestion of straw, without addition of enzymes was lower, *i.e.* 63 and 54% at TS contents of 13 and 21%, respectively.

Hence, the highest cellulose and hemicellulose degradations were achieved after simultaneous enzymatic hydrolysis and dry-digestion. The optimum temperature for the cellulolytic enzymes added is about 38ºC, which is very close to the mesophilic operation temperature of dry-AD in this study (32), supporting the action of the enzymes and making the first step of the digestion, the hydrolysis step, more efficient.

The extractives' content of wheat straw was 16.6% of the dry weight, and the degraded wheat straw with and without addition of cellulolytic enzymes showed 14 – 38% reduction in extractives (Figure 2C). Since the extractives include compounds, such as free sugars, oligomers and organic acids (33, 34), these compounds can be easily degraded and can potentially contribute to biogas production (35). The composition of extractives was not analysed in this study; however, further research on degradation of extractive compounds and their contributions in AD process would be interesting. The lignin content of the wheat straw remained intact before and after digestion (18.5% of dry weight), meaning that this fraction was not subjected to degradation during the digestion period applied.

The nitrogen content of wool textile waste was determined by the Kjeldahl method. The N-content of wool textiles was 14%, and a reduction of nearly 50% in the N-content was observed after digestion, with or without the addition of a protein degrading enzyme (Figure 3). The nitrogen content of the wool textile waste after 50-day degradation without and with addition of enzyme was 7.5 and 7.24% in reactors containing 13% TS, and respectively 9.4 and 8.2% when 21% TS was applied (Figure 3). However, these high degradation rates of the proteins in wool did not necessarily lead to higher methane productions (Figure 1). This might

be due to the accumulation of ammonium nitrogen as discussed earlier. A lag phase of 20 days was observed when wool textile waste was digested at TS of 13 and 21%, and this lag phase was prolonged. Moreover, the conversion of organic matter for gas production did not start up at all when the TS content was increased to 30%. However, the lag phase was shorter, and methane production rate was faster in the more diluted system, i.e. with TS of 6%. The ammonia levels in the digesters at the end of the digestion period were not measured; however, the obtained decrease in the N-content after the digestion on the one hand, and the low accumulated methane production determined on the other hand, can indicate an imbalance in the digestion system. Nevertheless, nitrogen is an important nutrient for microbial growth. However, high concentrations of nitrogen may lead to a severe disturbance in the performance of anaerobic digestion, which can cause a dramatic decrease in microbial activities (36). The result is decreased methane production rates and increased concentrations of intermediate digestion products, like VFAs, which finally will lead to a total termination of methanogenic activity (37, 38).

3.3. **Structural changes in wheat straw and wool obtained after dry-AD**

The cellulosic crystallinity of wheat straw was investigated with FTIR spectrometer. The crystallinity index of the native wheat straw and the digested straw obtained after dry-AD performed at TS concentrations of 13 and 21% and with and without the addition of enzymes is shown in Figure 4. The crystallinity index of the native straw was 0.62; however, throughout the 50-day long dry-AD process, the crystallinity index was increased to 1.3 – 1.5 when TS contents of 13 or 21% were applied, without or with the addition of cellulolytic enzymes (Figure 4). As previously mentioned, the crystallinity index is the absorbance ratio between crystalline cellulose I and the amorphous form of cellulose II (A_{1422}/A_{898}). As shown in Figure 4, there was a significant increase in crystallinity index after 50-day digestion

period, which was confirmed by statistical analyses (p-value = 0.000). Since the cellulolytic enzymes can target the less crystalline regions of cellulose fibres (39), this increase in crystallinity indicates that the amorphous cellulose part was initially subjected to biological degradation, subsequently leaving the crystalline cellulose fraction relatively intact.

The structural changes in the protein microstructure of wool textile waste were also studied by FTIR (Figure 5). The amide I (in the range between 1700 – 1600 cm^{-1}) and amide II (between 1545 – 1400 cm^{-1}) bands, as the most predominant vibrational bands of the protein backbone were investigated (data not shown) (15). These bands are mainly associated with the stretching vibrations of the peptide bonds. The most sensitive spectral region to the protein secondary structure is amide I; therefore, the de-convoluted amide I band was studied in detail to understand the changes in the secondary structure of protein in wool textile due to the digestion. The absorption regions of 1631 – 1621 cm^{-1} and 1694 – 1680 cm^{-1} show the secondary structures of β-sheet and the absorption regions of 1657 – 1651 cm^{-1}, and 1679 – 1670 cm^{-1} represent secondary structures of α-helix, and disordered regions, respectively. Originally, the wool textile waste contained 29.3% α-helix, 44.4% β-sheet and 17% disordered regions. There was a decrease (from 17% to 10%) obtained in these disordered regions after the 50-day long digestion period when TS of 13 or 21% was applied. Consequently, the degradation of wool textile waste led to an increase of α-helix conformation by 42.5 – 49.1% at both TS conditions, both with and without the addition of a protein degrading enzyme. Furthermore, there was a slight decrease in β-sheet conformation after 50-day dry-digestion (Figure 5). We can, therefore, conclude that as expected the disordered regions were the most affected regions during the digestion process. However, there is certainly a knowledge gap in anaerobic digestion of keratin rich substrates and their structural changes during the degradation process, which should be investigated further in the future.

4. Conclusions

Anaerobic digestion of straw and wool textile waste using dry digestion technology can be a promising option, compared to that using wet AD systems, when considering the higher methane yield and productivity, reduction in reactor volume and thus less energy consumption for heating and hence cheaper maintenance. However, the results of this study showed that the TS content applied must be properly adjusted to avoid overloading the system. However, it was also shown that the problems with overloading due to the high TS content could be solved by the co-digestion of these two substrates. Co-digestion of a carbon rich (wheat straw) and a nitrogen rich (wool textile waste) substrate can lead to higher methane productions than those calculated based of the methane potentials for the single substrates. Moreover, due to the synergetic effects and a better nutritional balance, the TS could be increased to 30% during co-digestion without causing serious problems to the digestion system. These findings can open up a new path for further optimisation and investigations of co-digestion of complex biomass fractions in dry-AD systems, since it can provide a cost effective opportunity for effective utilisation of these complex substrates.

Figure legends:

Figure 1: Accumulated methane production expressed in Nm^3CH_4/ kg VS for wheat straw (A), wool textile waste (B), and co-digestion of these two (C) obtained during 50-day AD, at TS contents of 6%, 13% 21% and 30%. The digestion conditions are described in the figure.

Figure 2: Degradation of cellulose (A), hemicellulose (B) and extractives (C) during 50-day dry-AD, based on initial loading of 100g dry matter of wheat straw. The error bars correspond to one-standard deviation.

Figure 3: Degradation of nitrogen content, based on initial loading of 100g dry matter of wool textile wastes. The error bars correspond to one-standard deviation.

Figure 4: Crystallinity index of wheat straw before and after 50-day dry-AD. The error bars correspond to one-standard deviation.

Figure 5: The secondary structure of wool textile protein (α-helix, β-sheet and disordered region), before and after 50-day dry-AD. The error bars correspond to one-standard deviation.

Table legend:

Table 1: Accumulated methane production (Nm^3CH_4/ kg VS), methane volumetric productivity (Nm^3 /m^3 Working Volume), and the expected calculated methane yield from the co-digested mixture, at TS contents of 6%, 13%, 21%, and 30%.

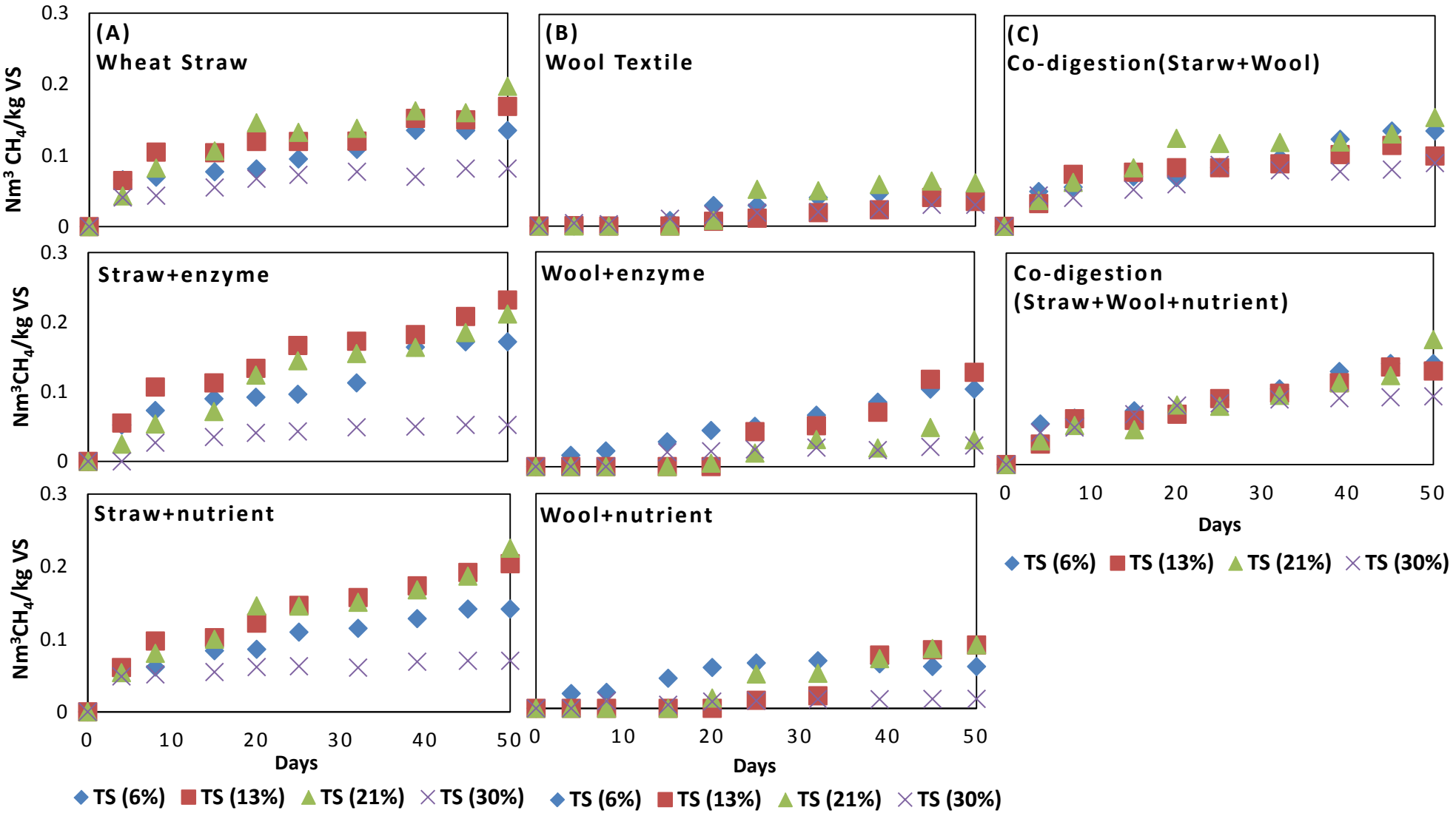
(A)
Wheat Straw
(B)
Wool Textile
(C)
Co-digestion(Starw+Wool)
Straw+enzyme
Wool+enzyme
Co-digestion
(Straw+Wool+nutrient)
Straw+nutrient
Wool+nutrient
Nm³ CH4/kg VS
Days
TS (6%)
TS (13%)
TS (21%)
TS (30%)

Figure 1.

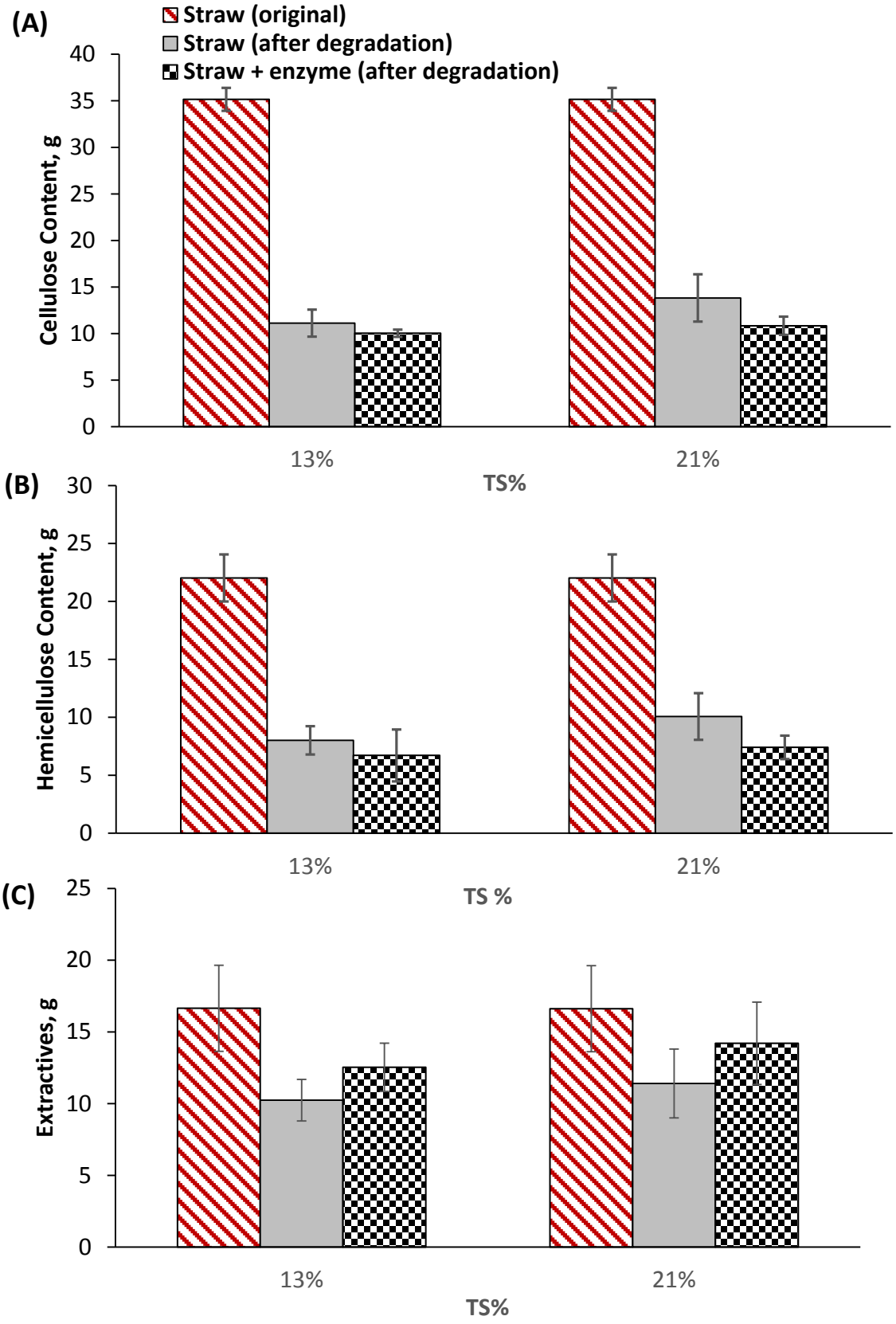
(A)
Straw (original)
Straw (after degradation)
Straw + enzyme (after degradation)
Cellulose Content, g
40
35
30
25
20
15
10
5
0
13%
21%
TS%
(B)
Hemicellulose Content, g
30
25
20
15
10
5
0
13%
21%
TS %
(C)
Extractives, g
25
20
15
10
5
0
13%
21%
TS%

Figure 2.

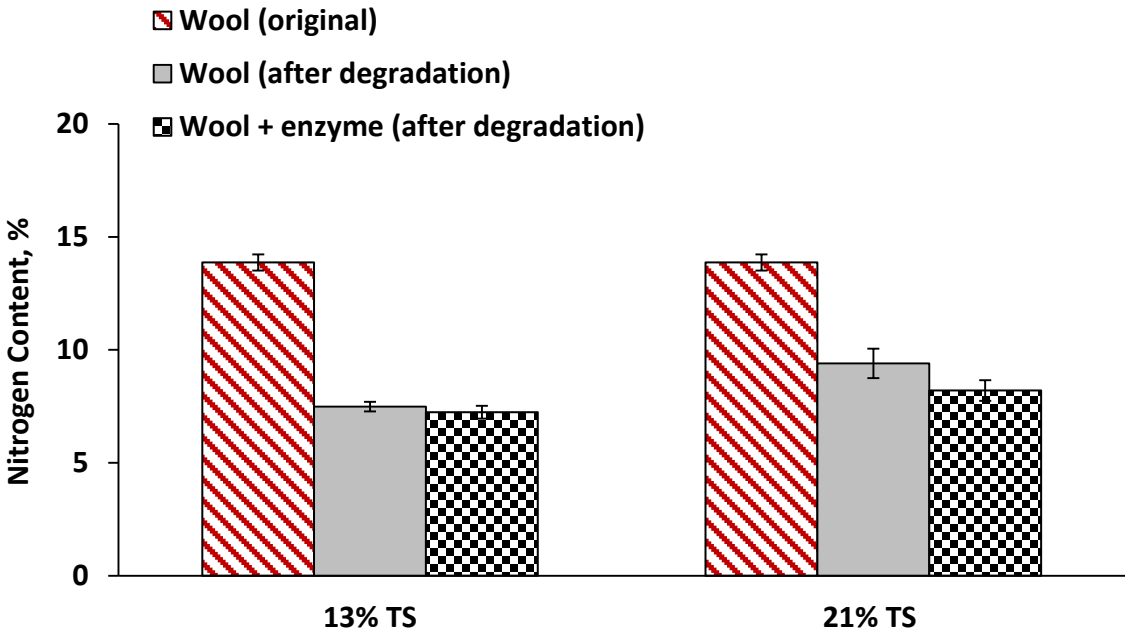
Wool (original)
Wool (after degradation)
Wool + enzyme (after degradation)
Nitrogen Content, %
20
15
10
5
0
13% TS
21% TS

Figure 3.

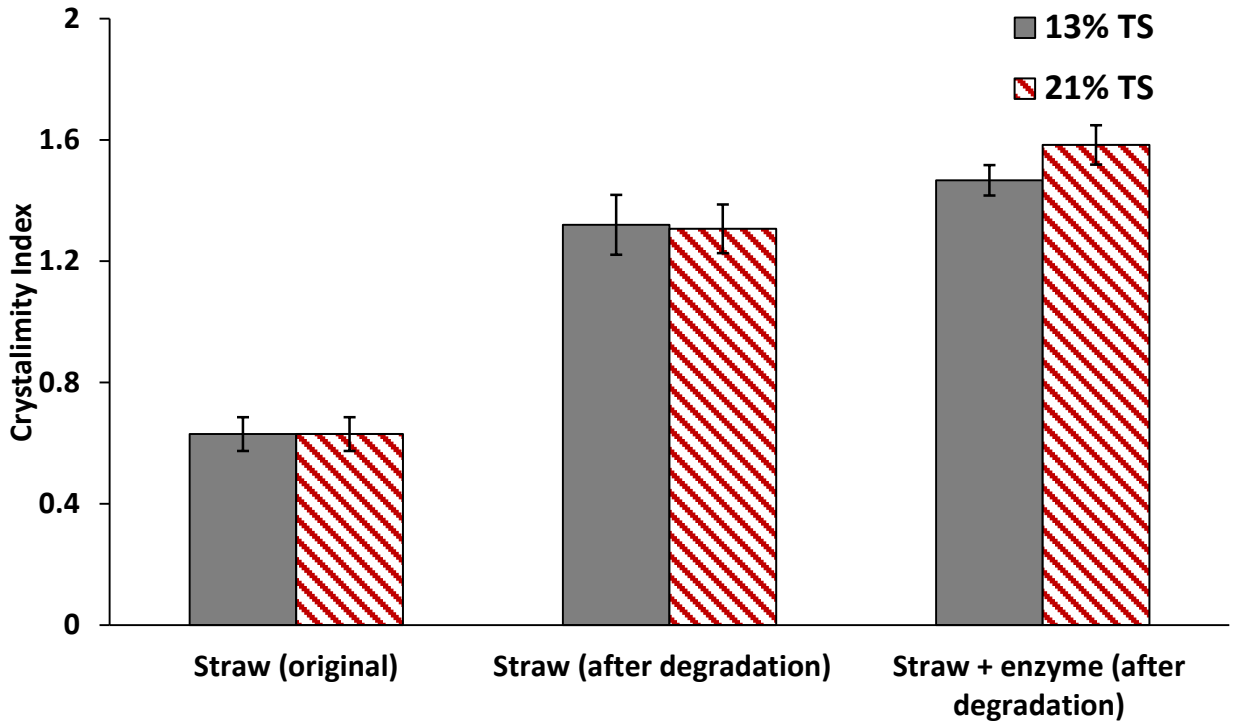
2
1.6
1.2
0.8
0.4
0
Crystalimity Index
13% TS
21% TS
Straw (original)
Straw (after degradation)
Straw + enzyme (after degradation)

Figure 4.

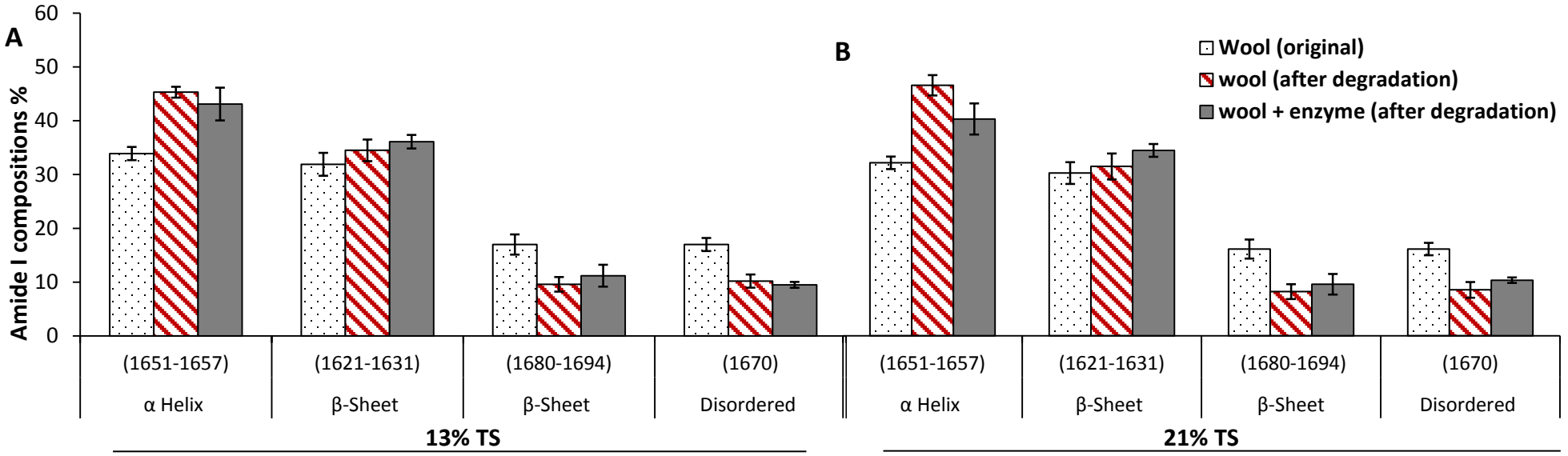
A
B
Amide I compositions %
60
50
40
30
20
10
0
Wool (original)
wool (after degradation)
wool + enzyme (after degradation)
(1651-1657)
α Helix
(1621-1631)
β-Sheet
(1680-1694)
β-Sheet
(1670)
Disordered
13% TS
21% TS

Figure 5.

Table 1.

	Feedstock	Accumulated methane yield (Nm^3 CH_4/ kg VS)	Volumetric productivity (m^3 CH4/m^3 Working Volume)	Expected methane yields of co-digested mixture (Nm^3 CH_4/ kg VS)
6% TS	Straw	0.135 ± 0.007	0.011	–
	Straw+ Enzyme	0.171 ± 0.030	0.014	–
	Straw+ Nutrient	0.141 ± 0.013	0.012	–
	Wool	0.048 ± 0.013	0.004	–
	Wool+ Enzyme	0.108 ± 0.005	0.009	–
	Wool+ Nutrient	0.058 ± 0.009	0.005	–
	Wool+ Straw	0.136 ± 0.009	0.014	0.091
	Wool+ straw + nutrient	0.143 ± 0.011	0.012	0.102
13% TS	Straw	0.170 ± 0.017	0.021	–
	Straw+ Enzyme	0.231 ± 0.011	0.029	–
	Straw+ Nutrient	0.204 ± 0.006	0.025	–
	Wool	0.035 ± 0.000	0.005	–
	Wool+ Enzyme	0.131 ± 0.005	0.016	–
	Wool+ Nutrient	0.088 ± 0.002	0.011	–
	Wool+ Straw	0.100 ± 0.010	0.012	0.102
	Wool+ straw + nutrient	0.133 ± 0.003	0.016	0.146
21% TS	Straw	0.200 ± 0.007	0.033	–
	Straw+ Enzyme	0.211 ± 0.002	0.035	–
	Straw+ Nutrient	0.225 ± 0.013	0.037	–
	Wool	0.061 ± 0.014	0.010	–
	Wool+ Enzyme	0.040 ± 0.007	0.000	–
	Wool+ Nutrient	0.089 ± 0.009	0.015	–
	Wool+ Straw	0.154 ± 0.001	0.026	0.130
	Wool+ straw + nutrient	0.177 ± 0.021	0.030	0.157
30% TS	Straw	0.081 ± 0.002	0.016	–
	Straw+ Enzyme	0.052 ± 0.002	0.010	–
	Straw+ Nutrient	0.070 ± 0.000	0.014	–
	Wool	0.030 ± 0.002	0.006	–
	Wool+ Enzyme	0.033 ± 0.003	0.006	–
	Wool+ Nutrient	0.013 ± 0.008	0.003	–
	Wool+ Straw	0.090 ± 0.005	0.020	0.055
	Wool+ straw + nutrient	0.097 ± 0.006	0.020	0.041

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Paper V

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Experimental and economical evaluation of bioconversion of forest residues to biogas using organosolv pretreatment

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HIGHLIGHTS

- Organosolv-pretreatment of forest residues for enhanced CH_4 yield was performed.
- The pretreatment increased the CH_4 yield by 50%.
- Acetic acid and ethanol pretreatments led to the highest methane yields.
- Techno-economical evaluation suggest, methanol pretreatment was more efficient.

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ABSTRACT

The methane potential of forest residues was compared after applying organic solvent, *i.e.*, acetic acid, ethanol, and methanol pretreatments using batch anaerobic digestion (AD). The pretreatments were performed at 190 °C with 50% (V/V) organic solvent for 60 min. The accumulated methane yields after 40 days of AD from pretreated forest residues were between 0.23 and 0.34 m^3 CH_4/kg VS, which shows a significant improvement compared to 0.05 m^3 CH_4/kg VS, from untreated forest residues. These improvements count up to 50% increase in the methane yields from the pretreated substrates based on expected theoretical yield from carbohydrates. Among the organic solvents, pretreatments with acetic acid and ethanol led to highest methane yields, *i.e.*, over 0.30 m^3 CH_4/kg VS. However, techno-economical evaluation showed, pretreatment with methanol was more viable financially. The capital investments of the plant operating 20,000 tons of forest residues varied between 56 and 60 million USD, which could be recovered in less than 8 years of operation.

1. Introduction

According to European Commission Energy, 2010, EU members aim to reach a 20% share of final energy consumption from renewable sources, with a binding target of 10% renewable energy in the transport sector by 2020. A development of renewable energy sources for production of heat and electricity is spread across the energy sectors; however, the production of renewable fuels for the transport sector is less established and requires a substantial progress to promote the contribution of biofuels for transportation (European Commission, 2011).

Among the renewable fuels, biogas is becoming an important part of the biomass-to-energy chain. Biogas is a renewable energy source, which is commercially produced from various organic waste materials such as wastewater sludge, municipal solid waste, and energy crops. Moreover, biogas has several advantages, since the overall energy efficiency is much higher in biogas production compared to that for bioethanol production (Murphy and Power, 2009) Furthermore since most of the industrial yeast species cannot assimilate pentoses, the utilization of pentoses remains one of the challenges in a lignocellulose to ethanol process (Karimi and Zamani, 2013). An alternative solution can be to produce biogas as only product from lignocellulosic biomass. Many industrial, municipal, agricultural, and forestry waste streams contain high portions of lignocelluloses, representing a great potential for biogas production. However, these energy-rich fractions are not commonly used yet for biogas production (Deublein and Steinhauser, 2008).

Due to the recalcitrant structure of the lignocelluloses, the crystallinity of the cellulose, and the presence of lignin, there is a limitation in the first step of the anaerobic degradation, *i.e.*, the hydrolysis step. Therefore, an appropriate pretreatment is essential, aiming to disrupt the cell wall barriers, to remove the lignin and to decrease the cellulose crystallinity, so that hydrolytic enzymes produced by facultative anaerobes acting during the

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anaerobic digestion process can easily access the biomass macrostructure (Fan et al., 1981; Zhao et al., 2009). Additionally, another limitation can occur even in the methanogenic step, where the methane is produced (Noike et al., 1985), since methanogenic bacteria have a low growth rate and are sensitive to the inhibitory products that might form during some specific pretreatment conditions. The application of the pretreatment methods, such as dilute acid pretreatment, steam explosion, and ammonia fiber explosion can lead to the formation of furans and phenolic compounds that negatively affect the balance of the anaerobic digestion process (Chundawat et al., 2007; Taherzadeh and Karimi, 2008). Therefore, applying a suitable pretreatment method that avoids these problems is of vital importance.

Among all the pretreatment methods, organosolv-pretreatment has been considered as one of the promising methods for pretreating lignocellulosic biomass and now being developed as part of a commercial lignocellulose biorefinery (Alvira et al., 2010; Pan et al., 2005). Pretreatment with an organic solvent partially hydrolyzes the lignin fraction and the bonds between the lignin and carbohydrates, aiming to remove the major barrier for an enzymatic attack (Hu et al., 2011; McMillan, 1994). Moreover, using organic solvents, like low molecular weight alcohols and organic acids for pretreatment of lignocelluloses, are not inhibitory for the methane producing microorganisms, since these easily-degradable compounds can be utilized by the microorganisms in the anaerobic digestion system.

So far, most of the studies carried out on investigating organosolv-pretreatment of lignocellulosic materials were focused on either the fractionation of biomass or the improvement of the enzymatic hydrolysis for bioethanol production. Pan et al. (2006) applied hot aqueous ethanol pretreatment on the hybrid poplar, which successfully improved the recovery of glucose by 85% after enzymatic hydrolysis. In another study, a combined treatment using sulfuric acid and ethanol on lodgepole pine (softwood) resulted in up to 97% conversion of cellulose to glucose (Pan et al., 2007). Nevertheless, no previous research has been reported for organosolv-pretreatment on lignocellulosic biomass aiming to improve biogas production.

In this study, forest residues were selected among the lignocellulosic wastes for the investigations, since they represent an abundant waste stream in Sweden. The tree tops and branches obtained from the Swedish forests counted up to about 1.6 M tons total solids in 2008. This amount is expected to increase to 3.5 M tons total solids/year by the year of 2018 (Teghammar et al., 2014), corresponding to a biomethane potential of 154 M m^3 CH_4/year.

One of the main problems faced in the biogas industry is its profitability as well as the supply chain of the raw materials (Rajendran et al., 2014). Given the fact that the volume of forest residues is estimated to grow in Sweden, the important question that arises is the profitability. In a previous study, process design and cost-estimation had been performed for co-digestion of the pretreated forest residues, using a cellulosic solvent, *i.e.*, *N*-Methylmorpholine-*N*-oxide (NMMO), with municipal solid waste (MSW) (Teghammar et al., 2014). The results showed that the cost of the solvent reflected the main part of the material costs; therefore, effective recovery of the NMMO was crucial for the feasibility of the industrial scale process. Whereas, when using low molecular weight organic solvents for the pretreatment they can be recovered and re-used easily, leading to a better economics of the process (Amiri et al., 2014; Zhao et al., 2009).

The aim of this study was to increase the digestibility of the forest residues using ethanol, methanol, and acetic acid pretreatments prior to biogas production. Specifically, to determine the biomethane potential of the pretreated forest residues using the above mentioned solvents in the presence and absence of catalysts such as sulfuric acid, hydrochloric acid, and acetic acid. The mass balances as well as the solvent recovery were accurately studied. Based on the experimental data, a process design was performed to estimate the overall energy needs and economics of the process.

2. Methods

2.1. Materials

Native forest residues (mixture of spruce, pine, bark, etc.) were obtained from the forests outside Borås in Sweden. These residues were dried at room temperature for a couple of days, followed by cutting, milling, and screening to achieve 2 mm particle size.

2.2. Organosolv pretreatment

Three different organic solvents including: ethanol, methanol, and acid acetic were used for the pretreatments. The pretreatments were carried out based on the process description by Pan et al. (2006). First, eight grams (dry weight) of the forest residues were mixed with 80 g (solid-to-liquid ratio of 1:10) of 50% (V/V) aqueous organic solvents. In the case of the ethanol and methanol pretreatments, the addition of 1% (W/W) (based on the forest residues dry mass) of two different acids (each separately), as catalysts were also investigated (*i.e.*, ethanol + sulfuric acid, ethanol + acetic acid, methanol + sulfuric acid, methanol + acetic acid). Moreover, the effect of the addition of two other catalysts, including sulfuric acid or hydrochloric acid (each added separately) was investigated in the case of the acetic acid pretreatment. The organosolv-pretreatments were carried out in sealed 150 ml stainless steel tubular reactors (Swagelok, U.S.A.), heated in an oil bath at 190 °C for 60 min. After the pretreatment, the reactors were taken out from the oil bath and placed in an ice chamber to cool below room temperature. The pretreated-forest residues were washed with 80 g aqueous organosolv (50% V/V) solution and then washed with 80 g of water. The washed biomass (*i.e.*, solid fraction) samples were allowed to dry prior to the composition analyses regarding carbohydrates and lignin. Finally, the materials were stored at 5 °C until use.

2.3. Anaerobic batch digestion assays

Anaerobic batch digestion assays at thermophilic conditions (55 °C) were performed in triplicates to investigate the effects of the different pretreatment conditions on the methane potential. The digesters used were serum glass bottles, each with a total volume of 118 ml, and closed with butyl rubber seals and aluminum caps (Hansen et al., 2004). The inoculum was obtained from an industrial 3000-m^3 municipal solid waste digester operating at thermophilic (55 °C) conditions (Borås Energi och Miljö AB, Borås, Sweden). Each flask contained 20 ml inoculum and 0.15 g VS (Volatile Solids) of untreated or treated substrate, and the final working volume was then adjusted to 25 ml with the addition of deionized water. Furthermore, a mixture of deionized water and inoculum was also prepared and used as a control to determine the gas production from the inoculum.

Finally, the headspace of each bottle was flushed with nitrogen to obtain anaerobic conditions. Gas samples were withdrawn regularly from the headspace of each bottle, and the accumulated methane production was determined using gas chromatography.

2.4. Analytical methods

The Total Solids (TS) and Volatile Solids (VS) were determined by oven drying the samples first at 105 °C until a constant weight was achieved and then by ignition at 575 °C in a furnace,

respectively (Sluiter et al., 2008a). The compositional analyses to determine the cellulose, hemicellulose, and lignin contents of the untreated and pretreated materials were performed according to the procedures described by Sluiter et al. (2008b). In this method, a two-step acid hydrolysis is applied first using concentrated and then diluted sulfuric acid to liberate the sugars. The acid-soluble and acid-insoluble lignin contents were determined using UV spectroscopy and after drying the samples at 575 °C, respectively. All the lignin and carbohydrate analyses were performed in triplicates.

The sugars, liberated from the cellulose and hemicelluloses, were analyzed using high performance liquid chromatography (HPLC), (Waters 2695, Millipore, Milford, U.S.A.), equipped with a refractive index (RI) detector (Waters 2414) and an ion-exchange column (Aminex HPX-87P, Bio-Rad, U.S.A.) at 85 °C using ultrapure water as eluent with a flow rate of 0.6 ml/min.

A gas chromatograph (Auto System Perkin Elmer, Waltham, MA) was used to measure the methane produced in the anaerobic digestion assays. The gas chromatograph was equipped with a packed column (Perkin Elmer, 60x1, 800OD, 80/100, Mesh) and a thermal conductivity detector (Perkin Elmer) with an injection temperature of 150 °C. The carrier gas was nitrogen, with a flow rate of 23 ml/min at 60 °C. Gas sampling was performed using a 250 μl pressure-tight gas syringe (VICI, Precision Sampling Inc., LA). After each gas measurement, the excess gas was released through a needle, to avoid overpressure in the head space of the flasks, and then a new sample was taken from the headspace and analyzed by gas chromatography (Teghammar et al., 2010). All the results of the methane volumes are presented at standard conditions.

To determine the initial methane production rates, a first-order kinetics model described previously by Jimenéz et al. (2004):

$$G = G_m\left(1 - e^{-K_0 t}\right), \quad (1)$$

where G is the accumulated methane volume (ml) after a time t (days), G_m is the final accumulated methane volume (ml), and K_0 is the observed specific rate constant of the overall process (days^{-1}). The specific rate constant (K_0) is calculated as follows:

$$Ln\left(\frac{G_m}{G_m - G}\right) = K_0 t$$

The significant differences between the methane productions using the pretreated forest residues with or without the addition of catalysts were verified by 2-sample t-tests using a software package MINITAB® (V 17.0).

2.5. Process description and economics

Based on the data obtained from the batch experiments and from the characterization studies, a process simulation was performed to evaluate the techno-economic feasibility of the biogas production from forest residues after using different organic solvents (*i.e.*, ethanol, acetic acid, and methanol) for the pretreatments. The simulation was performed using Intelligen Superpro® Designer program (V8.5, Massachusetts, U.S.A.). The plant was considered to have an annual operational capacity of 20,000 tons. Table 1 shows the list of materials and the assumptions considered to perform this study. The assumptions used were in accordance with those presented in previous studies (Forgács, 2012; Shafiei et al., 2013; Shafiei et al., 2011; Teghammar et al., 2014).

The forest residues arriving to the plant are first stored in a silo tank made of concrete, which can store an amount of material that is enough for five days. The stored material is then sent to a shredder made of carbon steel (CS), which can handle a throughput of 2.5 t/h. Furthermore, the shredded materials are then transported to the pretreatment reactor using a conveyor belt made of CS. The solvent (*i.e.*, ethanol, acetic acid, or methanol) used for the pretreatment is stored in a vertical tank with a volume of 35 m^3. Another vertical tank with a volume of 300 m^3 is used to store the water, which is used for different purposes within the plant. The solvent is then pumped to the pretreatment reactor using a centrifugal pump, made of stainless steel. In the pretreatment reactor, the solvent is kept in contact with the forest residues at 190 °C for 1 h. The pretreated material is then sent for filtration to a vacuum filter, which uses water and the solvent in equal volumes. The cake obtained from the filter is transported to the anaerobic digester, while the filtrate is sent to the evaporator to recover the solvent. The anaerobic digester has a working volume of between 2800 and 3600 m^3 depending on the pretreatment method, and is operated at 55 °C using a hydraulic retention time of 20 days.

Table 1
List of assumptions used in this study.

Material	Assumption
Annual processing capacity	20,000 tons fresh matter
Cost index	2014 1st quarter
Depreciation method	Straight line
Interest rate	7%
Lifetime of the plant	15 years
Salvage value	5%
Taxes	33%
Construction period	30 months
Start-up period	4 months
Electricity	0.1 USD/kWh
Steam	12 USD/MT
Water	0.001 USD/kg
Selling price of methane	1.81 USD/L (gasoline equivalent)
Lignin	3.0 USD/kg
Waste water treatment	0.0001 USD/kg
Cost of Forest residues	0.4 USD/kg
Ethanol	0.75 USD/kg
Acetic acid	0.70 USD/kg
Methanol	0.30 USD/kg

After the digestion, two different streams are generated, including a gas stream that goes to the biogas upgrading step and a liquid stream that is given to the farmers as fertilizers at no cost. The biogas produced in the anaerobic digester is initially compressed to a pressure of 7 bar using a centrifugal compressor made of CS prior to sending it to the upgrading column. The upgrading method applied is water scrubbing, where water comes in contact with the incoming biogas, making it possible to dissolve carbon dioxide, hydrogen sulfide, and other impurities. The upgraded gas then leaves the column at a pressure of 9 bar and is sent further to a centrifugal compressor for further compression to reach a pressure of 300 bar. The compressed methane is stored in a flat tank until it is sold or transported.

After the recovery of the solvent, the lignin rich filtrate is sent through a clarifier to remove the excess water. The sediment containing the lignin is centrifuged to remove the excess water, and the solids are separated. The cake from the centrifuge is sent to a drum dryer to remove the excess moisture, before it is stored in a storage tank in order to sell it as crude lignin. All the waste water streams, collected from the distillation, centrifugation, filtration, and drying steps, are sent to a waste water treatment plant. The treatment cost is assumed to be 1×10^{-3} USD/L.

For the evaluations, the biogas plant is assumed to have a lifetime of 15 years. Different profitability indexes, such as capital investment, payback period (PBP), return on investment (ROI), net present value (NPV), operating cost, and annual revenues combining the revenues generated from the methane and digestate were calculated. All the indexes were compared for each of the

different processes using the different solvents for the pretreatment of the forest residues. The base case was constructed for an annual capacity of 20,000 tons of forest residues (based on fresh matter); however, a sensitivity analysis was also performed using capacities ranging from 10,000 to 200,000 tons forest residues/year to study the effects of using different plant sizes and its impacts on the profitability indexes.

3. Result and discussion

Milled forest residues were subjected to organosolv-pretreatment using ethanol, methanol, or acetic acid with sulfuric acid, acetic acid as catalysts. After the pretreatment, the solid residues were chemically analyzed and subsequently used in batch anaerobic digestion assays.

3.1. Compositional analysis of the pretreated and untreated forest residues

The chemical composition of the pretreated and untreated forest residues is presented in Table 2. The acid insoluble lignin (AIL) and acid soluble lignin (ASL) contents in the untreated material were 41% and 3.7%, respectively. As a result of the organosolv-pretreatment, there was a slight decrease in the amount of AIL obtained, especially after pretreatment with ethanol using acetic acid as a catalyst (Table 2).

The results also showed that the organosolv-pretreatment with sulfuric acid as a catalyst was not effective to remove the lignin. These results are in agreement with previous works on organosolv-pretreatment using sulfuric acid as a catalyst (Pan et al., 2007). The reason for this observation might be the condensation of lignin, since it is released from the biomass structure in the early stages of the pretreatment process due to the high temperature; however, it can re-join with the cellulose and hemicellulose again at the end of the process. Therefore, the total lignin content in the biomass ultimately remains unchanged (Liu and Wyman, 2004). On the other hand, ASL was decreased nearly by 50% (*i.e.*, from 3.5% to 1.6%) after applying different organosolv-pretreatments on the forest residues (Table 2). Moreover, the results of the compositional analysis show that all the pretreatment conditions with different organic solvents resulted in an enrichment of the cellulose content compared to the native forest residues. The cellulose content of the untreated forest residues was 22.3%, and this value was considerably increased to between 31.2% and 43% after using organosolv-pretreatments. This improvement might be the result of partial solubilization of lignin and hemicelluloses.

The hemicellulose contents of the native forest residues were decreased by 14–43% following the organosolv-pretreatments. The main reduction in the hemicellulose content was observed for the pretreatment with ethanol, using sulfuric acid as a catalyst, which showed a major dissolution of hemicelluloses in the organic liquor that subsequently resulted in a major cellulose release.

The cellulose and hemicellulose loss during all the pretreatment conditions were between 1–3% and 3–5%, respectively.

3.2. Effects of organosolv-pretreatment on anaerobic digestion

The pretreated- and untreated forest residues were subjected to anaerobic batch digestion assays running at thermophilic conditions during 40 days. The results in Fig. 1 and Table 2 show that the organosolv-pretreatment could successfully increase the methane production of the forest residues, compared to that obtained from the untreated assays.

Table 2
Total carbohydrate and lignin contents and accumulated methane production with the pretreatment solvents and their recovery for the untreated and pretreated forest residues.

	AIL %	ASL %	Cellulose %	Hemicelluloses %	Total carbohydrate %	Accumulated m^3 CH_4/kg VS[b]	Solvent recovery %	K_0 $(day)^{-1}$[c]	Accumulated m^3 CH_4/kg VS[a]
Untreated	41.0	3.7	22.3	20.0	42.3	0.05 ± 0.006	–	–	0.05
Ethanol (without catalyst)	37.6	2.0	37.6	14.3	51.9	0.30 ± 0.009	97.2	0.0424	0.18
Ethanol and acetic acid	36.0	1.9	36.6	15.2	51.8	0.31 ± 0.009	97.2	0.0658	0.18
Ethanol and sulfuric acid	39.0	1.7	43.1	11.5	54.6	0.34 ± 0.001	97	0.0569	0.19
Methanol (without catalyst)	38.8	2.1	37.4	13.2	50.6	0.23 ± 0.005	98.2	0.0316	0.14
Methanol, acetic acid	38.4	1.2	39.0	15.2	54.2	0.31 ± 0.006	97.3	0.0320	0.21
Methanol, sulfuric acid	41.3	1.7	35.2	17.2	52.4	0.34 ± 0.014	97	0.0428	0.21
Acetic acid (without catalyst)	40.2	1.9	31.2	15.5	46.7	0.33 ± 0.009	96.3	0.0665	0.20
Acetic acid and hydrochloridric acid	39.3	1.6	32.2	16.2	48.4	0.28 ± 0.010	97.8	0.0292	0.18
Acetic acid and sulfuric acid	41.6	1.9	40.3	12.2	52.5	0.31 ± 0.001	97.5	0.0552	0.18

*1% (w/w) catalysts were added based on the dried weight of the forest residues.
[a] The accumulated methane yield obtained from the forest residues.
[b] The accumulated methane yield from the forest residues plus the remaining organic solvent in the reactor.
[c] The specific rate constant (K_0) is calculated for the first 8 days of the anaerobic digestion.

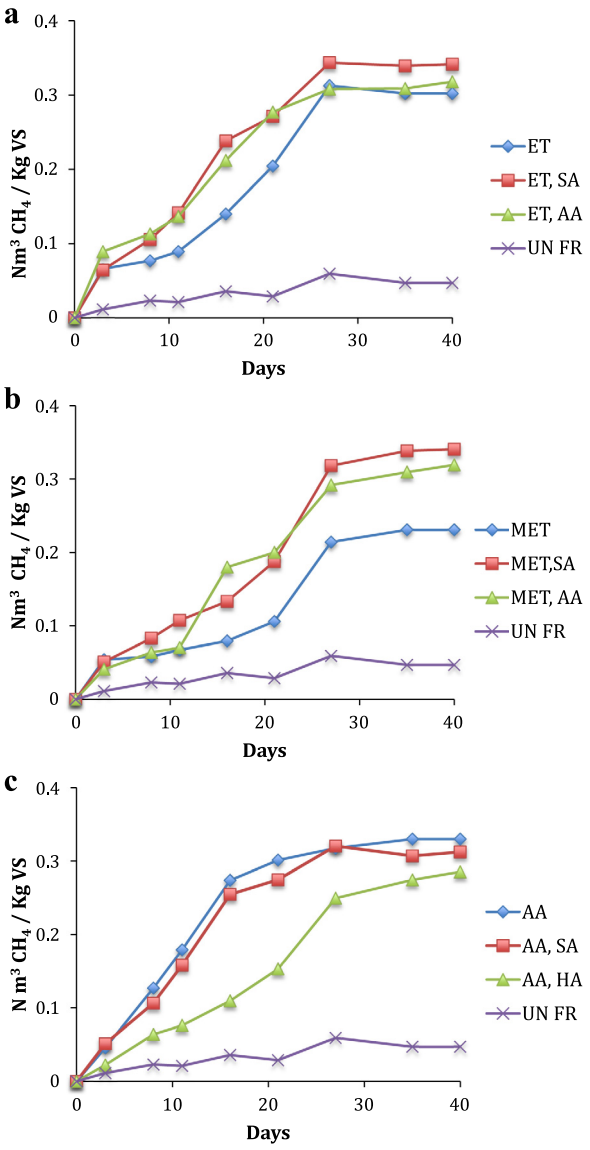

Fig. 1. Accumulated methane yield (Nm^3 CH_4/kg VS) obtained during the anaerobic batch digestion assays of untreated (UN FR) vs. pretreated forest residues: (a) ET, ethanol pretreatment, (b) AA, acetic acid pretreatment, and (c) MET, methanol pretreatment. The added catalysts were: SA: sulfuric acid, AA: acetic acid, and HA: hydrochloric acid.

The total methane yield, including the carbohydrate fraction and the organic solvent remaining in the samples after the pretreatment, was between 0.23 and 0.34 m^3 CH_4/kg VS. Most of the solvents (~96%) were recovered after the pretreatments; however, the remaining solvent in the substrate also accounted for the biogas production. The theoretical methane yield for acetic acid, ethanol, and methanol was 0.373, 0.486, and 0.525 m^3/kg VS, respectively. Based on these values the methane yield of the solvent remained in the substrate after the treatment can be calculated. Subtracting these methane yields of the remaining organic solvents from the obtained total methane yields will result in the methane yield only from the forest residues (Table 2). Hence, the methane production determined after applying the different organosolv-pretreatments on the forest residues were between 0.18 and 0.21 CH_4/kg VS, corresponding to up to 4 times higher methane yields compared to that of the untreated assay, which was 0.05 m^3 CH_4/kg VS (Table 2).

First order kinetics was used to determine the initial methane production rates (after the first 8 days of incubation) of the forest residues after applying the different organosolv pretreatments. Table 2 and Fig. 1 show that the initial digestion rate of the acetic acid pretreated forest residues is higher, with a specific constant of $K_0 = 0.066\ day^{-1}$, than those determined for the other applied organosolv-pretreatments. This is because acetic acid is the main intermediate in the anaerobic digestion process, and usually about 70% of the methane produced is formed through the acetoclastic pathway. There was also no lag-phase observed in the methane production from the ethanol-pretreated forest residues ($K_0 = 0.0424\ day^{-1}$), which is an indication that the microorganisms that are able to convert ethanol into methane were present in high quantities in the inoculum. In contrast, there was a long lag-phase in the methane production observed in the case of the methanol-pretreated assays ($K_0 = 0.0316\ day^{-1}$), since only a small group of the methanogens is able to use the methyl group as a precursor for the methane production, as stated in previous studies (Astals et al., 2013). However, the results indicate that toward the end of the process, the microorganisms in the digester showed the adaptability to use the methanol-forest residues mixture for the methane production (Fig. 1 and Table 2).

It can be stated that the pretreatment of lignocelluloses with low molecular weight alcohols or organic acids is beneficial, since they are intermediate products in the anaerobic digestion pathways and will ultimately result in higher methane yields in the digestion process. For that reason, pretreatments with the mentioned organic solvents do not require a high volume of processed water to filter and separate the solvent from the biomass prior to the anaerobic digestion, which is a great advantage compared to some other chemical pretreatment methods. It was previously shown that in the case of several chemical pretreatments, such as using alkaline, dilute acid, or *N*-methylmorpholine-*N*-oxide (NMMO), the chemical used for the pretreatment can be a source of inhibition for the methane producing microorganisms. The efficiency of those processes relies, therefore, on the consumption of a relatively high amount of water for washing the biomass in order to completely remove the chemical after the pretreatment (Chundawat et al., 2007; Kabir et al., 2013; Taherzadeh and Karimi, 2008).

A statistical analysis using 2-sample *t*-test was carried out to verify the significant differences between the methane yields obtained after the pretreatments using the different organic solvents, with or without the addition of a strong mineral acid as a catalyst. The results of the *t*-test indicate that there was no significant difference in obtained methane yields when using the different organosolvents for the pretreatments with the addition of different catalysts compared to those obtained when no catalysts were added (P-value > 0.05, data not shown) with one exception. A significant improvement was observed in the case of using methanol for the pretreatment with the addition of sulfuric acid (P-value < 0.05, data not shown). These results are in agreement with previous findings, which show that organosolv-pretreatments at elevated temperatures, *i.e.,* over 185 °C, do not necessarily require the addition of catalysts, since it is assumed that organic acids released from the biomass at these conditions can act as catalysts for the rupture of the lignocellulosic structure (Aziz and Sarkanen, 1989; Duff and Murray, 1996). However, the addition of a mineral acid will result in higher yields of xylose (Sun and Cheng, 2002), which is not required in the case of utilization of lignocellulosic biomass for biogas production. In general, the independent nature of the organosolv-pretreatment regarding the addition of strong acids is a great advantage for biogas production of lignocellulosic biomass, as the presence of a strong mineral acid can be corrosive and hazardous, thus, increasing the maintenance costs for the entire process.

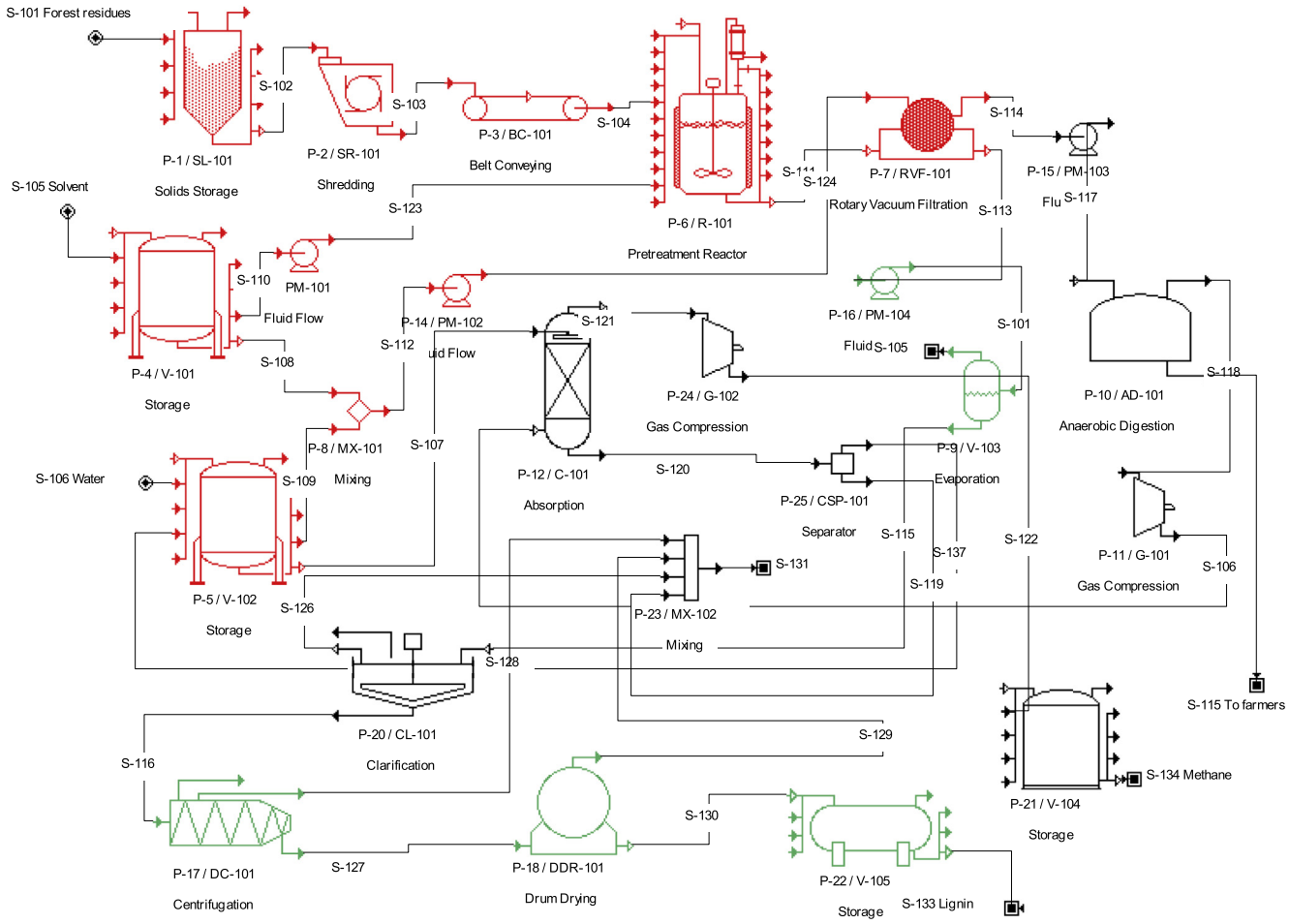

Fig. 2. Process flow-sheet of the biogas plant using the forest residues with ethanol/acetic acid/methanol pretreatments from Superpro designer.

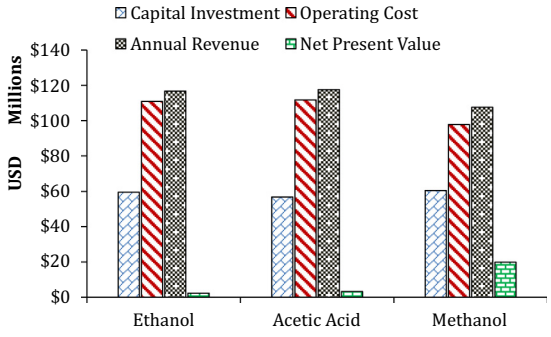

Fig. 3. Capital investment, operating cost, annual revenue, and net present value for the different pretreatment methods.

3.3. Techno-economic assessment

Fig. 2 shows the process flow-sheet of the biogas plant using forest residues as a feedstock and an organic solvent for the pretreatment step prior to the anaerobic digestion. The simulations were run separately for all the different pretreatments, *i.e.*, using ethanol, acetic acid, or methanol as a solvent for the pretreatment. The overall energy consumption of the plant was 6.95, 7.0, or 6.85 GWh/year, when applying ethanol, acetic acid, or methanol, respectively. In case of ethanol pretreatment, about 44% of the total energy was consumed for upgrading the biogas to obtain vehicle fuel, and that was 47% or 42%, respectively, when acetic acid or methanol was used. In comparison, the municipal biogas plant operating in Borås, Sweden, treating the organic fraction of the municipal solid waste has an annual processing capacity of 55,000 m^3 and consumes about 4.3 GWh energy per year, while about 52% of this is consumed in the upgrading step. However, the municipality plant in Borås operates under a different upgrading technology, which uses mono-ethanol amine. This technology has a higher energy demand compared to the water scrubbing system, applied in this study (Rajendran et al., 2014).

The net methane generation was the highest after the acetic acid pretreatment; counting up to 988 kg/h, while after the ethanol and methanol pretreatments, the methane generation was 954 kg/h and 890 kg/h, respectively.

Even though the overall amount of the methane generated was the highest after applying the acetic acid pretreatment, this process alternative was not economically attractive. Fig. 3 shows a comparison of the capital investments, operating costs, annual revenues, and NPVs for the three different pretreatment methods investigated. The capital investments for the processes applying the acetic acid, ethanol, or methanol pretreatments were 56.7, 59.4, or 60.5 million USD, respectively. In contrast, the NPV after 15 years of operation was 3.2 million USD for the process using the acetic acid for the pretreatment. Similarly, the NPV for the processes using the ethanol and methanol pretreatment methods were 2.3 and 19.9 million USD, respectively.

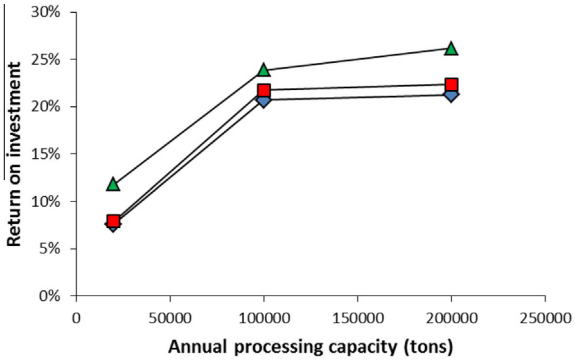

Fig. 4. Return on investment for the different operational capacities.

Since the NPV is the highest in the case of using methanol for the pretreatment, this makes this process alternative the most attractive one economically, compared to those alternatives which use ethanol or acetic acid for the pretreatment. The reason behind this is the low cost of the methanol, *i.e.*, 0.3 USD/kg. This affects the ratio between the annual revenue generated and the operating cost, which determines the net profit of the plant. This ratio was the highest (*i.e.*, 1.10) when using methanol for the pretreatment, and it was the lowest (*i.e.*, 1.05) when using ethanol. A similar study performed by Teghammar et al. (2014), evaluating biogas production from forest residues using the NMMO pretreatment, gave a revenue to operating cost ratio of 1.3 for a process with a capacity of 100,000 dry weight tons, which is 19.2% higher. Nevertheless, the assumptions between these two studies need to be considered, since the cost for the forest residues used in this study was higher compared to that in the study performed by Teghammar et al. (2014), to facilitate the transportation cost of the forest residues to the plant. In addition, the plant capacity in the study performed by Teghammar et al. (2014) was five times bigger, which can lead to additional profits. Moreover, the lifetime of the plant considered in the previous study was five years longer compared to that used in this study.

All the ROIs were calculated after taxes, yielding the final profit. The PBP and ROI were high, when using methanol for the pretreatment, which were 5.34 years and 11.8%, respectively. Considering the interest rate assumed (7%), using the methanol pretreatment leaves a 4.8% profit on investment. The PBP for the ethanol and acetic acid pretreatments were 7.05 and 6.9 years, respectively, which is almost half the life time of the plant.

3.4. Sensitivity analysis

The sensitivity analysis was carried out by altering the operational capacity of the plant from the base case, *i.e.*, 20,000 tons/year. The capacities considered were 10,000, 100,000, and 200,000 tons/year. Fig. 4 shows the ROI for the different annual operational capacities. The results suggest that the bigger the plant, the higher the profit when utilizing the forest residues to produce biogas for vehicle fuel; however, the cost of transportation also increased exponentially. The capital investments for a plant with 200,000 tons/year capacity varied between 472 and 514 million USD depending on which organosolv was used for the pretreatment (data not shown). The NPV, when using the methanol was high compared to that when the other two organosolvents were used for the pretreatments, achieving more than 25.5% or 20.3% higher NPV, respectively, compared to when ethanol or acetic acid was used. Increasing the plant size had a positive effect on the economics; however, reducing the plant capacity to 10,000 tons had a negative impact on the economical parameters. In this case, all the processes, independent of which kind of pretreatment methods was used, had a negative NPV, suggesting that a minimum of 20,000 tons/year capacity is required for the plant to be profitable.

4. Conclusion

Ethanol, methanol, and acetic acid were used for the pretreatment of forest residues prior to biogas production. The results of the laboratory experiments showed that higher methane yields and faster initial production rates could be achieved when acetic acid or ethanol pretreatments were applied, compared to those obtained after the pretreatment with methanol. However, interestingly, the economic analysis of the designed process alternatives for a large-scale process showed contradictory results, since the cost of methanol and its recovery is cheaper compared to the other two investigated solvents, which ultimately led to a more profitable process.

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