Innovative pretreatments of lignocellulosic biomass for biogas production

Bachelor Project

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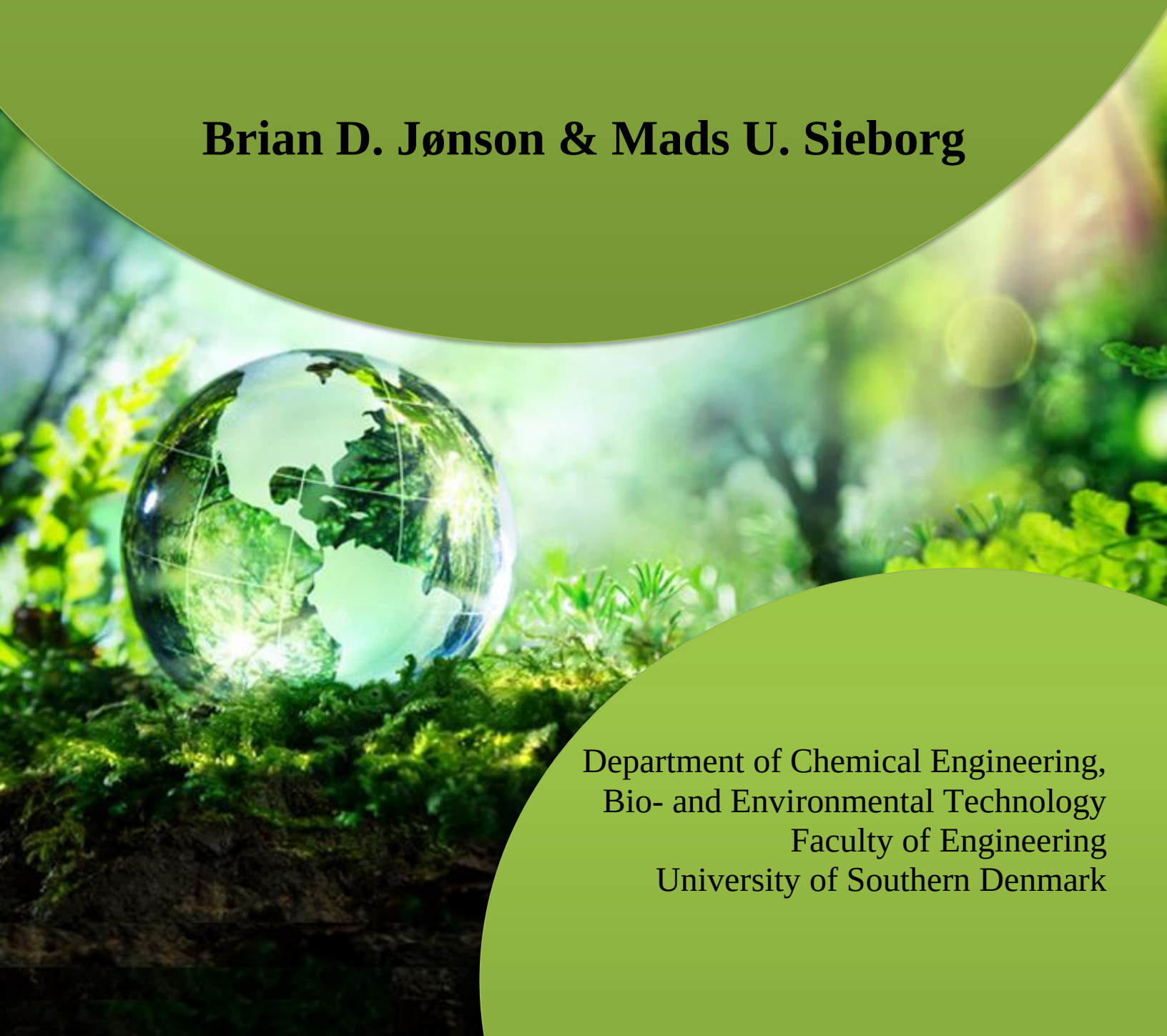

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Abstract

The present energy sources are mainly based on fossil fuels, which is not a long-lasting solution. Research in alternative energy source, as the biogas production with anaerobic digestion, are therefore in focus. The biogas production aims to utilize second generation biomasses as lignocellulosic straw, so the energy source becomes sustainable.

The objective of this thesis is to investigate innovative pretreatment methods to increase the biogas yield of lignocellulosic biomass. The thesis is composed of two distinct researches fulfilling the overall objectives. First, we investigated the co-ensiling effect of straw (S) and sugar beet (SB) at different ratios and ensiling periods. For the second activity, a combination of mechanical, chemical and hydrothermal pretreatment was attempted, while tannery waste water was alternatively applied to replace costly commercial chemicals.

We examined the influence of the physiochemical characteristics of the pretreated biomasses on the mono- and co-ensilage biomasses on the biodegradability (BD). The anaerobic digestion (AD) of the pretreated biomasses was investigated with biochemical methane potential (BMP) from batch experiments to determine the effect that the different pretreatments had on lignocellulosic biomass. The pretreatment effects were evaluated with synergistic effect and first order kinetics. Further was the effect of the pretreatment investigated with scanning electron microscope (SEM) and Fourier transform infrared spectres to evaluate the physical and chemical changes that the pretreatment made.

The physiochemical characteristics showed that co-ensilage resulted in lower mass losses than mono-ensilage. Mono-ensilage leaded to a loss of VS of 18% from 2 to 6 months and 30% from 6 to 8 months of ensilage. When sugar beet roots are ensiled alone, the fraction of VFA increased remarkably compared to co-silage samples. This result indicated that mono-ensiling of sugar beet roots causes active acetogenesis, which spoils optimal ensiling and causes high VS losses.

The results also showed that the concentration of lignocellulose had great impact of the rate of hydrolysis. The hydrolysis constant for mono-ensiled straw (2 month) was 0.035 day^{-1} and had a biodegradability of 28.3%, while the hydrolysis constant for mono-ensiled sugar beet (2 month) was 0.235 day^{-1} and had a biodegradability of 84.0%. Furthermore, did the results also show that co-ensilage would increase the BMP of the biomasses if the ensilage period was increased. This were observed from the synergistic results, which showed that the co-ensilage of 80:20 (SB:S) biomass for 8 months lead to a synergistic effect of 28%.

The further sequential pretreatment showed that only mechanical pretreatment had influence on the hydrolysis constant, while hydrothermal and chemical pretreatment had an increased effect of 29.7% in BMP compared to mechanical pretreatment of straw. The further sequential pretreatments showed no clear effect on co-ensiled samples, which were underlined by SEM analysis, where changes in surface structure could be observed for pretreated straw, but not for co-ensiled biomasses.

This thesis highlights that co-ensiling has a great potential to increase biogas production of lignocellulosic biomass such as cereal straw, and it confirms a potential of industrial wastewater as alternative pretreatment media replacing costly chemicals.

Resumé

De nuværende energi ressourcer er primært fossile brændstoffer, hvilket ikke er en bæredygtig løsning for fremtiden. Forskning fokuserer i alternativ energi, som f.eks. anaerob digestion. Formålet ved biogas er at kunne udnytte 2. generations biomasser som f.eks. strå, hvilket giver bæredygtig energi.

Formålet med dette projekt er at undersøge innovative forbehandlingsmetoder for at øge biogassen fra lignocellulosisk biomasse. Projektet er del op i to dele. I første del undersøges effekten af co-ensilering på forskellige blandinger af strå (S) og sukkerroe (SB), som har være ensileret i forskellige tidsperioder. Den anden del af projektet undersøges forskellige kombinationer af mekanisk, hydrotermisk og kemisk forbehandling af strå, her bruges garveri spildevand som et alternativ til dyre kemikalier.

Vi undersøgte virkningen af fysisk-kemiske egenskaber, af de forbehandlede biomasser på mono- og co-ensilering, på bionedbrydeligheden (BD) af dem. Anaerob digestion (AD) blev udført på de forbehandlede prøver, for at se effekten af biokemisk metan potential (BMP), efter forbehandlingerne, på lignocellulosisk biomasser. Effekten af forbehandlingerne blev vurderet ud fra synergieffekt og første-ordens kinetik. For at se de fysiske og kemiske ændringer i biomassen, blev der udført scanning elektron mikroskop (SEM) og fourier transform infrared spectres, hvor spektrene viste effekten.

De fysisk-kemiske egenskaber viste at co-ensilering resulterende i mindre masse tab end mono-ensilering. Ved mono-ensilering blev der mistet 18% fra 2 til 6 mdr. og 30% fra 6 til 8 mdr. Resultaterne viste at koncentrationen af lignocellulose havde en stor indflydelse på hastigheden af hydrolysen. Hydrolyse konstanten for mono-ensilering strå på 2 mdr. var 0.035 dag^{-1} og var bionedbrudt 28.3%. Modsat havde mono-ensilering af SB på 2 mdr. en hydrolyse konstant på 0.235 dag^{-1} og var bionedbrudt 84.0%. Resultaterne viste også at co-ensilering ville stige i BMP, sammen med tiden ensileringen var forgået i. Dette blev observeret ved synergieffekt, hvilket var på højest for co-ensilering af 80:20 (S:SB) som var 28%.

Anden del af projektet viste at det kun var mekanisk forbehandling der havde en indflydelse på hastigheden af hydrolysen, hvor at hydrotermisk og kemisk forbehandling øgede BMP med 29.7%, i forhold til den mekaniske forbehandling af strå.

Effekten af yderligere forbehandling på co-ensilering var utydelig, hvilket blev underbygget af SEM som kun viste ændringer i overflade strukturen for forbehandlet strå, men ikke for co-ensileret prøver.

Dette projekt sætter fokus på co-ensilerings store mulighed for at øge biogas produktionen af lignocellulosiske biomasser som strå. Det bekræfter også at industrielt spildevand som alternativ forbehandling til kemikalier, er muligt.

Preface

This bachelor thesis is conducted at Department of Chemical Engineering, Biotechnology and Environmental Technology (KBM), University of Southern Denmark (SDU) and corresponds to 15 ETCS points. The thesis was created from February 2017 to June 2017 and the experimental work was conducted in the same period, at the biogas laboratory outside of SDU and in the KBM laboratories.

A huge acknowledgement and thank should be given to our supervisor, professor Jin Mi Triolo, for her guidance and sharing of knowledge throughout the creation of the thesis. Her great interest in the thesis and guidelines have been a great help.

We are grateful for Søren Ugilt Larsen, at Aarhus University, for provided the co-ensiled samples.

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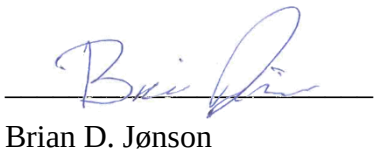
Lastly, thanks to Danny Kyrping, Department of Physics, Chemistry and Pharmacy (FKF), for helping and guidance in Scanning Electron Microscopy (SEM) experiment.

All data regarding physiochemical analysis and BMP in the bachelor thesis, is found in the zip-file which is uploaded with the thesis together with an ‘overview of all the results’.

The thesis was written by:

01-06-2017
Date



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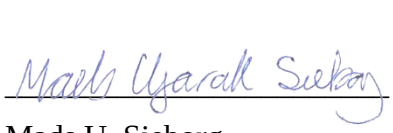

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Abbreviations

Name	Abbreviation / Symbol
First-generation	1G
Second-generation	2G
Third-generation	3G
Anaerobic digestion	AD
Biodegradability	BD
Biochemical Methane Potential	BMP
Cumulative CH_4 at the day of BMP test	CH_4 (X)
Chemical Oxygen Demand	COD
Continuous stirred-tank reactor	CSTR
Degree polymerization	DP
Estimated Biochemical Methane Potential	E-BMP
Fourier transform infrared spectroscopy-PAS	FTIR-PAS
Gas-chromatograph	GC
High-performance liquid chromatography	HPLC
Lactic acid bacteria	LAB
Not Available	NA
Root-Mean-Square Error	RMSE
Rotations per minute	RPM
Scanning Electron Microscope	SEM
Straw	S
Sugar Beet	SB
Synergistic effect	SE
Theoretical Biochemical Methane Potential	TBMP
Total Solids	TS
Total Solids corrected for easily degradable total solids	TS_{cor}
Volatile Fatty Acids	VFA
Volatile Solids	VS
Volatile Solids - Easily Degradable	VS_{ED}
Volatile Solids corrected for easily degradable volatile solids	VS_{cor}
Waste Water	WW
Wet weight	ww.
Weight	wt

Part I – Co-ensiling

Sample Name	Ratio [SB:S]*	Ensilage Time [Months]
S100:0-2M	100:0	2
S100:0-6M	100:0	6
S100:0-8M	100:0	8
S94:6-2M	94:6	2
S94:6-6M	94:6	6
S94:6-8M	94:6	8
S88:12-2M	88:12	2
S88:12-6M	88:12	6
S88:12-8M	88:12	8
S80:20-2M	80:20	2
S80:20-6M	80:20	6
S80:20-8M	80:20	8
S0:100-2M	0:100	2

*: Ratio of Sugar Beet (SB) and Straw (S) [SB:S] is based on wet weight.

Part II – Further pretreatment

Sample Name	Sample information	Pretreatment			
		Mechanical	Hydrothermal	Chemical & Thermal*	Combined**
S0:100-M	Milled Straw	X			
S0:100-T	Milled Straw	X	X		
S0:100-CT	Milled Straw	X		X	
S0:100-CCT	Milled Straw	X			X
S80:20-T	S80:20-8M		X		
S80:20-CT	S80:20-8M			X	
S80:20-CCT	S80:20-8M				X

*: The chemical part was conducted for 24 hours before the hydrothermal treatment was conducted.
**: The combined pretreatment is chemical and hydrothermal treatment at the same time.

Table of Content

1. Introduction

1.1. Project background

The advanced technologies that are available have increased the living standards and lifetime of the average person. This results in growth in the population of earth, which increases the demands for energy and food. The present energy sources based on fossil fuels are not a long-lasting solution, because the fossil fuels emit substantial amounts of greenhouse gases, which causes global warming. Furthermore, the fossil fuels are a limited energy resource, which only are estimated to last for the next 50-100 years if no alternatives are applied (McLamb, 2011). Therefore, lots of attention are drawn upon developing of new energy alternatives, as biogas, to the pollutive and limited fossil fuels.

1.1.1. The EU energy strategy

The European Commission (EC), and especially Denmark, are drawing a lot of attention towards finding a solution to these increasing demands, without the use of fossil fuels, and have therefore formulated different strategies, policies, programs and initiatives to change the EU countries to become more climate and environmental friendly. These strategies aim to reduce the greenhouse gasses and use more renewable energy sources. Of these strategies are especially the 2020, 2030 and 2050 strategies relevant.

Overall goals

The long-termed goal for the energy strategies of EU are the 2050 energy strategy, where the object of the strategies is to reduce greenhouse gas emissions by 80-95% compared to the levels from 1990 (European Union: European Commission, 2011a). But to achieve this goal, EU has set milestones at 2020 and 2030.

With the 2020 energy strategy has EU set an aim for its countries to:

- Reduce the greenhouse gas emissions by at least 20%.
- Achieve energy savings up to 20% or more compared to the levels in 1990.
- Increase the use of renewable energy to 20% (European Union: European Commission, 2010).

With the 2030 energy strategy are the target of EU to make its countries to:

- Reduce the greenhouse gas emissions by 40%.
- Increase the share of renewable energy consumption to at least 27%.
- Make energy savings corresponding to 27% (European Union: European Commission, 2011b).

Energy Scenarios

To accomplish these goals, many new initiatives have been made within the energy production section, including in the biogas production section. EU has created four different scenarios for the deployment of biogas production, which should be developed before 2030 (Kampman, Leguijt and Scholten, 2016). Common for all the scenarios are that they aim to replace fossil fuels with biogas/biomethane. The difference between the scenarios are the end-use of the biogas, which concerns the question about if the biogas should be used locally to make electricity for the grid and heat for local use, or upgraded to biomethane and transferred to the natural gas grid. Another difference between the constructed scenarios is the question of the rate of investments, the rate of biogas production and if it should be accelerated (Kampman, Leguijt and Scholten, 2016). There is no definitive conclusion to what scenario that should be deployed, but they give an overview of what direction that the biogas production should be advanced.

Biomass potential in EU

The feedstocks for the energy scenarios have also been evaluated in the report from EU. Fig. 1 gives an overview of the biomass potentials (Kampman, Leguijt and Scholten, 2016), which are based on a biomass report from EU (Elbersen *et al.*, 2014). These are based upon the current technologies that are available, and as illustrated in Fig. 1 agricultural residues are expected to be low compared to the other types of biomass. It is therefore very desired to find a cheap pretreatment, which can increase the biogas yield from agricultural residues as straw.

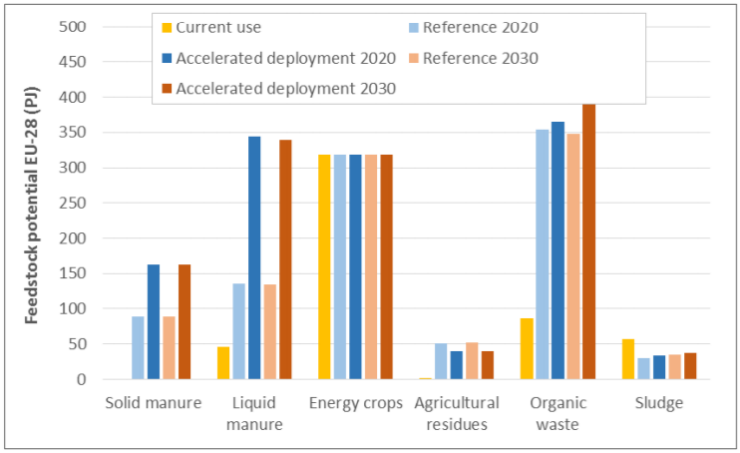

Fig. 1 - Overview of biomass potentials (Kampman et al., 2016)

The object for researching and developing biogas production for the countries are different. Many countries have increased their interest in the green transition, because of very few fossil fuels, very high pollution in their area, increased growth, utilization of residues or a vision of renewable and sustainable energy. EU is therefore focusing on this green transition, because they have realized that the climate changes are global, and not local. It is therefore important to advance possible sustainable and renewable energy sources.

1.1.2. The Danish energy strategy

For Denmark to comply with the EU aims and strategies, the Danish government has also put forward an energy strategy, where the object is to achieve independence from fossil fuels by 2050. The green transition will take a long time and preparation, and therefore different milestones and strategies are formulated for 2020, 2030, 2035 and 2050 to ensure the transition progress to the use of green and renewable energy sources (Breum, 2015).

- At 2020 should half of the traditional consumptions of electricity be covered by wind power.
- At 2030 should power plants, coal and oil-fired boilers be phased out from Danish power plants.
- At 2035 should the electricity and heat supply be covered by renewable energy.
- At 2050 should all energy supply (electricity, heat, industry and transport be covered by renewable energy).

During the green transition, it has been clear that Denmark have no single alternative green energy source, which is large enough to be able to provide the energy demands that Denmark have.

Specific potential scenarios

The Danish Government has therefore formulated five different energy scenarios with different combinations of alternative energy sources, where the former mentioned milestones are kept in schedule. In many of the scenarios, bioenergy is a competitor to the wind power. The problem with bioenergy is that the biomass for it, is a limited resource for a small country as Denmark. By comparing wind power and energy from biomass, wind power will have a high security of supply, because wind is an unlimited energy source. The wind power will at the same time have a low security of electricity, since it is not possible to regulate the amount of the wind and when it blows. Bioenergy have the opposite properties than wind energy, since it will have a low security of supply, because the available bio resources can be limited in a small country as Denmark. The security of electricity will therefore be high when using

bioenergy, because the energy can be stored in different energy carriers as biofuel. For example, the biogas can be stored and used when the energy demand cannot be satisfied by the alternative energy sources like wind power.

1.1.3. The Danish strategy for biogas production

The Danish government primarily focusses on wind power with support of other energy sources as bio-energy. The Danish Energy Agency has estimated that Denmark have a biomass potential of around 40 PJ of residue biomass available. However, a biogas taskforce from the Danish Energy Agency expected in 2014 an expansion of the biogas production, which corresponds to a double in the production from 4.3 PJ to 10 PJ before 2020 (Energistyrelsen, 2014).

With the "Energiaftalen" from the Danish Energy Agency, 2012, there are supposed to be some better framework conditions for the biogas production. To increase the expansion of the biogas production, the indenture is formulating that the existing support of using biogas for power planted heat should be increased. Furthermore, should different applications of biogas in the nature gas grid become more economical feasible (Energistyrelsen, 2012). Another advantage by using the production of biogas is that the organic residues, digestate, from the biogas plants can be used as fertilizer after it has been used for biogas production. This means that the utilization of the organic residues is even higher. The produced biogas can be upgraded to biomethane by combining them with e.g. electrolysis.

There are different barriers in the expansion of the biogas production and utilization in Denmark. These barriers are regarding the biomass, the gas deposition, the operational costs and the environmental regulations. If Denmark wants to replace the fossil fuels with CO_2 neutral biofuels, Denmark must import biomass to satisfy the current energy demand. And especially if the transport sector should be free of fossil fuels in 2050, then substantial amounts of the available biomass will be used in this sector. The energy should therefore come from various sources.

1.2 Anaerobic Digestion

Anaerobic digestion (AD) is a series of biological processes, where microorganisms break down biodegradable organic material in the absence of oxygen to produce biogas. This process can be divided into four (4) processes. These processes are called; hydrolysis, acidogenic phase, acetogenic phase and methanogenesis and are shown in Fig. 2.

For the anaerobic digestion to occur, it needs biomass and inoculum to produce the biogas. Inoculum is the effluent from an active digestion that contains all the necessary microorganisms for the production of biogas in an anaerobic digestion. The advantage from using inoculum from an active digestion, is that the microorganisms are more stable, and can start a digestion with new substrate fast.

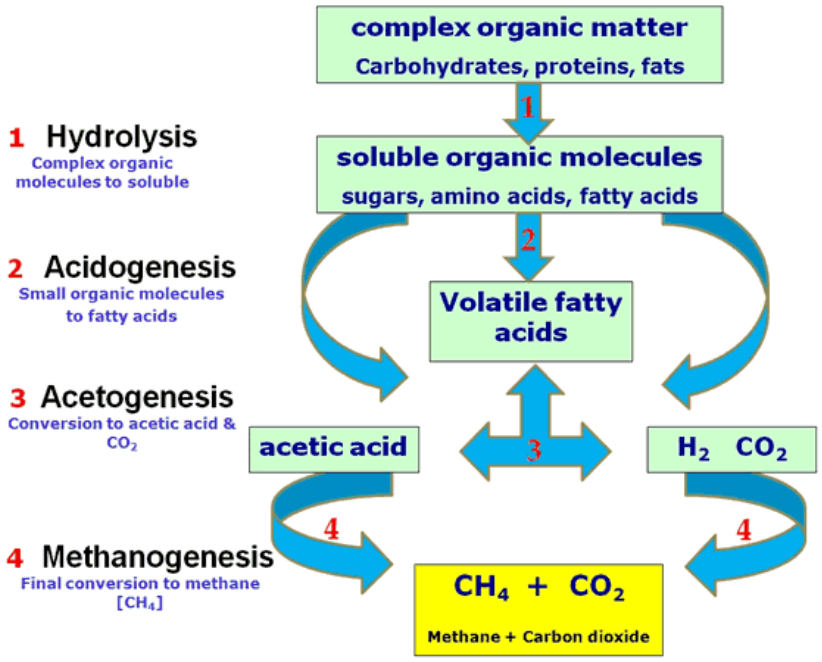

Fig. 2 - Processes of anaerobic digestion (Amaya, Barragán and Tapia, 2013)

1.2.1. Definition of biomass

Biomass is a term for all material of an organic origin. Biomass therefore covers a large variety of sources like animal manure, vegetable oil, sewage sludge, straw, etc. The large variety of biomass sources entails a wide variety of physical and chemical properties.

Biomasses is divided into three groups, where two of them are the major groups. These groups are referred to as generations. The first generation (1G) is food-based biomass, such as sugar beet. The issues with using 1G biomasses, is that the increasing population around the world lead to bigger demand for

food (Global Agriculture towards 2050, 2009). This issue is not present when using second generation (2G) biomasses. These biomasses are non-food biomasses as corn stover, straw etc. The main part of 2G biomasses are lignocellulosic biomasses. Advantages for using 2G biomasses are that these are not edible and since it is waste product, the cost is low. The problem with lignocellulosic biomasses, are discussed later. The newest and least used is third generation (3G) biomasses, which can be crops as algae. Algae is good to produce biofuels, since they use a wide range of carbon sources. The downside is the cost expenses of producing algae. The amount of fertilizer used for growing algae, will overcome the energy saved by using algae as biofuel (Lee and Lavoie, 2013).

1.2.2. Biodegradability

Biodegradability is a measure for the substrates ability to be disintegrated by microorganisms. The composition of the substrates is important for the speed of disintegration. More accessible carbohydrates as cellulose or hemicellulose will result in a faster disintegration. Structures like lignin or lignocellulose would lower the degradability. This is one of the biggest factors that can affect the production of biogas. Lignin is a polymer which is made of aromatic alcohols, and is insoluble in water and alcohol. Lignin reinforces the structure of cellulose and hemi-cellulose (described further in section 1.3.3.) therefore lignocellulosic biomasses are hard to disintegrate. The problem with lignin is that it is insoluble and thereby strengthens the structure, making it harder to hydrolyse the biomass. Other factors that have an influence on the biodegradability is the crystallinity, accessible surface area, degree of polymerization etc. (See section 1.3.4).

Easier degradable biomass can be sugar beet. This biomass does not contain lignin, so the molecules will be more accessible for the microorganisms, which will ensure a faster disintegration. Another factor is the structure of sugar beet. This biomass does not have a fixed cell structure as lignocellulosic biomasses, this can be seen in section 1.3.4.

1.2.3. Processes for anaerobic digestion

First process – Hydrolytic phase

The first step of AD is hydrolysis. Via hydrolysis, microorganisms (facultative and obligatory anaerobic bacteria) transform the macromolecule compounds into monomers using water and catalysed by exoenzyme produced by bacteria. Hydrolysis is a chemical reaction splitting macromolecular using water (See Fig. 2). The enzymatic hydrolysis is split up into three phases. During the first phase the enzymes are absorbed from the liquid phase and applied to the available surface of the cellulose. Afterwards the cellulose biodegrades into monomers and oligomers. The last phase is the desorption of the enzymes from the surface area of the cellulose to the liquid phase (Taherzadeh and Karimi, 2008). For example, protein, carbohydrates and fats are split into long chain organic weak acids e.g., amino acids, sugars and fatty acids. An example of this, is the formation of glucose from cellulose:

$$(C_6H_{10}O_5)_n + nH_2O \rightarrow nC_6H_{12}O_6 \quad (1)$$

Second phase – Acidogenic phase

In this phase, the acidogenic microorganisms and enzymes, converts the monomers to short chained volatile acids, also known as volatile fatty acids (VFA), along with alcohol, CO_2 and H_2.

VFAs are short chained fatty acids, with C2 to C5. These VFAs are; propionic acid (CH_3CH_2COOH); butyric acid ($CH_3CH_2CH_2COOH$); acetic acid (CH_3COOH) and formic acid ($HCOOH$).

Three typical acidogenic reaction from glucose are presented in Eq. 2-4, in which formations of ethanol, propionate and acetic acid occur (Deublin and Steinhauser, 2008).

$$C_6H_{12}O_6 \leftrightarrow 2CH_3CH_2OH + 2CO_2 \quad (2)$$

$$C_6H_{12}O_6 + 2H_2 \leftrightarrow 2CH_3CH_2COOH + 2H_2O \quad (3)$$

$$C_6H_{12}O_6 \rightarrow 3CH_3COOH \quad (4)$$

Third phase – Acetogenic phase

The products from the acidogenic phase are turned into acetic acid, H_2 and CO_2 by hydrogen producing acetogenic bacteria. Homoacetogenic microorganisms reduce H_2 and CO_2 into acetic acid (Deublin and Steinhauser, 2008).

$$2CO_2 + 4H_2 \leftrightarrow CH_3COOH + 2H_2O \quad (5)$$

Symbiosis between acetogenic- and methanogenic microorganisms

The production of H_2 interferes with the efficiency of the acetogenic microorganisms. These microorganisms only produce the acetate when the partial pressure of H_2 is low, and since they produce H_2 – the partial pressure rises. Due to this, there must be a symbiosis between the acetogenic microorganisms and the methanogenic microorganisms.

Methanogenic microorganisms are efficient when the partial pressure of H_2 is high, therefore the symbiosis is important. The methanogenic microorganisms remove the H_2 from the acetogenic microorganisms, by doing this the acetogenic microorganisms are efficient with the low H_2 partial pressure and the methanogenic microorganisms with the high H_2 partial pressure.

If the partial pressure of H_2 is high for the acetogenic microorganisms, they will not produce the acetate, but produce some acids, i.e. butyric acid and propionic acid. The acetogenic phase is the limiting phase for degradability for the last step, methanogenic phase. This is due to the anaerobic conversion in this phase is on expense of the methanogenic phase – where the substrates (H_2, CO_2 and acetic acid) goes to the methanogenic phase.

Fourth phase – Methanogenic phase

The last phase is the methanogenic phase. This is the phase, where the methane production occurs. The phase requires anaerobic conditions, since the microorganisms, which degrade the substrates, are strictly anaerobic (Botheju and Bakke, 2011). There are different methanogenic microorganisms depending on the different substrates; CO_2-, methyl- and acetate substrates. But common for all of them is that it is essential that the methanogenic and acetogenic phases are in symbiosis. Under some circumstances the acetogenic microorganisms can be in symbiosis with other microorganisms than methanogenic. This means that the methanogenic microorganisms will get less H_2, and therefore make less methane.

During the methanogenic phase, the methane is produced by two reactions. The first is an oxidation of acetic acid:

$$CH_3COOH \leftrightarrow CH_4 + CO_2 \qquad (6)$$

From the reduction of acetate is ~70% of the methane produced by acetoclastic microorganisms (eq. 6). The last ~30% of methane production (eq. 7), comes from the reduction of CO_2 and H_2 by hydrogenotrophic microorganisms (Deublin and Steinhauser, 2008).

$$CO_2 + 4\,H_2 \leftrightarrow \ CH_4 + 2H_2O \qquad (7)$$

1.2.4. Parameters for anaerobic digestion

The biological processes in AD is dependent of a lot of parameters. The microorganisms need a certain environment to live and grow, and therefore many parameters are important to consider.

An advantage for the digestion could be a two-stage digestion, where acidogenic phase will be the first stage, and methanogenic phase in the second stage. This is because the acetogenesis and methanogenesis is more sensitive to the environment, than the hydrolysis and acidogenesis (Ward *et al.*, 2008). The processes in the second stage is also dependent on each other, as described above in the processes. If the digestion is done in a one-stage system, the parameters must be prioritised to the methanogenesis, for making sure the microorganisms can survive in the environment, since they are more sensitive in this process.

The following parameters are some that is used in this project, and have influence on the digestion.

Temperature

Temperature is one of the major parameters that needs to be controlled in order for the temperature dependent methanogenic microorganisms to produce methane. If the temperature is to low, or to high, the microorganisms will not be optimized to produce methane.

There are three optimal temperature ranges for the acidogenic bacteria; physcrophilic, mesophilic and thermophilic. Mesophilic range is 32-42°C, this is where the mesophilic microorganisms have the optimal digestion. Thermophilic range is 48-55°C, this is where the thermophilic microorganisms have the optimal digestion (Deublin and Steinhauser, 2008).

Most of the methanogenic microorganisms are mesophilic, because they are more stable. Thermophilic methanogens are very sensitive to rapid changes in temperature, even a minor change in temperature can have a significant impact at the methane production (Zinder, Anguish and Cardwell, 1984).

The temperature affect the amount of free ammonia (FA), which can inhibit the methanogenic process. FA is the main cause of inhibition since it will accumulate VFAs. When this happens, the pH falls, which decreases the concentration of FA. This will end up in an ‘inhibited steady state’, where the digestion operates with lower methane yield.

When the temperature rises, so does the concentration of FA produced. Therefore, the thermophilic digestion (55°C) is more easily inhibited than mesophilic digestion (37°C) (Chen, Jay and Kurt, 2008).

pH

Another factor, which can inhibit the microorganisms in the digestion is the pH. The optimal pH for AD is 6.8-7.2, which is narrow. The narrow pH optimum is because of the hydrolysis/acidogenic phase and the methanogenic phase. The methanogenic phase has an optimal pH at 7 and the hydrolysis/acidogenic at pH 5.5 (Ward *et al.*, 2008). Therefore, a two-stage digestion can be preferred.

If the pH drops below 6.6 the methanogens growth is reduced significantly, and if the pH become alkaline, this will lead to destruction of microbial granules and subsequent failure of the methanogenic process.

The pH is held in the range of 6.8-7.2, because of natural procedures and two natural buffer systems. The first ensures that a strong acidification will not take place. This is based on the CO_2, hydrogen and carbonate in the system. Based on the pH increasing or decreasing, the CO_2 will wither dissolve in the substrate or dissolve and form carbonic acid. The second system, is the ammonia-ammonium buffer system. This works the opposite way, to avoid a too weak acidification. If the pH is increasing, ammonium is formed and by that releases hydroxyl ions, but if the pH decreases more ammonia is formed instead.

Hydrogen partial pressure

The hydrogen partial pressure is an important parameter. Because of the needed symbiosis between acetogenesis and methanogenesis, where the partial pressure of H_2 is a limiting factor to have these two processes to work optimal. Too much hydrogen around the acetogenic microorganisms will lead to inhibition of the acetogenic microorganisms. Furthermore, a too low hydrogen partial pressure would lead to lower methane production by the methanogenic microorganisms (Cazier *et al.*, 2015).

Type of substrate

The type of substrate determines the rate of the degradation. The metabolism of the microorganisms can stop if the microorganisms do not have access to more of the organic components.

Sugar will be one of the fastest components to be degraded. On the other hand, lignin and cellulose will take longer time to degrade, and therefore the time for the digestion must take longer time.

Light

Light is a factor that can prevent the methanogenic microorganisms to produce methane. Light is not necessarily lethal for the microorganisms, but will inhibit the production of methane.

Nutrients

The most important about nutrients is the carbon to nitrogen ratio (C/N-ratio) of the substrate. The most optimal ratio is 20-40. If the ratio becomes too low, there will become an inhibition of methane formation, because of the increased ammonia production. Contrary if the ratio is too high, the microorganisms cannot work properly.

The ratio is only an indicator because it depends on the structure of the substrate. Substrates with lignin, can have a higher concentration of nitrogen, since nitrogen can be bound to lignin. This will make the C/N-ratio lower, but it does not indicate there will be inhibition.

1.2.5. Inhibitors

Inhibitors are compounds that are formed during the digestion, which can limit the process, or even be toxic if the concentration is high enough. The amount of inhibition is depending on the substrate and the bacteria used in the digester.

Oxygen

Oxygen is an inhibitor. For the two first phases, the bacteria are *facultatively* anaerobic. This means they do not use the oxygen, but they can still live and operate with oxygen present. The last two phases are *obligatorily* anaerobic, which means they cannot operate or live with oxygen present.

Based on this, the entire process usually is anaerobic, with no oxygen present. But if the process is two-staged there can be oxygen present in the first phase.

Sulphur compounds

Sulphur compounds are mostly found in industrial waste water. Sulphate can convert into H_2S, which can inhibit the methanogenesis. Since sulphate-degrading microorganisms need less energy and no symbiosis, these are favoured if sulphate is present.

H_2S is a gas, and will be removed together with the biogas. But, if the pH is decreasing the amount of dissolved H_2S in the substrate rises, which works as poison to the other microorganisms. At too high concentrations it will work as an inhibitor. The upper limit of sulphide concentration, to maintain a stable AD, is in the range of 100-800 mg/L for dissolved sulphide or 50-400 mg/L for undissolved H_2S (Parkin *et al.*, 1990).

During this acetogenic phase, hydrogenic sulphur is formed as a precipitate from organic nitrogen and other sulphur compounds. All this is possible because ammonia is produced in the process as well. There are different reactions for sulphate to be reduced, this can be done because of the sulphate-reducing bacteria (Deublin and Steinhauser, 2008). The reactions of this can be seen in eq. 8 and 9.

$$SO_4^{2-} + CH_3COOH \rightarrow HS^- + CO_2 + HCO_3^- + H_2O \quad (8)$$

$$SO_4^{2-} + 2CH_3CHOHCOOH \rightarrow HS^- + 2CH_3COOH + CO_2 + HCO_3^- + H_2O \quad (9)$$

1.3. Pretreatment

1.3.1. Objectives of using pretreatments of biomass for biogas production

Biogas is produced by anaerobic digestion of organic materials in the absence of oxygen. The biogas yield and the rate of anaerobic digestion depend on biodegradability of organic materials. Not all the organic materials are transformed to biogas at the same rate, so to enhance biogas yield, pretreatments may be needed to increase the rate and yield of the hydrolysis (e.g., physical-, chemical- or biological-pretreatment methods etc.) (Zheng *et al.*, 2014).

The pretreatment therefore have two objectives: To increase rate of anaerobic digestion and to increase the yield. In Fig. 3, there are three different scenarios illustrated. The black line illustrates the anaerobic digestion of substrate without pretreatment, while the red line illustrates a pretreatment of substrate, which increases the rate of anaerobic digestion and the blue line illustrates a pretreatment, which increases the biogas yield. By implementing these pretreatment processes, the digestibility of the biomass can be enhanced (Montgommery and Bochmann, 2014).

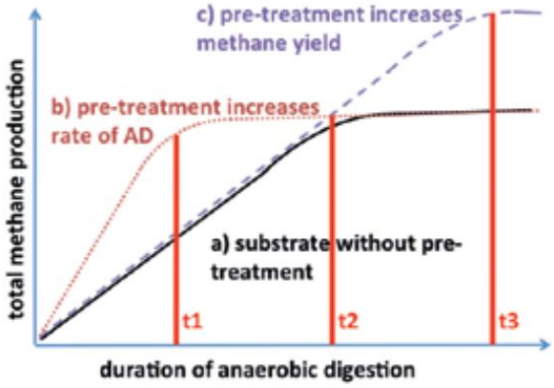

Fig. 3 - Scenarios for pretreatment (Montgommery and Bochmann, 2014).

1.3.2. The object of utilising lignocellulosic biomass for biogas production

The biomass, which is the raw material for a biogas production, must be renewable, which means that it should be naturally replenished at a fast rate and therefore includes most organic materials. Furthermore, the biomass should also be sustainable, which preclude most of the 1G biomasses, since most the 1G biomasses are crops for food. The 1G biomasses are therefore not suitable for substituting fossil fuels, since it will be at the expense of potential food. The interest in 2G biomasses are therefore increased, and especially for lignocellulosic biomasses. Lignocellulosic biomasses are for example forest residues,

straw, energy crop, food crop residues etc. Lignocellulosic biomasses also have an economically advantage over other agriculturally feedstocks, because it can be produced quickly and at a significantly lower cost than food crops. Furthermore, lignocellulosic biomasses are often residues, which can be utilised by converting them into biofuels as biogas (Zafar, 2015).

1.3.3. Structure of lignocellulosic biomass

The main components of lignocellulosic biomass are lignin, cellulose and hemicellulose. The cellulose is linear polysaccharides, which are attached to each other by hemicellulose. The cellulose and hemicellulose are protected by the lignin. This structure makes cellulose resistant to biological and chemical treatments (Taherzadeh and Karimi, 2008). The structure of cellulose, hemicellulose and lignin are described with the lignocellulose matrix, which can be seen in Fig. 4.

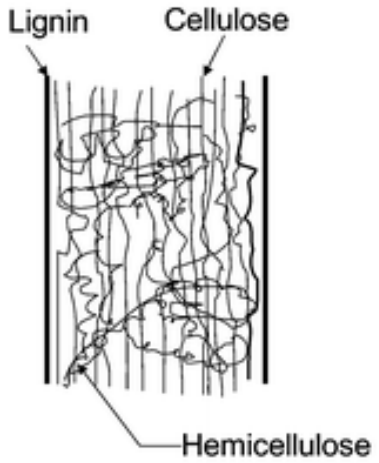

Fig. 4 - Lignocellulosic matrix (Tadesse and Luque, 2011).

The structure of lignin makes it non-degradable in anaerobic environments, so the higher amount of lignin in the biomass, the less biodegradable it is (Fernandes *et al.*, 2009). The lignin structure is very irregular and contains randomly cross-linked phenolic polymer. Hemicellulose also inhibits the hydrolysis of the cellulose, because hemicellulose has a complete amorphous structure, which is very weak compared to the crystalline structure of cellulose. The amorphous structure of hemicellulose is one of the reasons that the hydrolysis of hemicellulose is relatively easier compared to the hydrolysis of cellulose (Singh and Harvey, 2010). The principal of the pretreatment processes is therefore to reduce the mechanical and chemical lignocellulosic strength e.g. by breaking down physical and chemical linkages between

these lignocellulosic polymers and loosen the lignocellulosic matrix to facilitating the hydrolytic enzymes' access to cellulose.

The main factors that affect the rate of the biodegradation of lignocellulosic material by enzymes are:

- The crystallinity of cellulose.
- The accessible surface area.
- The protection of lignin and hemicellulose.
- The degree of cellulose polymerization.

The effect of the pretreatment can be seen in Fig. 5.

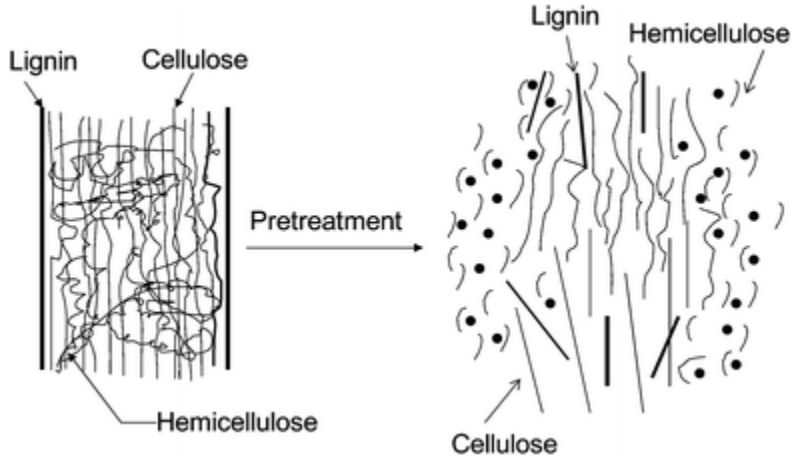

Fig. 5 - Effect of pretreatment on structure (Tadesse and Luque, 2011).

1.3.4. Rate limiting steps in biogas production

Crystallinity of cellulose

The crystallinity of cellulose is one of the reasons of low biodegradability of cellulosic biomass. Cellulose chains contain many hydroxyl groups, which can form inter- and intra-molecular hydrogen bonds, which support the crystalline structure of cellulose. These interactions in crystalline structure are hydrophobic, which makes cellulose insoluble in normal aqueous solutions (Chaplin, 2002). It is therefore more difficult to hydrolyse cellulose. Cellulose contains both amorphous regions and crystalline regions, whereas the amorphous regions are hydrolysed much faster than the crystalline regions (Yang *et al.*, 2011). One of the factors, which increases the hydrolysis of hemicellulose compared to cellulose, is that the amorphous region is soluble and easily accessed by the enzymes (Li, 2014), in opposite of the crystalline structure of cellulose, where the molecules are fixed in a crystalline array due to its hydrogen-

bond (Fig. 6). This limit the number of enzymes that can access the cellulose, and thereby make cellulose more resistant to hydrolysis (Hendriks and Zeeman, 2009). These regions are illustrated in Fig. 6. The major part of cellulose has a crystalline structure, which corresponds to around 2/3 of the cellulose (Chum *et al.*, 1985).

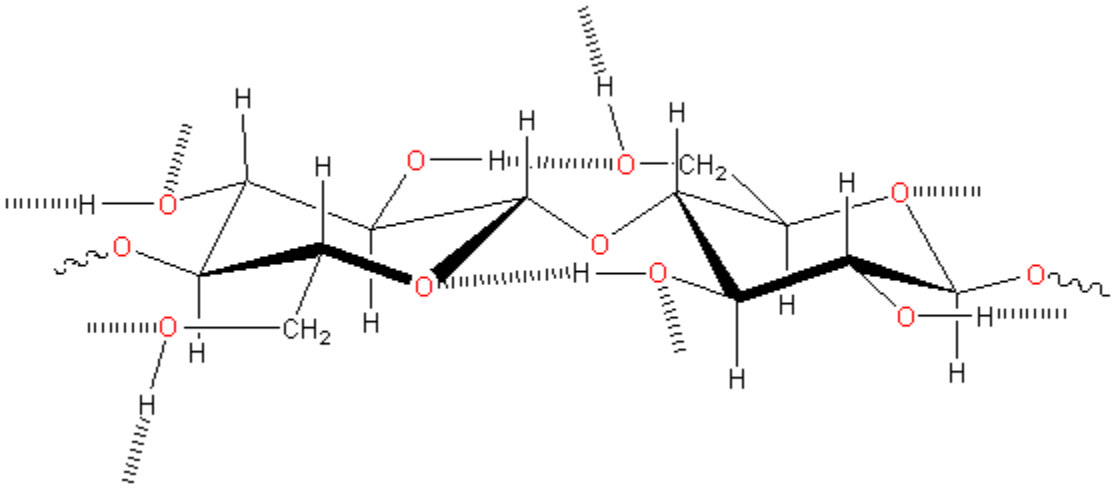

Fig. 6 - Regions for enzymes to access cellulose (Chaplin, 2002).

Protection by lignin and hemicellulose

The hemicellulose and lignin inhibits the hydrolysis of the cellulose, which means that a larger amount of hemicellulose and lignin will lower the biogas yield from the anaerobic digestion. Lignin is non-degradable and blocks hydrolysis of cellulose and hemicellulose. Lignin is therefore one of the limiting factors in the hydrolysis and anaerobic digestion. The lignin molecules are bonded to the hemicellulose and cellulose with covalent, ether and ester linkages, but these bonds can be broken if the lignocellulosic biomass is pretreated with diluted acid or alkaline (Lee, 2008).

Accessible surface area

Another factor which affects the biodegradability of the biomass is the accessible surface area. Several studies have reported a good correlation between the accessible surface area and the anaerobic digestion of lignocellulosic biomass, and that the accessible surface area is not an individual factor, since it correlates with the crystallinity (Taherzadeh and Karimi, 2008). The surface area can be divided into two types of surface area:

- The external surface area, which is related to the shape and size of the particles.
- The internal surface area, which is related to the capillary structure of cellulosic fibrils (Taherzadeh and Karimi, 2008).

The accessible surface area is related to enzymatic hydrolysis because this is where the liquid enzymes adsorb to catalyse the hydrolysis reaction. This means that the accessible surface area will be able to interact and adsorb more enzymes if it is larger, and thereby increase the rate of hydrolysis (Taherzadeh and Karimi, 2008).

The surface area of the substrate correlates to the type of the substrate. If the substrate is lignocellulosic, the area is smaller than the area of sugar beet due to the structure of lignocellulosic. Pictures below shows the difference in the structure for a lignocellulosic- and a non-lignocellulosic biomass.

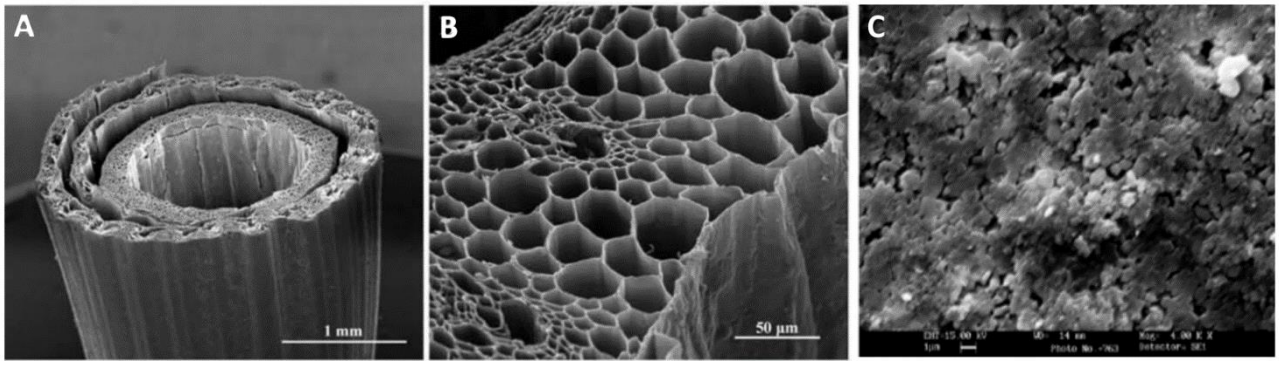

Fig. 7 - A & B - Untreated wheat straw (Kristensen et al., 2008). C - Untreated sugar beet sample (Afshar et al,. 2014).

As shown in Fig. 7, the lignocellulosic biomass has a structure, where the sugar beet looks more mashed. Thereby it can be seen, that the microorganisms have more access to the substrate when there is no structure. Therefore, the type and the surface area of the substrate is important.

Most of the available pretreatments have effect on the accessible surface area and increases this. By implementing a mechanical pretreatment will decrease the size of the particles and thereby increase the external surface area. By implementing a chemical alkaline pretreatment will induce swelling of the plant cell walls, which increases the internal and external surface area. Furthermore, will the structural changes during a hydrothermal pretreatment lead to an increase in the accessible surface area of the cellulose,

when the relocation of lignin and partial dissolution of hemicellulose occur (Nitsos, Matis and Triantafyllidis, 2012).

The degree polymerization of cellulose and hemicellulose

The degree of cellulose polymerization (DP) is describing the length of polymer and oligomer molecules. It is defined as the number of monomeric glucose molecules in a polymer or oligomer chain. The DP of cellulose contributes to mechanical- and chemical strength and stability. There is correlation between the particle sizes, which are bigger, when the DP is higher. It is preferred that the particles have a smaller particle size, so the accessible surface area is increased (Karimi and Taherzadeh, 2016). Wheat straw has a degree of polymerization of 2660 (Hallac and Ragauskas, 2011). Based on increasing the yield and rate of anaerobic digestion, it is desired to reduce the degree of polymerization. And studies show that treating the biomass with lime as a chemical pretreatment reduces this polymerization of cellulose (Hallac and Ragauskas, 2011).

1.3.5. Physical pretreatment

To increase biodegradability, the rate of biogas production, and the enzymatic hydrolysis, the biomass can undergo a physical pretreatment. A physical pretreatment can be conducted with different methods e.g. milling and irradiation. The type of physical pretreatment, which is being examined in this thesis is milling.

Mechanical pretreatment (Milling and grinding)

Milling is a method to reduce the size of the substrate to improve the biodegradability by rupturing the cell walls of the substrate and making the biodegradable components more accessible to enzymes and microorganisms. When the size is reduced, the surface area of the biomass is increased, which results in the large molecules being broken into smaller ones. The accessibility to the residue particles is therefore increased for the microorganisms and enzymes, so the physical pretreatment will increase the speed and efficiency of the hydrolysis, and thereby the production of biogas. The digestibility is also increased by physical pretreatments as milling because it disrupts the crystalline structure of cellulose (Yoshida *et al.*, 2014).

Studies reported that the hydrolysis yield (reduced sugars) of lignocellulosic rich wheat straw were increased from 6% to 34% if a sieve based grinding pretreatment were implemented to reduce the particle sizes (Silvia *et al.*, 2012). The increased hydrolysis yield depends on the size of which the particles are

reduced to. The reduction of the particle size increases the accessible surface area, which favours the enzymatic adsorption, and thereby increases the rate limiting step in the biogas production: The hydrolysis.

Another crucial factor, which the physical pretreatment process impacts, is the viscosity. It decreases the viscosity and thereby makes the biomass easier to mix inside a digester (Taherzadeh and Karimi, 2008). This can be an important parameter when operating a CSTR reactor or a biogas plant.

The physical pretreatment also generates disadvantages, because of the high-energy consumption, when using milling as pretreatment. This can make the pretreatment unsuitable compared to the large expenses to conduct the pretreatment. Another disadvantage of physical pretreatment is that the effect of physical pretreatment is limited without combining other pretreatments (e.g., thermal or chemical pretreatment), since it is unable to disrupt the lignocellulosic structure in the biomass, which inhibits the hydrolysis and lessens the access of the enzymes to the cellulose (Taherzadeh and Karimi, 2008).

A physical pretreatment is therefore often combined with other pretreatment methods to increase the efficiency and yield of the production of biogas.

1.3.6. Hydrothermal pretreatment

Hydrothermal pretreatment is another pretreatment method, which can be applied to increase the digestibility and biodegradability of the biomass. The hydrothermal pretreatment uses high temperatures, pressure and autoionization of water to generate hydrogen ions, and therefore reduces the pH to acidic levels. This is beneficial to hydrolytic reactions, which are favoured in the removal of hemicellulose. The hydrothermal pretreatment can be applied as a pretreatment for the biomass in favour of causing auto hydrolysis of the hemicellulose and transformation of the lignin.

The temperature and pressure are a widely-discussed subject. It varies from 120-160°C. Studies (Bougrier, Delgenès and Carrère, 2008) reported that aggressive hydrothermal pretreatments with high temperatures (Above 180°C) decreased the biodegradability and biogas production. A study tested BMP of organic waste after autoclaving it for 15-30 min at 2 bar at 134°C (Percorini *et al.*, 2016). Another study tested BMP of lignocellulosic biomass after autoclaving it four times at 95°C and 1 bar (Heerah *et al.*, 2008). Furthermore, did a study test segregated food waste at 160°C and 6.2 bar (Tampio, 2014).

Common for all these studies were that there was an increase in soluble chemical oxygen demand (COD) and an increase in methane production.

The hydrothermal pretreatment leads to partial solubilisation of the substrate. The first polymer to solubilize is the hemicellulose followed by lignin. This reduces the digester volume and enhances biogas production (Mudhoo, 2012). Treating the biomass with hydrothermal pretreatment will also reduce the viscosity, which is an important parameter if you operate a CSTR or a biogas plant. Studies reported that hemicellulose in the solid phase of sunflower oil cake would solubilize at temperatures above 150°C, and lignin would partially solubilize at temperatures above 150°C, while cellulose remained in the solid phase. The hemicellulose fraction would change from 13% of the fibres to 7% of the fibres by increasing the temperature of the hydrothermal pretreatment from ambient temperature to 150°C (Fernández-Cegrí *et al.*, 2012). Another study reports a similar trend, that hydrothermal pretreatment reduces the hemicellulose fraction in the solid phase (Kaparaju and Felby, 2010). Thereby is the protection of cellulose, by lignin and hemicellulose, reduced.

The object with the hydrothermal pretreatment is to increase the rate limiting step of hydrolysis in the anaerobic digestion of lignocellulosic biomass. A study reported that after a 72h of hydrolysis of untreated wheat straw would have a cellulose to glucose conversion of 20.1%. This study also reported that a 72h hydrolysis of hydrothermal pretreated wheat straw would have a cellulose to glucose conversion of 79.9% (Kaparaju and Felby, 2010). The rate of the hydrolysis does therefore seem to be increased when a hydrothermal pretreatment is implemented in anaerobic digestion of lignocellulosic biomass.

Temperature levels above 200°C are expected to promote an inhibitory effect on the digestion process, since the microbial cell components are hydrolysed (Mudhoo, 2012). These are the DNA and RNA which are used in the polymerization of sugars and amino acids (Mudhoo, 2012). When these are hydrolysed, the concentration of phosphorus and nitrogen increases. The hydrothermal pretreatment also affects sugars, since it destroys the xylan fraction and causes incomplete disruption of the lignin-carbohydrate matrix. Another disadvantage is that the thermal pre-treatment generates inhibitory compounds. Thermal pretreatment may cause an inhibitory or toxic environment for the bacteria when lignin and hemicellulose are solubilized, since phenolic compounds will be produced.

1.3.7. Chemical pretreatment

Object for chemical pretreatment

The objective of the chemical pretreatment is to alter the lignocellulosic structure to make easily digestible carbohydrates, and increase the enzymatic accessibility of the cellulose. This will increase the yield of biogas and increase the rate of anaerobic digestion. A study reports that chemical pretreatment with lime can increase the yield of anaerobic digestion with 25% (Heiszwolf, Dobbe and Mear, 2008). Chemical pretreatment is a method to disrupt the lignocellulosic structure by using chemicals instead of energy, which is used in hydrothermal and physical pretreatments. It is therefore of great interest to find a chemical pretreatment with a cheap and recoverable chemical, so the expenses of the pretreatment can be reduced. Chemical for pretreatment could be oxidizing agents, alkali, acids or salts.

Alkaline pretreatment

Alkalis and acids are commonly used for increasing the solubilisation of hemicellulose and lignin in biomass. This will furthermore increase availability for enzymatic attacks. Studies show that acids are more effective to solubilize the hemicellulose, while alkalis are found more effective in the removal of lignin (Rodriguez, 2017). Alkaline pretreatment alters the structure of lignin and increases the partial decrystalization of cellulose, the partial solvation of hemicellulose, the porosity and internal surface area of the biomass by swelling, and it decreases the polymerization of the feedstock.

Furthermore, alkaline chemical pretreatment also induces the saponification and cleaves the lignin-carbohydrate linkages. When comparing the acid and alkaline pretreatment processes, the alkaline pretreatment has less sugar degradation. When sugars are degraded, the degradation compounds will be present in the biomass. These degradation compounds can be furans, different carboxylic acids and phenol derivatives, which all have an inhibitory effect on the digestion. It is therefore very important to control the pH, temperature, residence time and pressure to lower this degradation as much as possible (Behera *et al.*, 2014).

Studies show that alkaline pretreatment compared to acid pretreatment provides the most effective method to break ester bonds between the lignocellulosic materials as lignin, cellulose and hemicellulose (Gáspár, Kálmán and Réczey, 2007). This leads to a lower fragmentation of hemicellulose polymers, which means that the formation of inhibitors can be avoided.

One of the commonly used alkaline compounds for chemical pretreatment is lime, $CaCO_3$, which is one of the cheapest chemicals in proportion to the amount of biomass that gets treated that are available (Brodeur *et al.*, 2011). Alkaline pretreatment with lime removes acetyl groups, and various uronic acids in the hemicellulose, which reduces the steric hindrance to the sites for the enzymes adsorption. This increases accessibility of hemicellulose and cellulose for the enzymes which cause hydrolysis.

Furthermore, alkaline pretreatment causes saponification, which leads to swelling. When the biomass is swollen, the internal surface area is increased, the lignin structure is disrupted, the crystallinity and degree of polymerization are decreased and the linkage between lignin and carbohydrates is broken. All these parameters increase digestibility of the biomass (Behera *et al.*, 2014). Studies (Kaar and Holtzapple, 2000) reported that pretreatment of corn stover with lime would increase the enzymatic hydrolysis by 9 times compared to untreated corn stover. This were done with a loading rate of 0.075 g $Ca(OH)_2$ (g dry biomass)$^{-1}$ and by heating for 4h at 120°C. Other studies reported that the retention time of chemical pretreatment vary with the temperature, so the retention time could be reduced by increasing the temperature, but the chemical pretreatment could also be done with similar effect at ambient temperatures at longer retention time (Playne, 1984).

The alkaline chemical pretreatment can also be used to prevent pH drops. During the acidogenesis volatile fatty acids, which lower the pH value, will be made. The residual alkalis will be able to prevent any possible pH drop during the acidogenesis phase, which otherwise would have been toxic or inhibitory for the bacteria.

Benefits and disadvantages of chemical pretreatment

Chemical pretreatment of biomass is very relevant, because of the low energy consumption related to the pretreatment method. The chemical pretreatment method is however considered economically unattractive, because of the expenses of alkalis and acids for the chemical pretreatment. Furthermore, are the corrosive and toxic environment from the acids a drawback for the chemical pretreatment, since it requires extra resistant materials of the reactors. If the expenses of chemicals could be reduced, then this pretreatment method would be very feasible (Brodeur *et al.*, 2011).

Therefore, it could be interesting in utilising chemicals in waste water to do this pretreatment. Another disadvantage with chemical pretreatment is the generation of inhibitors and toxic materials, which harms

the downstream process, where the biomass is processed. These inhibitors are phenolic compounds, furfurals, salts and aldehydes, but a lot of research are done in using lime as alkaline catalyst for chemical pretreatments, since it can be neutralised with CO_2, which is a cheap method for neutralisation. During this neutralisation, the chemicals can be converted into residue salts, which also should be separated from the biomass to avoid inhibitory effects. The overall effect on the biomass of the different pretreatments are illustrated in Fig. 8.

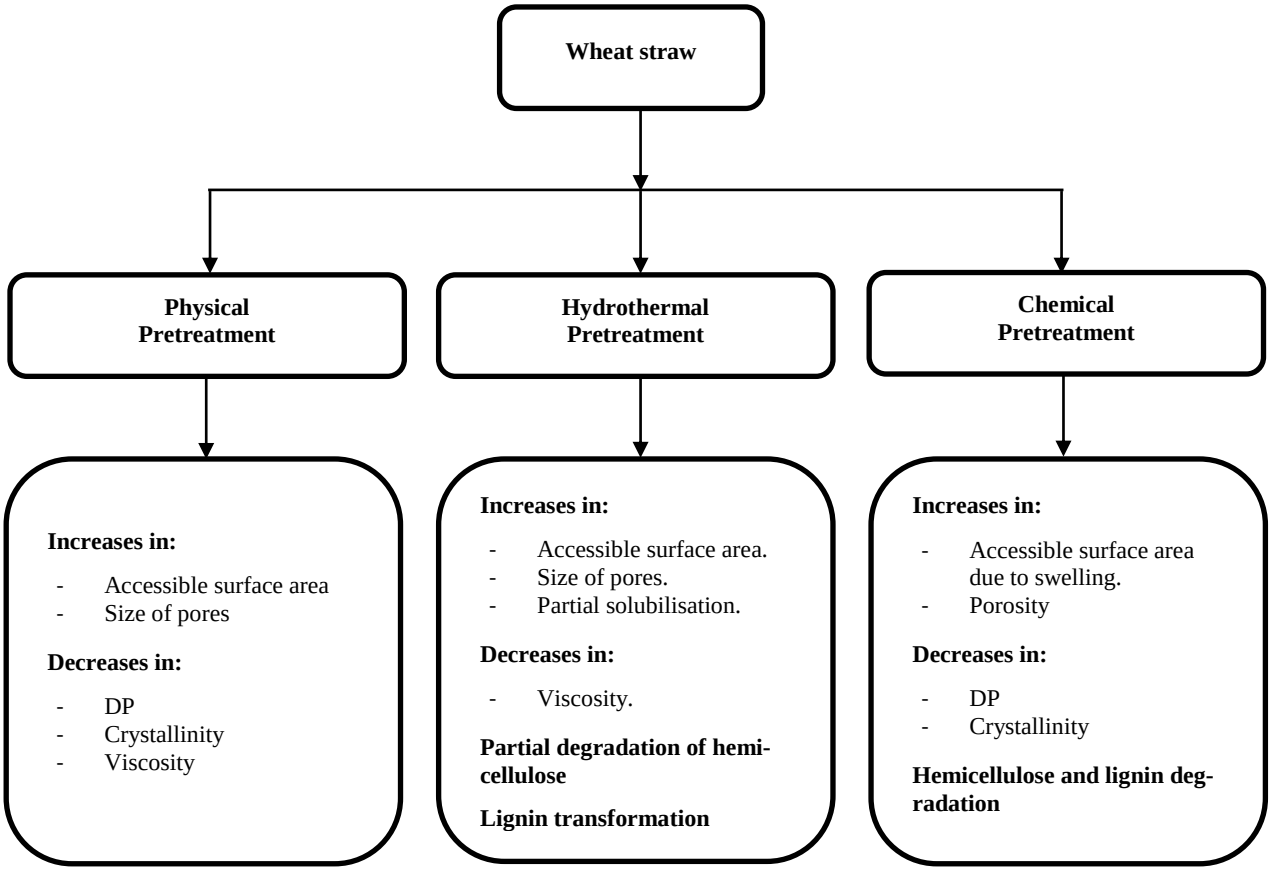

Fig. 8 - Pretreatment effects on biomass (Talebnia, Karakashev and Angelidika, 2010).

1.3.8 Ensilage storage and pretreatment

Ensiling is a storage method of plant biomasses, which can be used as a pretreatment method to improve hydrolysis of slow degradable lignocellulosic biomass such as cereal straw. The ensiling process can be divided into 4 phases (Elferink *et al*., 2000).

The 1st phase: The 1st phase is the aerobic phase. When the biomasses are loaded into a silo, then the silo is sealed to exclude the air, and generate an anaerobic environment. The available oxygen in the ensilage container are used from the plants respiration and aerobic microorganisms.

The 2nd phase: The 2nd phase is the fermentation phase, where the silage becomes anaerobic and the lactic acid bacteria (LAB) will become dominant in the silage. This will start a fermentation with different LAB as Lactobacillus, which produces lactic acid, volatile fatty acids and alcohols that accumulates in biomass and decreases the overall pH (Herrmann, Heiermann and Idler 2011). When the anaerobic environment is kept, the activity of aerobic microorganisms will be inhibited, which is what is preferred with the storage technique (Weinberg and Ashbell, 2003).

The 3rd phase: The 3rd phase is the stable phase, where the activity of the microorganisms starts to become inhibited and the microorganisms decreases in numbers because of the acidic environment that they have created. This phase will last until air is exposed to the silage sample.

The 4th phase: The last phase is when the silage is exposed to air and the aerobic microorganisms starts to become active again. This is mainly acetic acid bacteria and yeasts, which leads to spoilage of the biomass.

The stages describe the storage technique of ensiling, but in proportion to ensiling as a pretreatment method, the 2nd and 3rd phase are in focus. The sugars, VFA and alcohols are easily degradable components that can be used directly of the acidogenic-, acetogenic- and methanogenic bacteria in the anaerobic digestion. So, when the concentration of VFA, alcohols and sugars increase, the rate of the anaerobic digestion will also increase, because they can be easily degraded. Especially the acetic acid can be directly converted to biogas fast by the acetoclastic methanogens. The concentrations of different acids depend on the type of biomass and the silage conditions. Lactic acid should be the major acid in an optimal ensilage of biomass compared to acetic acid, butyric acid and propionic acid (Jensen *et al*., 2014).

1.4. Aim of the project

1.4.1. Objective of the study

Due to the demand of energy, and the primary energy sources being fossil fuels, there is an increasing interest in utilizing sustainable energy sources. The biomasses used for biofuels can be categorised in generations (G). 1G is biomasses used in, e.g. food, and is therefore not ideal to use for energy. 2G biomasses is non-food crops, such as straw, these would be ideal to use for energy. The problem with 2G biomasses is the content of lignocellulose. Due to the lignocellulosic structure, which reinforces the cellulose, the biomass cannot be utilized properly without pretreatment.

Pretreatments to enhance biogas yields are often not economically viable, and therefore there is a need to develop alternative pretreatment methods. Co-ensiling is an easy and cheap method to both storage and pretreatment of the biomasses. Tannery waste water can be used as a chemical pretreatment due to its characteristics, e.g. content of chemicals. This is also a cheap product, since it is waste water from a tannery.

The objective of this study is to increase the biogas yield of straw by anaerobic digestion by applying different pretreatment methods. To do this, the project is divided into two parts, which contains different pretreatment methods. One part is containing co-ensiled samples, where different ratios of sugar beet and straw have been stored for different time periods. The storage will pretreat the straw by fermentation of sugar beet. The other part is further pretreatment which contains mechanical-, hydrothermal- and chemical pretreatment of samples which is both co-ensiled and non-treated.

1.4.2. Hypothesis/Problem statement

We hypothesize that;

- Co-ensiling will increase biodegradability of straw and thereby methane potential of BMP.
- Co-ensiling may reduce the risk of VS loss, due to the minimizing acetogenesis during the ensiling period.
- Combination of chemical and hydrothermal pretreatment may increase methane production for straw and co-ensilage.
- Tannery waste water may replace alkaline chemical pretreatment and improve methane potential for 2G biomass.

1.4.3. Specific objectives

To fulfil the overall objective, the project is carried out with specific objectives.

- Conduct physiochemical analysis on the co-ensiled samples. To investigate sugars, alcohols and VFAs of co-ensilage.
- Investigate VS loss affected by different co-ensiling ratios and duration.
- Examine the biochemical methane potential (BMP) of both co-ensiled and further pretreated samples.
- Carry out first-order kinetic analysis to test the pretreatment effects on methane production speed.
- Carry out combination of hydrothermal and chemical pretreatment.
- Observe the pretreatment effect by scanning electron microscope (SEM) and Fourier transform infrared spectroscopy (FTIR) spectra.

2. Materials and Method

The project is divided into two parts. The first part is co-ensiling and the second part is pretreatment. Below is an overview of the difference between the analyses from Part I and Part II.

Table 1 - Analyses difference between parts

Overview of difference between Part I and Part II							
	Physiochemical analysis	TS/ VS	VFA	Sugar/alcohols	Pre-treatment	BMP test	Quantitative Analysis
Part I	X	X	X	X		X	X
Part II					X	X	X

As shown in Table 1, the BMP test and quantitative analyses is present for both parts. Therefore, these parts are only described in Part I. Any difference in these parts, will be mentioned.

2.1. Part I – Co-ensiling

2.1.1. Samples used

Co-silage sample

The samples used in this part of the project was ensiled samples from the Danish Technological Institute in Aarhus. These samples have been ensiled, at room temperature in 1 litre vacuum bags, for different time periods (2, 6 and 8 months) and consisted of sugar beet and straw. The different samples can be seen in table 2. The composition of the samples was based on wet weight. The environment was controlled and similar to each other.

Table 2 - Samples for Part I.

Composition [Sugar beet:Straw]	Ensiled [Months]	Amount [No. of samples]
100:0	2	2
	6	2
	8	2
94:6	2	2
	6	2
	8	2
88:12	2	2
	6	2
	8	2
80:20	2	2
	6	2
	8	2
0:100	2	2

The samples were kept in a freezer at -18°C until sample preparation.

Physiochemical analyses

Since the samples for the project was duplicated, they were mixed into one sample, to ensure uniform samples.

Total solids and Volatile solids determination

Physiochemical analyses were performed on the samples. The first analysis was measurement of the TS (Total solid[1]), where 2-3 grams of biomass sample was dried in an oven, overnight, at 105°C. The next analysis measured the volatile solids content in the biomass that corresponds to the organic solids, which evaporates after combustion at 550°C. The samples were put into an oven at 550°C for 2 hours following standard procedure (APHA, 2005). From the VS results, the amounts of substrate and inoculum for the BMP test can be determined.

[1] Total solid = Dry Matter (DM)

Volatile solids like VFA and alcohol are lost during the determination of the total solids (TS). It is therefore necessary to correct the TS and VS content for the lost volatile solids. If the volatiles are not considered in the calculations of VS and TS, it will lead to an underestimation of VS and an overestimation of the BMP (Weißbach and Strubelt, 2008). Therefore, analyses of the VFA and alcohol content were made so the TS and VS concentration could be corrected.

Determination of alcohols and sugars

To analyse for the alcohols; sorbitol, glycerol and ethanol, and for the sugars; glucose, maltose and fructose in the samples, HPLC (High Performance Liquid Chromatography) (Agilent 1100, Agilent Technologies Deutschland GmbH & Co. KG, Waldbronn, Germany) was used. The analyse was performed on the untreated sample. A small amount of sample was diluted in ultra-pure water, then centrifuged at 5000 rpm for 15 min, and then filtered through a 0.45 µm membrane filter into a vial prior the analysis.

VFA analysis

The VFA concentration were analysed on GC (gas-chromatograph) (7890B, Agilent Technologies, Santa Clara, CA, USA) with a flame ionization detector and $30\,\mathrm{m} * 0.25\,\mathrm{mm} * 0.25\,\mu\mathrm{m}$ column (HP-INNOWax, Agilent Technologies, Santa Clara, CA, USA). For GC analysis, the preparation of sample was almost the same as for HPLC. The difference was a small amount of phosphoric acid was added together with water to correct the pH to around 2. A standard curve was made for VFA on GC. This includes concentrations from 0.25 to 104 mM of phosphoric acid.

2.1.2. Biochemical Methane Potential test

The BMP test was carried out according to the German Standard (VDI 4630, 2006). Substrate and inoculum was mixed in 500 mL infusion bottles. Headspace in the bottles is set to 70%. Based on the VS results from the preparation, the amount of substrate and inoculum can be determined for each substrate. The ratio between inoculum and substrate was 3:1 based on VS. A blank test only containing inoculum was carried out to ensure the gas measured could be corrected to only be from the substrate. A standard bottle containing microcrystalline cellulose as substrate and inoculum was also prepared. This was used as reference to see how good the digestion was, since microcrystalline cellulose is easily degradable. Each sample was made as duplicates.

After the substrate and inoculum were mixed in the bottles, these were closed airtight with butyl rubber stoppers and aluminium crimps. All the bottles were then flushed with N_2 for one minute to ensure O_2 was not present in the bottle. After flushing the bottles, the needles would stay in for an extra minute to ensure there was no overpressure in the bottles.

The flushed bottles were placed in an incubator at 37°C for 30 days, to ensure optimal temperature for mesophilic digestion.

Gas was measured volumetric each day for the first 14 days. This was done by using a manometer to determine the overpressure, and removing the overpressure by using a syringe with a needle. The bottles were shaken before gas measurement to ensure there were no gas pockets, which could affect the daily gas amount. To determine the composition of the removed gas, the gas was put on vacuum-vials once a week for GC-analysis. The GC for gas-analysis (7890A, Agilent Technologies, Santa Clara, CA, USA), were equipped with a thermal conductivity detector and a $30\ m * 0.320\ mm$ column (J&W 113-4332, Agilent Technologies, Santa Clara, CA, USA).

2.1.3. Quantitative evaluation of BMP

Gas correction

The first analysis, which were made on the gas measurements, were to correct it to dry gas at standard conditions (273.15 K and 101.325 kPa). This was done by using the formula from the German Standard (VDI 4630, 2006).

$$V_0^{dry} = \frac{V[(P-P_w)*T_0]}{P_0*T} \quad (10)$$

V_0^{dry} is the volume of dry gas in standard conditions (NL); V is measured biogas; P is the pressure of the gas, when extracted from the bottle (kPa); P_w is vapor pressure at the temperature (37°C) (kPa); T_0 and P_0 refers to the normal temperature of 273.15 K and pressure at 101.3 kPa; T is the temperature of the incubator.

After this correction, the gas from the inoculum was extracted from the measured biogas. This gave the biogas produced only by the substrate. Final calculations, was to calculate the biogas measured to methane produced (NL CH_4 / kg VS) from the GC-analysis of the biogas.

2.1.4. Scanning Electron Microscope

Another analysis used to see if the co-ensiling had any effect, was to see if the structure of straw has changed after co-ensiling with sugar beet. To see this, a Scanning Electron Microscope (SEM) analysis was made to observe surface and morphology and topography (Lima *et al.*, 2013). Preparation for this analysis was to dry untreated sample in oven at 65°C for 24 hours. After this, the dried samples were grounded in a coffee grinder and kept in airtight bags. Since the samples were non-conductive, the electrons could not detect anything. Therefore, all samples were coated with gold, before the SEM analysis began.

2.1.5. Fourier Transform Infrared Spectroscopy (FTIR)

The chemical changes were examined with Fourier transform infrared spectroscopy (FTIR-PAS). The samples were pretreated prior to the spectroscopy by drying at temperature of 60°C, and afterwards grinding of the biomass to powder in a coffee grinder. These samples were analysed in a Nicolet 6700 spectrometer (ThermoScientific, USA) equipped with a PA-301 photoacoustic detector (Gasera Ltd, Finland) to record a FTIR-PAS spectrum. The samples were packed in ring cups with a diameter of 10 mm, which were placed in the detector chamber. The chamber and samples were purged with helium before and during the analysis to reduce the effect of the moisture evaporating from the samples during the measurement. For the samples were 32 scans in the infrared region between 4000 and 400 cm^{-1} at a resolution of 16 cm^{-1} were recorded and averaged (Bekiaris *et al.*, 2015).

2.1.6 Calculation of theoretical BMP

Theoretical biochemical methane potential (TBMP) is a stochiometric estimate of the amount of methane that the substrate can produce. This can be based on the elements of the substrate. For this, an elementary analysis was made to determine C, H, O, N and S in the substrate. For the elemental analysis (vario MACRO cube, Elemtar analysensysteme GmbH, Germany) were the untreated samples of biomasses packed in small tin packages for C, H, N and S-analysis and silver packages for O-analysis. The results were weight based and provided a percentage of the different elements. These percentages must be corrected to remove the water in the biomasses from the percentages.

Buswells equation (eq. 11) is used to describe the conversion of substrate into different biogas composition with varying concentrations of CO_2, CH_4, NH_3 and H_2S (Symons and Buswell, 1933).

$$C_aH_bO_cN_dS_e + \left(a - \frac{b}{4} - \frac{c}{2} + \frac{3d}{4} + \frac{e}{2}\right)H_2O \rightarrow \left(\frac{a}{2} + \frac{b}{8} - \frac{c}{4} - \frac{3d}{8} - \frac{e}{4}\right)CH_4 + \left(\frac{a}{2} - \frac{b}{8} + \frac{c}{4} + \frac{3d}{8} + \frac{e}{4}\right)CO_2 + dNH_3 \quad (11)$$

This could be rewritten into (Nielfa and Fdz-Polanco, 2015):

$$B_{th} = \frac{\left(\frac{a}{2}+\frac{b}{8}-\frac{c}{4}-\frac{3d}{8}-\frac{s}{4}\right)*22.4}{12a+b+16c+14d+32s} * 1000 \left[\frac{NL\ CH4}{kg\ VS}\right] \quad (12)$$

From these equations, the could the TBMP be calculated, when knowing the VS content of the different compounds in the substrate. The stoichiometric method to assess methane potential does not consider the microbial growth and biodegradability. Which means that VS is totally anaerobically digested and is converted to biogas production.

2.2. Part II – Pretreatment

2.2.1. Samples

Samples for this experiment were one co-ensiled and one pure milled straw. The milled straw (S0:100), is considered to have the same physiochemical properties as S0:100-2M, which was used in Part I. The co-ensiled sample is S80:20-8M, which was also used in Part I.

For these experiments, tannery waste water (WW) has been used, which was collected from a tannery in Denmark.

2.2.2. Pretreatment Procedure

The samples were already prepared from the previous experimental procedure. Physiochemical analyses were also made from the previous experiment for the substrate. New TS and VS analyses were performed on the new inoculum and the WW. TS and VS were the same for the inoculum. And for the WW was so low it would not affect the ratio between substrate and inoculum, which were based on VS.

For this experiment, sequential pretreatments was to be investigated. To do this, the substrate had to be pretreated differently. The pretreatments were mechanical, chemical and hydrothermal as shown below in Fig. 9. The mechanical part was only for the pure straw, which as a sample were milled beforehand. The chemical pretreatment used WW as a replacement of conventional chemicals. There were two kind pretreatments, where the WW was included. In the first part were the biomass and WW mixed and kept for 24 hours at 37°C in 500 mL infusion bottles. This was done for both milled straw and ensiled sugar beet and straw.

The other kind of chemical pretreatment, was a combined chemical and hydrothermal pretreatment. This was made in 500 mL infusion bottles. Hydrothermal pretreatment was accomplished by putting the samples with either WW or water (if they were not pretreated with WW), into an autoclave where an *'open-liquids'* program was premade and used. After the hydrothermal pretreatment, all the samples were ready for BMP test. All samples were done in duplicates.

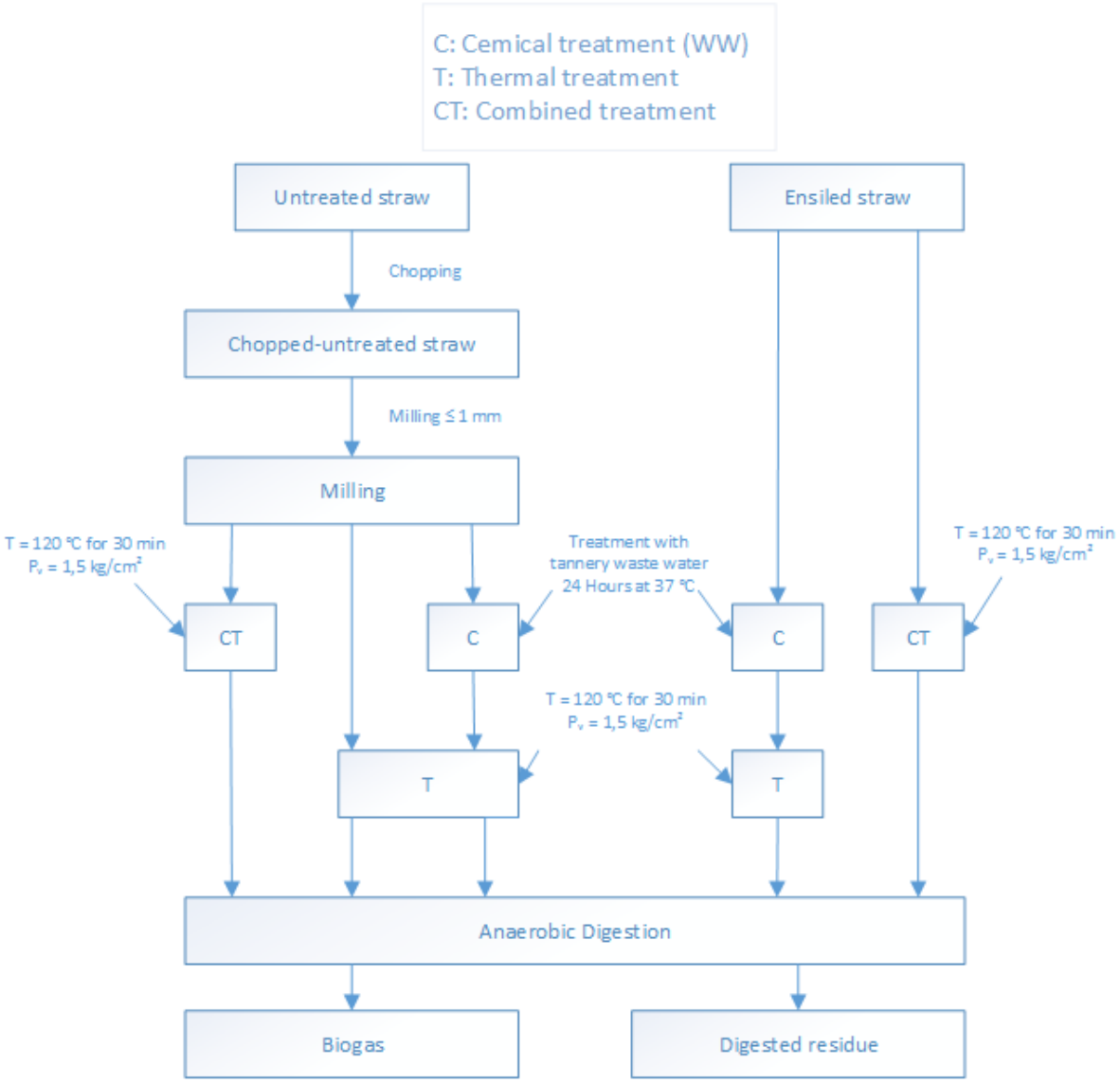

Fig. 9 – Pretreatment procedures.

2.2.3. Biochemical Methane Potential test

The BMP test was the same experimental procedure as for Part I. There are two blank tests in Part II. One with only inoculum, and a second with inoculum and WW. Both are used as before in the quantitative analysis based on the sample containing WW or not.

2.2.4. Quantitative Evaluation of BMP test

TBMP was not conducted, since the samples used in part II were the same as from part I.

3. Data analysis

3.1. Part I – Co-ensiling effect

3.1.1. Theoretical biochemical methane potential

The theoretical biochemical methane potential (TBMP) of co-ensiled samples were determined by conducting an elemental analysis. The elemental analysis was separated into analysis of carbon (C), hydrogen (H), nitrogen (N) and sulphur (S) with one column and oxygen (O) with another column. The results of these analysis are shown in Table 3.

Table 3 – Elemental analysis results for Part I.

Sample Name	Mass [g]	C [wt%]	H [wt%]	N [wt%]	S [wt%]	Sample Name	Mass [g]	O [wt%]
S100:0-2M	0.01284	7.76	4.135	0.19	0.502	**S100:0-2M**	0.00714	63.143
S100:0-6M	0.01034	7.14	3.960	0.19	0.248	**S100:0-6M**	0.00741	51.498
S100:0-8M	0.01336	5.16	2.857	0.16	0.145	**S100:0-8M**	0.00539	68.894
S94:6-2M	0.01187	8.56	3.368	0.21	0.142	**S94:6-2M**	0.00392	64.060
S94:6-6M	0.01489	7.97	3.101	0.18	0.097	**S94:6-6M**	0.00366	64.235
S94:6-8M	0.01320	8.57	3.080	0.22	0.112	**S94:6-8M**	0.00458	64.235
S88:12-2M	0.01149	8.96	4.223	0.19	0.118	**S88:12-2M**	0.00504	63.815
S88:12-6M	0.01182	13.10	5.071	0.22	0.115	**S88:12-6M**	0.00470	65.539
S88:12-8M	0.01367	12.29	5.084	0.19	0.087	**S88:12-8M**	0.00485	64.210
S80:20-2M	0.01160	12.63	4.727	0.27	0.098	**S80:20-2M**	0.00414	64.681
S80:20-6M	0.01206	14.25	5.070	0.24	0.085	**S80:20-6M**	0.00429	66.400
S80:20-8M	0.01321	13.56	4.754	0.22	0.076	**S80:20-8M**	0.00334	60.264
S0:100-2M	0.01253	41.71	4.571	0.71	0.102	**S0:100-2M**	0.00531	51.465

The results of the elements were not only from the solids in the biomasses, but also from the water in biomasses. The percentage of H and O is therefore too high, and the results has therefore been corrected so the water is not included in the elements of the samples.

With the element ratios of the C, H, N and O in a specific mass is it possible to calculate the moles of the different elements. With the moles of the different elements in a specific mass of the biomass can the rewritten Buswell's equation (eq. 13) (VDI 4630, 2006) be used to calculate the TBMP.

$$B_{th} = \frac{\left(\frac{a}{2}+\frac{b}{8}-\frac{c}{4}-\frac{3d}{8}-\frac{s}{4}\right)*22.4}{12a+b+16c+14d+32s} * 1000 \left[\frac{NL\ CH_4}{kg\ VS}\right] \quad (13)$$

The B_{th} corresponds to the TBMP and the value of 22.4 is the volume in litres of 1 molar gas at standard conditions.

The TBMP results can be seen in section 4.1.1.

3.1.2. Total solid and volatile solid determination

The correction of TS and VS is extra important when the substrate is ensiled sugar beet, since the sugar beet have fermented some of its sugars into alcohol and VFA during the ensilage. The concentration of volatile solids, which are not included in the determination is therefore even higher. It is not relevant to correct the volatile solids for 100% straw, since the lignocellulosic structure reduces the fermentation during the ensilage. There will therefore only be generated very low amounts of VFA and alcohol, and does therefore not have to be corrected. In Table 4 is corrected TS and corrected VS presented.

Table 4: Correction of TS and VS from the total VFA and alcohol concentration.

	Total VFA and alcohols	TS_{cor}	SD	VS_{cor}	SD	VS of TS	Ash content
	[g/kg]	[g/kg]		[g/kg]		[%]	[g/kg]
S100:0-2M	2.96	196.1	0.71	187.1	0.65	95.37	9.08
S100:0-6M	2.86	162.4	0.38	153.5	0.36	94.50	8.93
S100:0-8M	2.65	139.0	0.04	130.6	0.02	93.94	8.42
S94:6-2M	6.78	246.5	1.64	232.8	1.58	94.45	13.68
S94:6-6M	3.42	177.7	0.76	163.5	0.64	92.00	14.23
S94:6-8M	7.81	240.5	0.24	226.8	0.30	94.28	13.77
S88:12-2M	7.73	260.5	0.88	244.5	0.80	93.86	16.00
S88:12-6M	5.82	271.2	0.69	252.2	0.44	93.00	18.98
S88:12-8M	9.19	282.0	0.57	262.8	0.52	93.19	19.21
S80:20-2M	9.14	350.0	0.12	324.4	0.07	92.70	25.56
S80:20-6M	8.46	338.8	0.91	313.4	0.99	92.49	25.44
S80:20-8M	8.15	313.9	0.44	279.8	1.66	89.13	34.11
S0:100-2M	NA	916.0	0.05	848.6	0.29	92.64	67.40

3.1.3. Physiochemical data

Generation of sugars during co-ensiling

The measured concentrations of the easily degradable sugars; glucose, maltose and fructose were extracted from the chromatograms from the HPLC and GC. The concentrations of glucose and maltose can be seen at Table 5.

Table 5 - Concentrations of sugars depending on co-ensiling proportion and ensiling period.

	S100:0-			S94:6-			S88-12-			S80:20-		
	2M	6M	8M	2M	6M	8M	2M	6M	8M	2M	6M	8M
Glucose [g/L]	1.66	1.69	1.17	5.11	2.14	5.88	5.93	4.24	7.13	7.17	6.52	6.23
Maltose [g/L]	0.40	0.37	0.22	0.89	0.37	0.98	1.01	0.75	1.21	1.19	1.01	0.96
Total sugar concentration [g/L]	2.06	2.07	1.39	6.00	2.51	6.86	6.94	4.98	8.34	8.36	7.53	7.14

It has only been possible to identify glucose and maltose in the biomasses, but this may be because of the fructose peak has a retention time of 11.142 min with the used method, while sorbitol has a retention time of 11.419 min, which means the peaks will be very close. It must therefore be expected that the area from fructose has been integrated as sorbitol.

Generation of organic acids during co-ensiling

The concentrations of the volatile fatty acids (C2-C5) e.g. acetic acid, propionic acid, iso-butiric acid, butyric acid, iso-valeric acid and valeric acid were determined by using HPLC. Only acetic acid and butyric acid were found in the silage samples, while the other VFA's were below detection limit. The concentrations of acetic acid and butyric acid can be seen in Table 6.

Table 6 – VFA content in samples for Part I.

	S100:0-			S94:6-			S88-12-			S80:20-		
	2M	6M	8M	2M	2M	6M	8M	2M	2M	6M	8M	2M
Butyric acid [g/L]	0.08	0.03	0.00	0.13	0.00	0.10	0.12	0.07	0.11	0.11	0.10	0.09
Acetic acid [g/L]	0.82	0.76	1.26	0.66	0.91	0.85	0.68	0.77	0.74	0.68	0.84	0.92
Total VFA conc. [g/L]	0.90	0.79	1.26	0.79	0.91	0.95	0.80	0.84	0.85	0.79	0.93	1.01

Although lactic acid is one of the major organic acids from co-ensiling, because of time limitation, determination of lactic acid was not included in this project.

Generation of alcohols during co-ensiling

The concentration of the alcohols; sorbitol, glycerol and ethanol were determined with HPLC to examine the effect of the ensilage. The concentrations of the different alcohols can be observed at Table 7.

Table 7 – Alcohol contents in samples for Part I.

	S100:0-			S94:6-			S88-12-			S80:20-		
	2M	6M	8M	2M	6M	8M	2M	6M	8M	2M	6M	8M
Sorbitol [g/L]	10.23	6.14	3.66	11.89	6.20	14.50	11.58	9.54	14.26	11.93	12.55	10.91
Glycerol [g/L]	0.11	0.20	0.75	0.11	0.94	0.43	0.12	0.09	0.07	0.12	0.34	0.36
Ethanol [g/L]	0.10	0.40	0.68	0.03	0.54	0.43	0.12	0.09	0.07	0.12	0.34	0.36
Total alcohol concentration [g/L]	10.43	6.75	5.09	12.03	7.68	14.97	11.73	9.83	14.36	12.08	12.92	11.29

The sorbitol concentration is much higher than glycerol and ethanol, which is because of the poor separation of the retention times between fructose and sorbitol.

Total easily degradable volatile solids

The sugars, organic acids and alcohols are easily degradable volatile solids. The identified and quantified sugars, organic acids and alcohols are added up and introduced in Table 8 based on the concentration and on the fraction of the total VS.

Table 8 – Total easily degradable VS for samples in Part I.

	S100:0-			S94:6-			S88:12-			S80:12-		
	2M	6M	8M	2M	6M	8M	2M	6M	8M	2M	6M	8M
Total VS_{ED} [g/L]	13.39	9.61	7.74	18.8	11.10	22.78	19.46	15.65	23.55	21.22	21.38	19.44
Total VS_{ED} [% of VS]	7.51	6.57	6.23	8.52	7.15	10.77	8.37	6.46	9.48	6.79	7.11	7.25

3.1.4. Synergistic effects

To see the effect of co-ensilage on the samples, estimations of VS and BMP were conducted based on the comparison between the measured methane and an estimate for the same compositions methane production.

The estimations were based on the pure samples (e.g. S100:0 and S0:100). From these, new VS were conducted by considering the ratio for the estimated sample (e.g. S94:6-6M).

The measured data used for estimations are shown below in Table 9.

Table 9 – The measured data used for estimations of BMP and VS.

Sample Name	VS [g/kg]	BMP_{30} $\left[\frac{NL\ CH_4}{kg\ VS}\right]$
S100:0-2M	187.1	369.4
S100:0-6M	153.5	412.5
S100:0-8M	130.6	443.6
S0:100-2M	848.6	186.7

Estimation of new VS is based on the composition and the VS known from the pure samples.

The formula for estimating VS is shown in eq. 14.

$$VS_{Estimated} = VS_{SB} * Ratio_{SB} + VS_{Straw} * Ratio_{Straw} \quad (14)$$

Example for estimating for composition S94:6-6M:

$$VS_{Estimated} = (153.5 * 0.94)\frac{g}{kg} + (848.6 * 0.06)\frac{g}{kg} = 195.2\frac{g}{kg} \quad (15)$$

Calculations for VS is carried out on all compositions and months as shown before. All results are shown below in Table 10.

Table 10 – New estimated VS concentrations.

	S94:6-			S88:12-			S80:20-		
	E-2M	E-6M	E-8M	E-2M	E-6M	E-8M	E-2M	E-6M	E-8M
Months	2	6	8	2	6	8	2	6	8
E-VS [g/kg]	226.8	195.2	173.7	266.4	236.9	216.7	319.4	292.5	274.2

Now is it possible to estimate methane production for the known ensiled compositions. The estimate is based on the VS and BMP from the measured pure samples. This is all divided with the estimated VS, shown in eq. 16.

The BMP results for the samples, which are used for the estimations are shown in Table 11.

$$BMP_{Estimated}\left[\frac{NL\ CH_4}{kg\ VS}\right] = \frac{\left(Ratio_{SB}{*}VS_{SB}\left[\frac{g}{kg}\right]{*}BMP_{SB}\left[\frac{NL\ CH_4}{kg\ VS}\right]\right)+\left(Ratio_{Straw}{*}VS_{Straw}\left[\frac{g}{kg}\right]{*}BMP_{Straw}\left[\frac{NL\ CH_4}{kg\ VS}\right]\right)}{VS_{Estimated}\left[\frac{g}{kg}\right]} \quad (16)$$

An example for BMP estimation, for 30 days, is shown for S94:6-6M, eq. 17.

$$BMP_{E-S94:6-6M} = \frac{\left(0.94{*}153.5\left[\frac{g}{kg}\right]{*}412.5\left[\frac{NL\ CH_4}{kg\ VS}\right]\right)+\left(0.06{*}848.6\left[\frac{g}{kg}\right]{*}186.7\left[\frac{NL\ CH_4}{kg\ VS}\right]\right)}{195.2\left[\frac{g}{kg}\right]} \quad (17)$$

$$BMP_{E-S94:6-6M} = 353.6\left[\frac{NL\ CH_4}{kg\ VS}\right]$$

The same calculations were conducted on all compositions and months as before, which can be seen in Table 11.

Table 11 – Estimated and measured BMP of compositions after 30 days.

	S94:6-			S88:12-			S80:20-		
	E-2M	E-6M	E-8M	E-2M	E-6M	E-8M	E-2M	E-6M	E-8M
E-BMP$_{30}$	328.4	353.6	368.3	299.6	315.4	322.9	272.3	281.5	284.6
BMP$_{30}$	304.5	411.1	354.1	291.2	307.4	312.9	253.9	244.3	298.6

To see the effect of co-ensilage of SB and straw, the synergistic effects are found between the estimations and the measured BMP results. This gives the difference between the estimated and the measured. High synergistic effect indicated a good effect from co-ensilage on the biodegradability. Estimation gives an idea of the expected BMP results, and if the estimated BMP is higher than measured BMP, it indicates there is no synergistic effect. Synergistic effect is calculated by the difference between the estimated and the measured, and then divided by the estimated. The equation for synergistic effect is shown in eq. 18.

$$Synergy\ Effect\ [\%] = \frac{BMP_{Measured}-BMP_{Estimated}}{BMP_{Estimated}} \quad (18)$$

The synergistic effect for S94:6-6M, is shown in eq. 19. The measured BMP is known, and the estimated BMP have been calculated before.

$$Synergy\ Effect_{S94:6-6M} = \frac{411.1\left[\frac{NL\,CH_4}{kg\,VS}\right] - 353.6\left[\frac{NL\,CH_4}{kg\,VS}\right]}{353.6\left[\frac{NL\,CH_4}{kg\,VS}\right]} * 100\% = 16.3\% \qquad (19)$$

The calculations are carried out on all the samples and the results can be seen in section 4.1.4.

3.1.5. Kinetic analysis

Kinetics were calculated to see the speed of the digestion, with eq. 20 (Pham *et al.*, 2013):

$$B_t = B_0 * (1 - e^{-k*t}) \qquad (20)$$

In eq. 20, B_t is cumulative methane yield at time *t*, $\left[\frac{NL\,CH_4}{kg\,VS}\right]$; B_0 is ultimate methane potential (BMP) $\left[\frac{NL\,CH_4}{kg\,VS}\right]$; *K* is the hydrolysis constant which describes the speed of which the VS is degraded per day [day^{-1}], and *T* is time [day].

By using eq. 20, the measured data were estimated based on *k* and B_0 which depended on the Root-Mean-Square-Error (RMSE), giving the most precise estimations. All this were done by first setting a *k* and B_0 and getting the first estimations. After this, the 'Solver' in excel would set *k* and B_0 to values, that gave the lowest RMSE and thereby the most precise estimations.

Usage of kinetics

Kinetics can be used to see the speed of the digestion, because the first-order constant (*k*) is the hydrolysis constant. The speed depends on the biodegradability of the biomasses. Lignocellulosic biomasses will have a much slower speed (lower constant) than a biomass containing cellulose or mono sugars.

The formula for first-order kinetics:

$$B_t = B_0 * (1 - e^{-k*t}) \qquad (21)$$

Eq. 21 can be used to show how much of the biomass is degraded by a certain time. E.g. if the digestion is 20 days, and the *k* and B_0 is known, B_t can be calculated. After this, the percentage of biomass degraded can be calculated as well with eq. 22 (Triolo, 2011).

$$BD = \frac{B_t}{B_0} \qquad (22)$$

The first-order equation can also be used to calculate the time it takes for the biomass to be degraded by, e.g. 90%. To do this, $B_t = 0.9 * B_0$ and then the equation can be solved for t, which will give the time for the digestion to degrade the biomass by 90%.

If this should be used in the real world, e.g. a biogas plant. This formula would not be the best. This formula is used because the data is from a batch experiment, and not from a CSTR. Due to the hydrolysis constant (k day^{-1}) is obtained from a batch condition, where the amounts of microorganisms are limited, the production of methane cannot be compared to a CSTR.

3.2. Part II – Further pretreatment effect

3.2.1. Physiochemical data

The samples used in Part II, is straw (S0:100) which have been mechanical treated and milled to 1 mm and S80:20-8M, which also were used in Part I. Assumed that straw does not change during mono-ensiling, due to its composition, the physiochemical results for S0:100-2M is used for the milled straw.

The physiochemical results for the inoculum and tannery WW were conducted the same way, as the physiochemical results in Part I. Although, only TS and VS were measured. The results are shown below in Table 12.

Table 12 - Physiochemical results for samples in Part II.

Sample Name	TS [g/kg]	SD	VS [g/kg]	SD	VS of TS [%]	Ash [g/kg]
S80:20	302.4	0.43	268.3	1.65	88.72	34.10
S0:100	916.0	0.05	848.6	0.29	92.64	67.40
Inoculum	83.9	0.15	48.4	0.10	57.86	35.50
Tannery WW	8.70	0.01	0.9	0.00	10.86	7.70

As seen in Table 12 the VS of inoculum nearly differs from the inoculum used in Part I (VS of 48.8 g/kg). The tannery WW has a VS of 0.9 g/kg, this is extremely low, and have nearly no effect on the BMP results, based on VS.

4. Results and discussion

4.1. Part I – Co-ensiling effect

4.1.1. Theoretical biochemical methane potential

The TBMP of the biomasses with different mixing ratios and ensiling periods were determined based on the elementary analysis experiment of the different samples. The results of these can be seen in Table 13.

Table 13 - Theoretical Biochemical Methane Potential.

Sample Name	TBMP $\left[\frac{NL\ CH_4}{kg\ VS}\right]$
S100:0-2M	439.61
S100:0-6M	447.32
S100:0-8M	468.25
S94:6-2M	566.99
S94:6-6M	NA
S94:6-8M	549.52
S88:12-2M	NA
S88:12-6M	621.31
S88:12-8M	649.97
S80:20-2M	596.05
S80:20-6M	640.27
S80:20-8M	NA
S0:100-2M	660.44

The TBMP of S100:0 samples were in the interval 439.61 $\left[\frac{NL\ CH_4}{kg\ VS}\right]$ to 468.25 $\left[\frac{NL\ CH_4}{kg\ VS}\right]$ for the different ensiling periods and the TBMP of S0:100-2M were determined to be 660.44 $\left[\frac{NL\ CH_4}{kg\ VS}\right]$. The TBMP of the different mixing ratios between sugar beet and straw increases when the ratio of straw is higher. A study (Liu *et al.*, 2016) reports that TBMP of straw is 454.9 $\left[\frac{NL\ CH_4}{kg\ VS}\right]$ compared to sugar beet, which has a TBMP of 442.4 $\left[\frac{NL\ CH_4}{kg\ VS}\right]$. The TBMP of sugar beet is close to what is referred in the literature, but the determined TBMP of straw is higher than literature reports. The lignin inside the lignocellulosic structure

of straw has a TBMP of 727.1 $\left[\frac{NL\ CH_4}{kg\ lignin}\right]$ (Triolo *et al.*, 2011). The lignin is non-biodegradable, but is still included in the TBMP. This is the reason for the higher TBMP when the fraction of lignin increases, since sugar beet not contain lignin. The TBMP is dependent on harvest time, storage and ensiling too so this may cause a difference.

The TBMP of sample S94:6-6M, S88:12-2M and S80:20-8M were not available, because of elementary analysis of these sample were not reliable. This may be because of too high inhomogeneity in the analysis of these samples.

4.1.2. Physiochemical results

The influence of ensilage on alcohols, volatile fatty acids and sugars

Table 14 is an overview of physiochemical results where the quantified easily degradable volatile solids (VS_{ED}), and generation of total VFAs and alcohols are presented.

Table 14 - An overview of the physiochemical results.

	S100:0-			S94:6-			S88:12-			S80:20-			S0:100-
	2M	6M	8M	2M	6M	8M	2M	6M	8M	2M	6M	8M	2M
TS_{corr} [g/kg]	196.1	162.4	139.0	246.5	177.7	240.5	260.5	271.2	282.0	350.0	338.8	313.9	91.6
VS_{corr} [g/kg]	187.1	153.5	130.6	232.8	163.5	226.8	244.5	252.2	262.8	324.4	313.4	279.8	84.9
Total VS_{ED} [g/L]	13.39	9.61	7.74	18.82	11.10	22.78	19.46	15.65	23.55	21.22	21.38	19.44	NA
Total VS_{ED} [% of VS]	7.51	6.57	6.23	8.52	7.15	10.77	8.37	6.46	9.48	6.79	7.11	7.25	NA
Total sugars [g/L]	2.06	2.07	1.39	6.00	2.51	6.86	6.94	4.98	8.34	8.36	7.53	7.14	NA
Total alcohols [g/L]	10.43	6.75	5.09	12.03	7.68	14.97	11.73	9.83	14.36	12.08	12.92	11.29	NA
Total VFAs [g/L]	0.90	0.79	1.26	0.79	0.91	0.95	0.80	0.84	0.85	0.79	0.93	1.01	NA

The concentration of easily degradable volatile solid during the ensilage

The concentrations of VFA's, alcohols and sugars can be observed in Table 14. The fraction between these easily degradable volatile solids are interesting, because it can be used to determine the effect of the ensilage fermentation. These fractions are illustrated in Fig. 10.

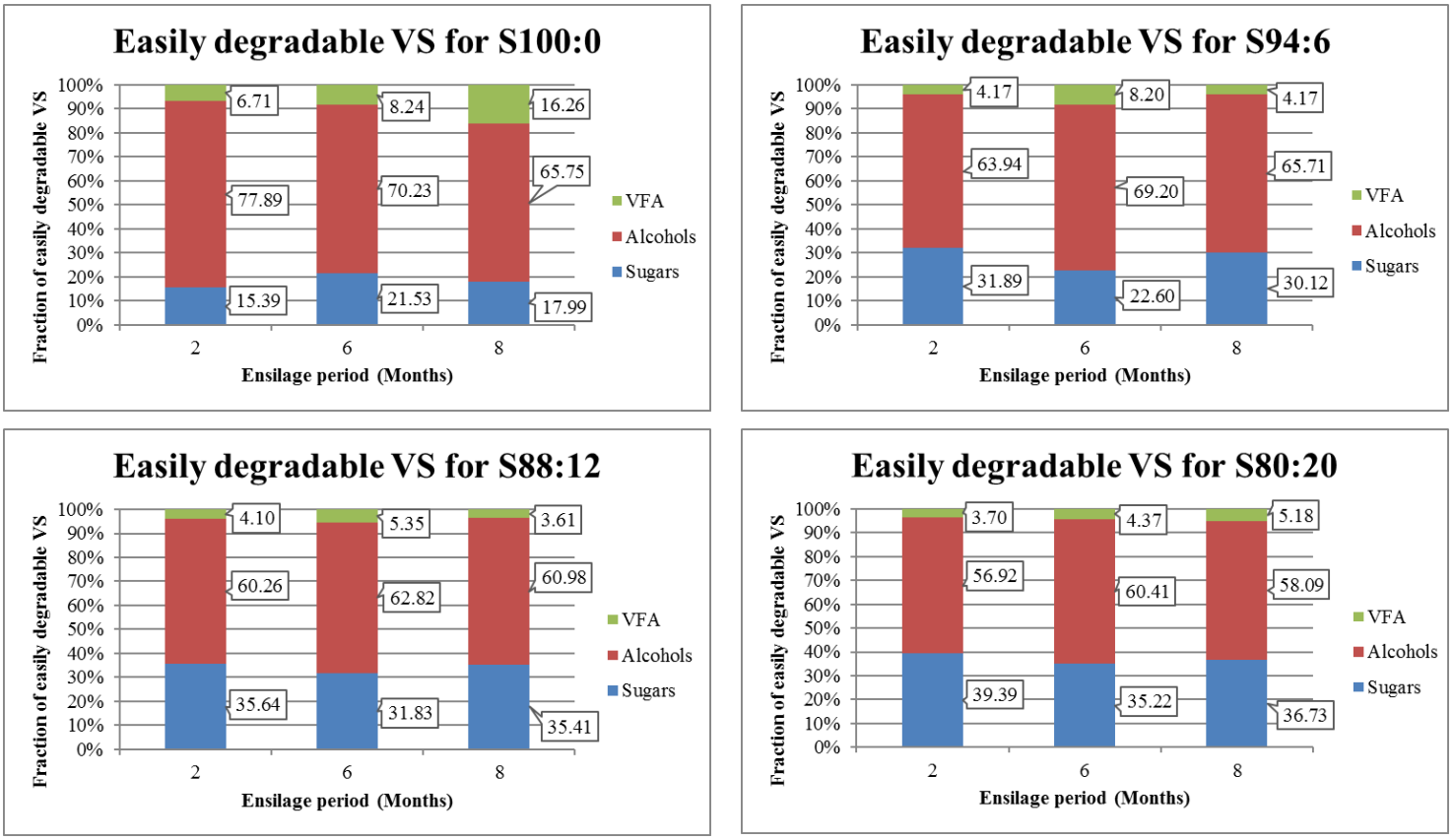

Fig. 10 – Diagrams of the different fractions of easily degradable VS compared to the ensilage period. VFA is volatile fatty acids.

It can be seen on the results from Fig. 10 that the VFA fraction increases and the alcohol fraction decreases for sample S100:0 during the mono-ensilage. This indicates that the acetogenesis phase has been active during the mono-ensiling of sugar beet roots, since the alcohols are converted into acetic acid, H_2 and CO_2. The H_2 and CO_2 will be lost VS, that will leave the silo when it is opened, so it is less desired to have the acetogenesis phase occurring, because it would result in mass loss. The only two detectable types of VFA that could be identified and quantified from the biomass with the GC were acetic acid and butyric acid. The VFA's after 8 months of ensilage for sample S100:0 may therefore be expected to come from the degradation of alcohols in the acetogenesis among others. The concentration of lactic acid was

not determined when the concentrations of different VFA were determined, so it must be expected that this leads to a small overestimation of the BMP.
The fraction of sugar is generally increasing when the ratio of straw increases, while the fraction of alcohol and VFA decreases. This could indicate that the rate of the fermentation during the ensilage has been decreased because of the co-ensiling between sugar beet and straw compared to the mono-ensiling of pure sugar beet.

A method to increase the concentrations of alcohols, produced during the anaerobic fermentation while being ensiled, could be to add different specific biological additives like bacteria and enzymes to the biomass before ensilage to increase the biological degradation and the fermentation. The increased biological degradation would make the hydrolysis faster in the anaerobic digestion when producing biogas. This is the rate limiting step, so it is therefore relevant to find solutions, which increases the hydrolysis during the anaerobic digestion (Vervaeren *et al.*, 2010). And co-ensiling could be part of a solution for this.

Volatile solid loss during ensiling

A disadvantage to the ensilage is the energy loss, which would reduce the BMP of the biomass. It is desired to have the fermentation be carried out by LAB to produce lactic acids and acetic acid, because that results in the lowest losses of DM and energy from the biomass during the storage (Kreuger *et al.*, 2011). The acetic acid fermentation and homolactic fermentation, which produces lactic- and acetic acid losses no DM, because these acids can be used directly in the anaerobic digestion. The only energy that is lost will be energy that the microorganisms uses for their growth. Other fermentation processes may cause higher mass and energy losses as e.g. the butyric acid fermentation. The butyric acid fermentation is carried out by the Clostridia bacteria and are undesired because the fermentation causes a mass loss of 51% (Kreuger, Nges and Björnsson, 2011) and an energy loss of 18.4% (Sakhawat, 2011). The mass loss is from the products from the fermentation, because butyric acid is produced along with CO_2 and H_2. These will disappear when the silo is opened. The loss of VS corresponds to loss of energy, since a smaller amount can be converted into biogas. It is therefore relevant to consider the loss of VS compared to the easily degradable volatile solids that are produced during the ensilage, so it can be determined if it is feasible to use ensilage as a pretreatment and not only a storage method for biomasses.

The VS loss has been determined by calculating the difference in VS content in the different periods of ensilage.

Table 15 - Overview of loss of VS.

S100:0 samples		S88:12 samples		S80:20 samples	
Loss of VS from 2 to 6 months of ensilage.	Loss of VS from 6 to 8 months of ensilage.	Loss of VS from 2 to 6 months of ensilage.	Loss of VS from 6 to 8 months of ensilage.	Loss of VS from 2 to 6 months of ensilage.	Loss of VS from 6 to 8 months of ensilage.
18%	30%	-3%	-7%	3%	14%

Data for loss of VS from 2 to 6 months and from 6 to 8 months for the samples with the ratios; S100:0, S88:12 and S80:20. For the S100:0 samples the amount of lost VS is 18% based on [g/kg] from 2 to 6 months of ensilage, while the amount of lost VS increases to 30% from 6 to 8 months of ensilage. The trend of increasing loss of VS in proportion to the ensilage period also occurs for S80:20 samples, but with a smaller effect. For S88:12 samples the VS loss is negative, which means that a higher VS concentration could be determined in the samples, which were ensiled for a longer period. This could be because of the non-homogeneous samples and experimental errors. The determined VS are close to each other for S88:12 samples, so it indicates that the VS loss are low for that sample.

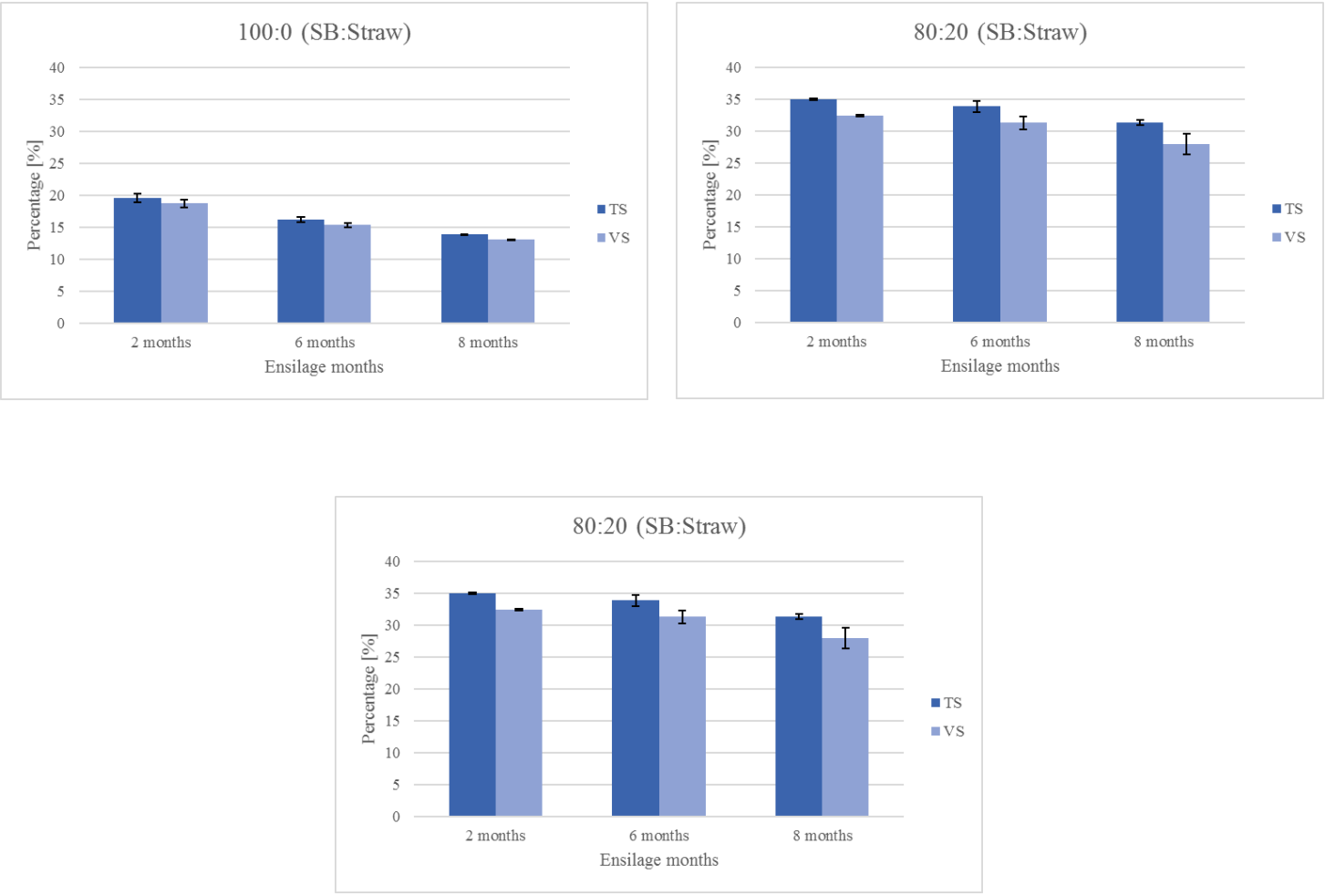

Fig. 11 – The VS loss during the ensilage period. TS is total solids; VS is volatile solids; e.g. 80:20 is the ratio of straw and sugar beet.

Different approaches have been investigated by others to overcome the loss of VS during ensilage. Studies have reported that a way to reduce the VS loss is to supress and inhibit the activation of hydrogen and CO_2 producing bacteria like Clostridium and other enterobacteria, since they are reported to cause higher VS losses than lactic acid bacteria (Sakhawat, 2011). The loss is lower for the co-ensiled samples than for the mono-ensiled samples as it can be seen in Fig. 11, which may be because of the dry matter concentrations. The growth of Clostridium bacteria is very dependent of the dry matter concentration and are reported to be inhibited when the dry matter approaches 300 g/kg (McDonald *et al.*, 2010). This is because the Clostridia bacteria are less sensitive to lower water availability than LAB, so by increasing

the DM concentration, then the undesired Clostridia bacteria can be inhibited (Elferink *et al*., 2000). Furthermore, Clostridia is inhibited at low pH, which means that a sufficient and rapidly drop in pH during the ensilage can inhibit the Clostridia and thereby decrease the mass and energy loss during the ensilage.

The results from the physiochemical analysis therefore indicates that mono-ensiling of sugar beet roots causes remarkably activation of acetogenic microorganisms, which spoils optimal ensiling and causes high VS losses, compared to co-ensiling of straw and sugar beet.

4.1.3. Overview of BMP of silage samples.

Table 16 shows the BMP results of all the samples including inoculum (blank) and microcrystalline cellulose as reference material. The results shown are from day 10, 20 and 30 of BMP test.

Table 16 - Overview of BMP results for Part I.

Sample Name	CH_4 (10) $\left[\frac{NL\ CH_4}{kg\ VS}\right]$	CH_4 (20) $\left[\frac{NL\ CH_4}{kg\ VS}\right]$	BMP_{30} $\left[\frac{NL\ CH_4}{kg\ VS}\right]$	SD
S100:0-2M	322.6	363.4	369.4	5.3
S100:0-6M	369.0	405.8	412.5	4.0
S100:0-8M	388.0	440.2	443.6	29.3
S94:6-2M	247.1	289.2	304.5	14.2
S94:6-6M	321.1	384.2	411.1	1.9
S94:6-8M	268.3	334.8	354.1	46.0
S88:12-2M	226.3	268.3	291.2	16.1
S88:12-6M	240.7	284.7	307.4	0.3
S88:12-8M	230.1	288.4	312.9	15.4
S80:20-2M	192.5	233.6	253.9	15.5
S80:20-6M	180.6	221.0	244.3	6.6
S80:20-8M	207.6	265.0	298.6	39.6
S0:100-2M	85.9	145.3	186.7	8.5
Cellulose	364.1	414.2	424.0	6.4

The BMP results ranged from 244 $\frac{NL\ CH_4}{kg\ VS}$ to 444 $\frac{NL\ CH_4}{kg\ VS}$. The SD ranges from 0.1 to 46, which is high. As seen in Table 16, BMP shows that longer durations of ensilaging increased methane potential, besides

S94:6-6M, all samples followed this trend. The BMP increased due to the improvement of biodegradability, which corresponds to the co-ensiling and the time.

Cellulose, which was used as reference material, had a BMP of 424 $\frac{NL\ CH_4}{kg\ VS}$. Considering TBMP of microcrystalline cellulose is 414 $\frac{NL\ CH_4}{kg\ VS}$, the BMP of microcrystalline cellulose was slightly higher than BMP (2-3%).

Fig. 12 show the cumulative methane production curves, which clearly shows that ensilage influenced the production of methane production rate and final methane yield at 30 days.

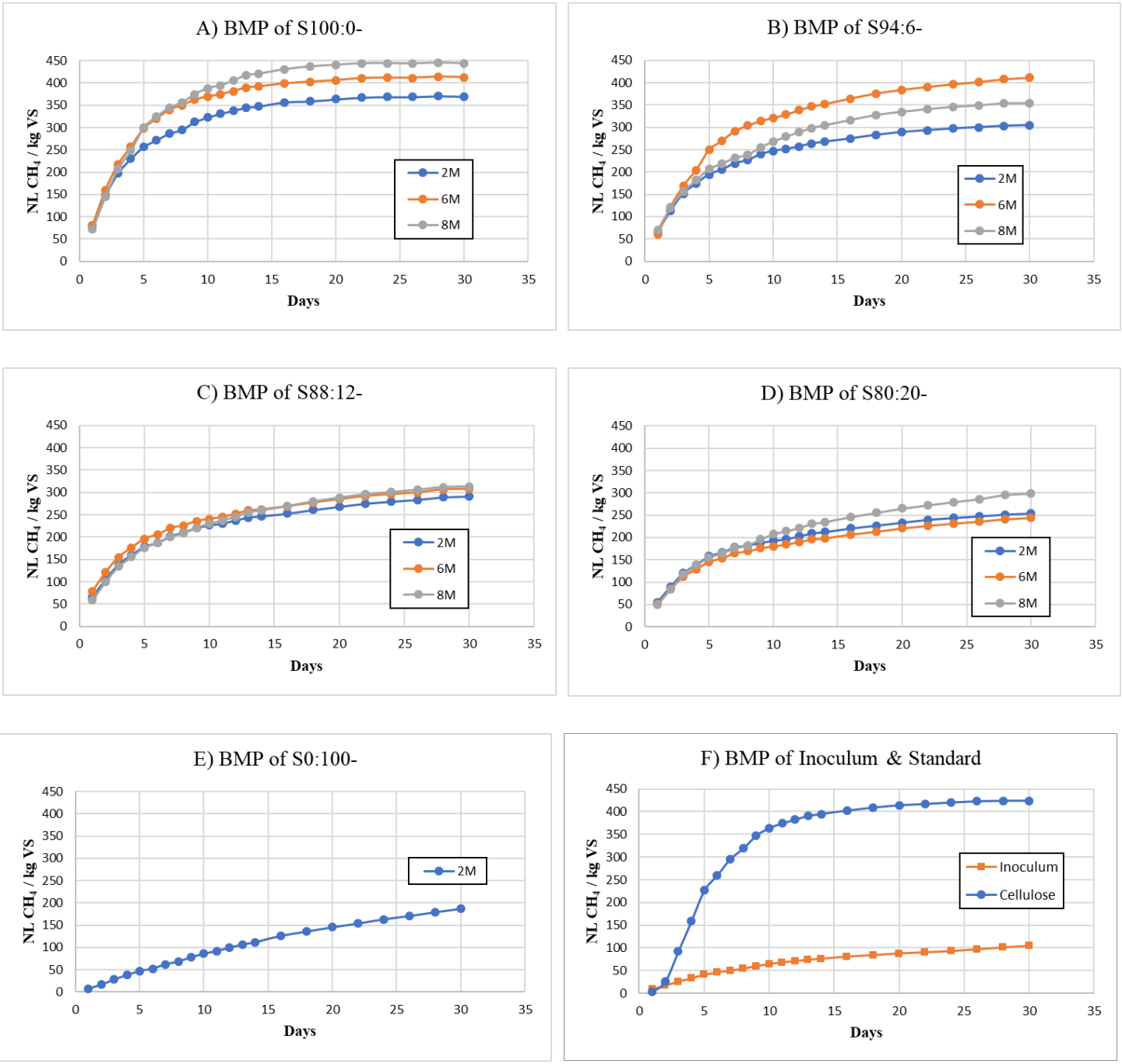

Fig. 12 - Visualisation of cumulated methane results for samples in Part I.

In Fig. 12D, S80:20-8M has a higher BMP than that of S80:20-2M and S80:20-6M. When comparing methane production from S100:0 (Fig. 12A), where the graph stabilises, and for Fig. 12E, which is 100% ensiled straw, the cumulative methane curve is not flattened out, which indicates that a BMP test on 30

days is not time enough to access the ultimate methane potential of ensiled straw. For Fig. 12B a clear difference between six (6) months of ensilage and 2-8 months of ensilage is shown. Compared to the other parts of Fig. 12, the trend is that 6 months should end between 2 and 8 months.

4.1.4. Synergistic effect of silage samples.

Below in Table 17, an overview of synergistic effect is shown.

Table 17 - Overview of synergistic effects (SE).

	S94:6-			S88:12-			S80:12-		
	2M	6M	8M	2M	6M	8M	2M	6M	8M
BMP_{30}	304.5	411.1	354.1	291.2	307.4	312.9	253.9	244.3	298.6
SD of BMP_{30}	14.2	1.9	46.0	16.1	0.3	15.4	15.5	6.6	39.6
E-BMP_{30}	328.4	353.6	368.3	279.5	291.4	295.1	233.2	236.0	233.3
SE [%]	-7.3	NA	-3.9	4.2	5.5	6.0	8.9	3.6	28.0

The synergistic effect ranged from -8 to +28%. Based on the measured BMP and the estimated BMP, the results are as expected. All but one result is shown, see further discussion on this in section 5.1. In Fig. 13, a visualisation of the synergistic effect is illustrated.

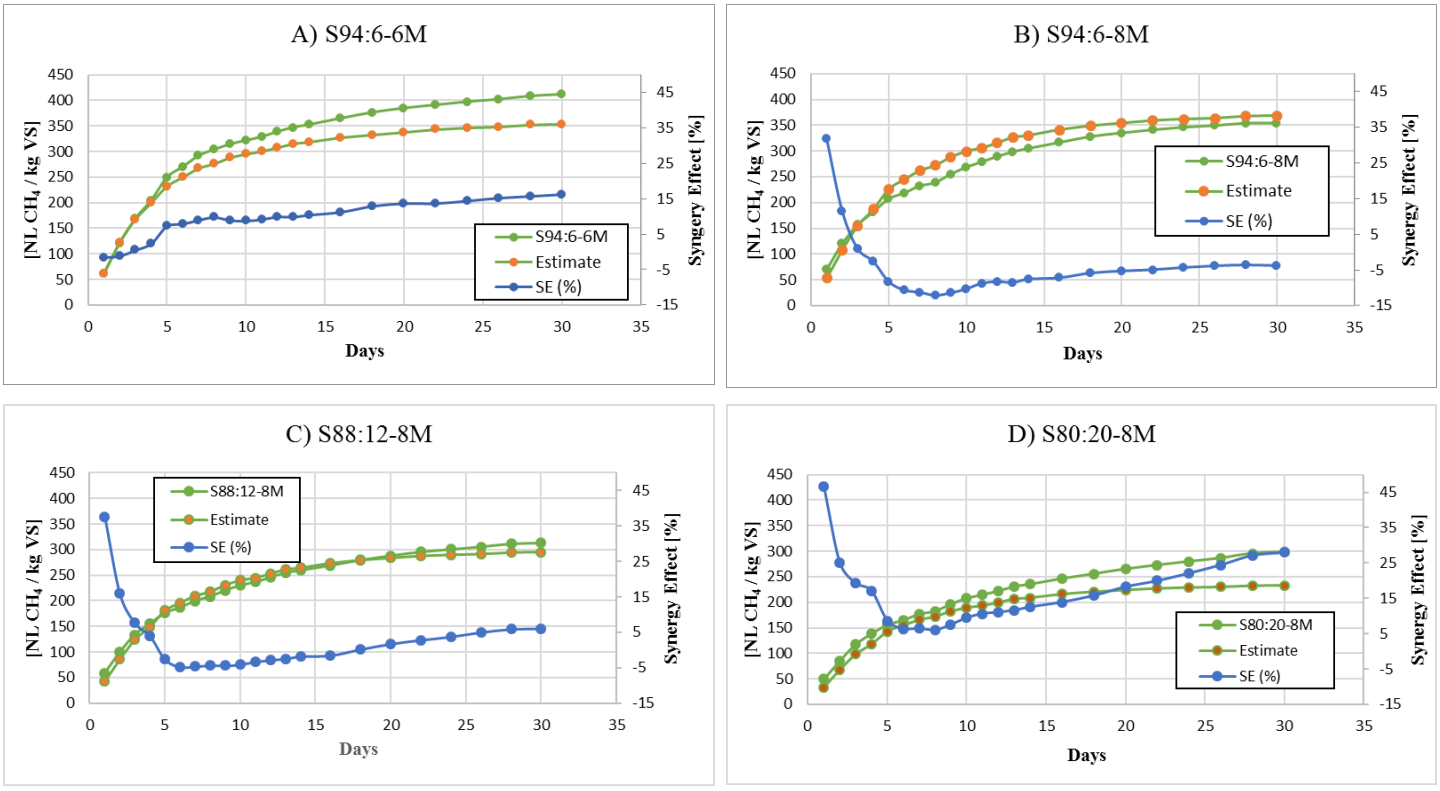

Fig. 13 - Comparison of synergistic effect. S (full green) is the cumulated BMP results; Estimate is the estimations from the first-order kinetics; SE is synergistic effect.

The 8 months ensiled samples are compared to see what effect the longest ensiling had on the different compositions. S94:6-6M is shown even though it is not a reliable result.

When looking at the Fig. 13B-13D it is clearly to see a small difference for 13B and 13C in the estimated and measured. This can also be seen on the synergistic effect, which is respectively –4% and 6%. These effects are seen as no effect, due to an experimental error of 10%. Therefore, these effects are not clear. Fig.15(D) shows a clear difference between the measured and estimated results. This corresponds to a synergistic effect of 28%. This is high, which indicates that S80:20-8M had beneficial effect of co-ensiling.

4.1.5. First-order kinetics analysis results of silage samples.

The first-order kinetics describes the speed of the biogas production through the hydrolysis constant (k day^{-1}). The constant describes the fraction of VS, which will disintegrate each day of the digestion. Therefore, the higher the constant is, the faster the digestion is. In Table 18 the results from the kinetics is shown. The constant is shown together with the Root-Mean-Square Error (RMSE), which shows how well the estimated data fits to the measured data. RMSE have the unit $\frac{NL\ CH_4}{kg\ VS}$, because it gives the average error of NL CH_4 per day.

Table 18 – First-order kinetics hydrolysis constant for Part I.

Sample Name	k (day^{-1})	RMSE $\left(\frac{NL\ CH_4}{kg\ VS}\right)$
S100:0-2M	0.235	1.383
S100:0-6M	0.247	0.892
S100:0-8M	0.207	0.953
S94:6-2M	0.206	1.875
S94:6-6M	0.177	1.818
S94:6-8M	0.165	2.322
S88:12-2M	0.195	2.172
S88:12-6M	0.215	2.676
S88:12-8M	0.158	2.166
S80:20-2M	0.193	2.086
S80:20-6M	0.183	2.146
S80:20-8M	0.137	2.188
S0:100-2M	0.035	0.282
Inoculum	0.090	0.310
Cellulose	0.139	5.996

The kinetic constant (*k* day^{-1}) varied widely from 0.035 to 0.247 day^{-1}. The higher kinetic constants were found in S100:0-2M, S100:0-6M and S100:0-8M and the lowest constant was pure wheat straw S0:100-2M, which was only xx (day-1). Furthermore, the results of kinetic analysis clearly indicate that when the biomass contains lignocellulose, the degradation of the biomass is slower, than if it only consists of mono sugars. The RMSE ranges from 0.282 to 5.996 $\frac{NL\ CH_4}{kg\ VS}$, which is considered reliable, indicating most of the estimations fit well to the estimated values. Visualisation of the constants are shown in Fig. 14.

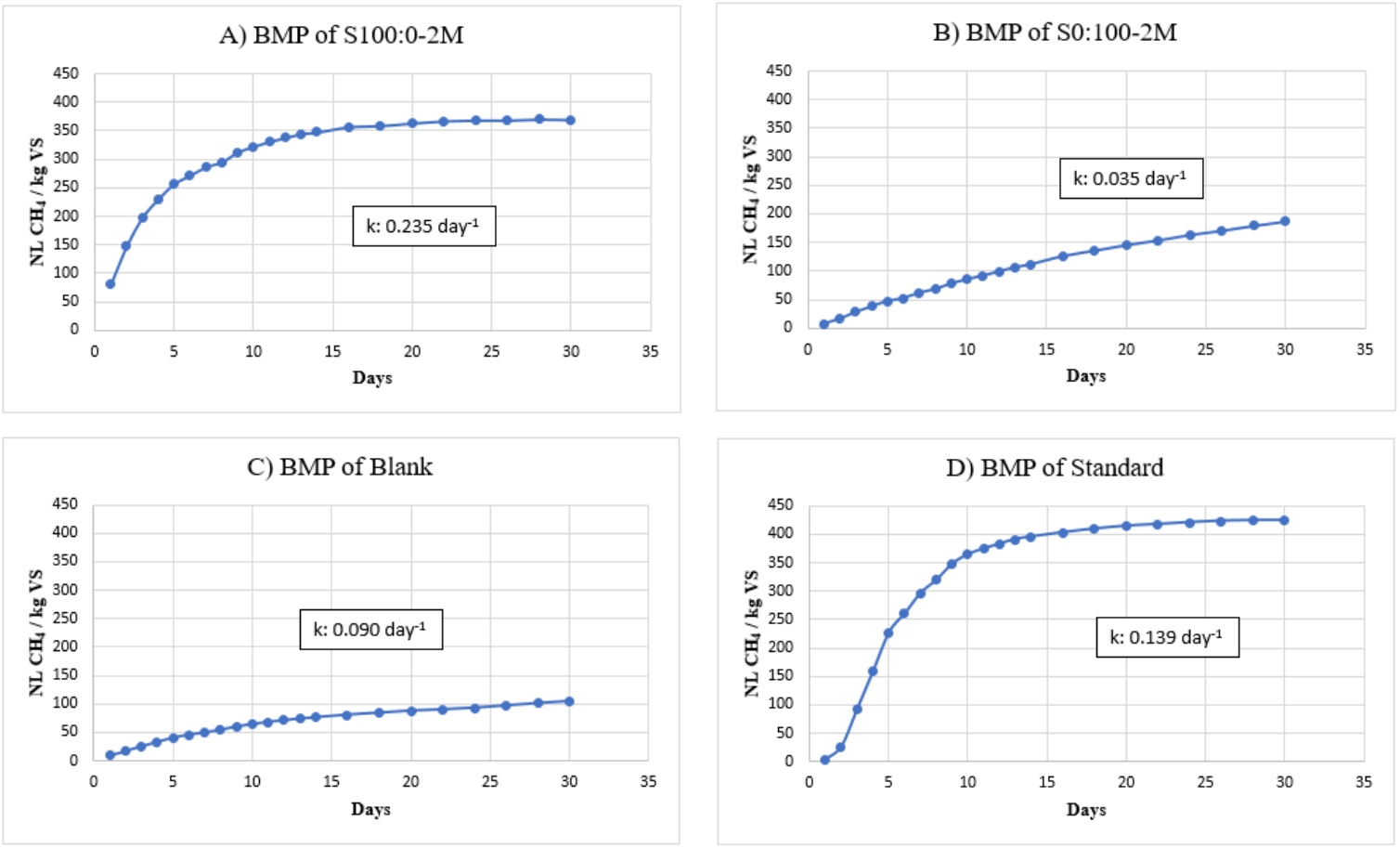

Fig. 14 – A) BMP of S100:0-2M. B) BMP of S0:100-2M. C) BMP of inoculum, standard bottle. D) BMP of microcrystalline cellulose, standard bottle.

Visualisation shows that the constant gives a good estimation of the speed of the biogas production. For Fig. 14A the constant is much higher than for Fig. 14B, which corresponds to the difference in the biomass. The reason that cellulose (Fig. 14D) have a high RMSE of 6 $\frac{NL\ CH_4}{kg\ VS}$, compared to Fig. 14B of 0.3 $\frac{NL\ CH_4}{kg\ VS}$, is the lag phase microcrystalline had in the beginning. This phase makes the estimations slightly less precise, but still very reliable.

4.1.6. Biodegradability of silage samples.

The effect of the ensilage storage on the different co-substrates has been examined with the biodegradability (BD). The biodegradability is listed in Table 19, and is based upon the difference in BMP and TBMP.

Table 19 - The biodegradability (BD) results from BMP/TBMP.

Sample Name	BMP $\left[\frac{NL\ CH_4}{kg\ VS}\right]$	TBMP $\left[\frac{NL\ CH_4}{kg\ VS}\right]$	BD [%]
S100:0-2M	369.40	439.61	84.03
S100:0-6M	412.52	447.32	92.22
S100:0-8M	443.58	468.25	94.73
S94:6-2M	304.49	566.99	44.41
S94:6-6M	411.07	NA*	59.95
S94:6-8M	354.07	549.52	64.43
S88:12-2M	291.16	621.31	46.86
S88:12-6M	307.36	NA**	49.47
S88:12-8M	312.88	649.97	48.14
S80:20-2M	253.93	596.05	42.60
S80:20-6M	244.34	640.27	38.16
S80:20-8M	298.58	NA***	46.63
S0:100-2M	186.68	660.44	28.27

*: This TBMP data is not available, so to access the BD has the data from S94:6-2M been used.

**: This TBMP data is not available, so to access the BD has the data from S88:12-2M been used.

***: This TBMP data is not available, so to access the BD has the data from S80:20-6M been used.

The BD increases when the ensilage period is increased from 2 months up to 8 months for sample; S100:0 and S94:6. For sample S88:12 is the BD increasing from 2 to 6 months for ensilage, while it decreases by a small amount from 6 to 8 months. The BD of sample S80:20 decreases from 2 to 6 months and increases again from 6 to 8 months.

A trend that can be observed in the BD is that the BD decreases, as the ratio of lignocellulosic straw increases in the co-substrate. Lignocellulosic biomasses as straw has a very low biodegradability, as explained in section 1.3.3, compared to biomasses as sugar beet. By comparing the BD of S100:0-2M and S0:100-2M, a difference of 56% can be observed, which underlines the low biodegradability of straw. Furthermore, the effect of the ensilage can be observed in the BD. The trend in the BD that can be observed for S100:0 and S94:6, where the BD increases when the ensilage period is increased, indicates

that the ensilage is effective for biomasses that contains large fractions of sugar beet. The same trend cannot be observed when the fraction of lignocellulosic biomass increases. The ensilage does therefore seem to have the highest effect of easy degradable biomasses.

4.2. Part II – Further pretreatment effect

4.2.1. Characterisation of tannery waste water

Tannery WW is a waste product from a tannery production. The water comes from the tannery production, where it has been used for hair removal of the hides. In the tanning process has lime ($CaCO_3$) been added to the tannery water, which increases the pH to 11-12. The tannery WW can be collected before it is cleansed, and used instead of chemicals in a pretreatment of lignocellulosic biomass due to its similarity to conventional used alkaline chemicals. Another factor that would also increase the biodegradability and BMP of the biomass is the concentration of enzymes that the tannery WW contains. These enzymes are protease, lipase, amylase and other industrial enzymes, which are added for the processing of the hides. Pretreatment with tannery waste water does therefore have potential as both a chemical pretreatment because of the pH and the content of lime and as a biological pretreatment because of the enzymes that it contains (Vazifehkhoran, 2016)

4.2.2. Overview of BMP for further pretreated samples.

Table 20 shows the BMP results of the pretreated samples, including two inoculums as a blank, to see the inhibition effect of tannery WW. The results are shown for day 10, 20 and 30 of BMP.

Table 20 - Overview of pretreatment BMP results from Part II.

Sample Name	CH_4 (10) $\left[\frac{NL\ CH_4}{kg\ VS}\right]$	CH_4 (20) $\left[\frac{NL\ CH_4}{kg\ VS}\right]$	BMP_{30} $\left[\frac{NL\ CH_4}{kg\ VS}\right]$	SD
S0:100-M	117.6	164.2	180.7	4.4
S0:100-T	129.4	185.4	214.8	11.7
S0:100-CT	135.0	195.7	223.6	18.3
S0:100-CCT	140.8	203.4	234.4	4.2
S80:20-8M*	207.6	265.0	298.6	10.1
S80:20-T	209.0	295.7	327.7	24.0
S80:20-CT	201.5	278.0	299.1	5.3
S80:20-CCT	213.9	298.2	327.5	1.4
Inoculum	34.1	50.5	63.5	0.1
Inoculum w. WW	33.0	49.7	63.9	4.4

*: BMP results from S80:20-8M used in Part I.

The BMP results from the pretreatment ranges from 180.7 $\frac{NL\ CH_4}{kg\ VS}$ to 327.7 $\frac{NL\ CH_4}{kg\ VS}$. The SD ranges from 0.1 to 24, which is lower than the SD for Part I, but still a bit high. As seen on BMP_{30} for S0:100-M to S0:100-CCT, there is a clear difference on the BMP, which also can be observed for S80:20-T to S80:20-CCT, which indicates that the pretreatment influenced the BMP.

A visualisation of the results is shown in Fig. 15, where the same sample with different pretreatments are compared, including the inoculum containing tannery WW.

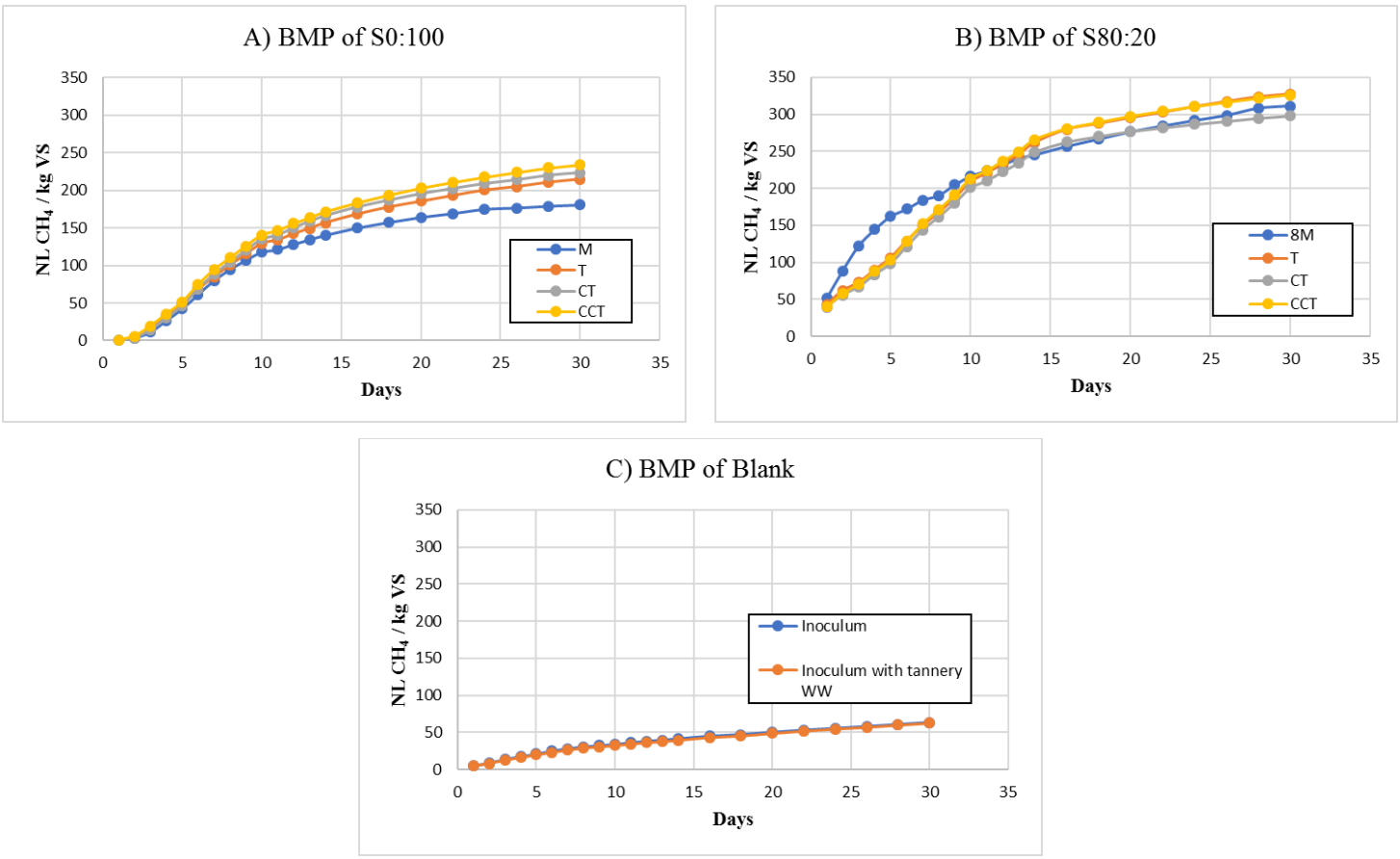

Fig. 15 – Cumulative methane production of (A) Straw; (B) Co-ensiled samples. (M) Milled; (T) Hydrothermal; (CT) Chemical before Hydrothermal; (CCT) Combined Chemical and Hydrothermal.

In Fig. 15, the combined pretreatment (CCT) is the one with the highest BMP both for the straw and the co-ensiled samples. This indicates that the combined pretreatment increased the BMP the most.

For the straw samples, the mechanical pretreatment (milling) is the one with the lowest BMP after 30 days. This is due to milling not affecting BMP, but mostly affects the production rate. The other pretreatment are clearly higher than this, which indicates the effect of further pretreatment than mechanical treatment will result in higher BMP.

Co-ensilage samples are not the same scenario as straw samples. The BMP for S80:20-8M, is put in to compare with. The effect of pretreatment on co-ensilage sample is not clear. The BMP seems to be alike, indicating these pretreatments (hydrothermal and chemical) may have similar effect as the co-ensilage.

Applying both pretreatments could result in needless pretreatment, but further experiments is needed to give clear results.

4.2.3. Effect of further pretreatment

In Table 21 the effect, based on the reference samples, is shown together with the BMP.

Table 21 - Overview of the pretreatment effects on BMP.

Sample Name	BMP $\left[\frac{NL\ CH_4}{kg\ VS}\right]$	SD	Effect [%]
S0:100-M	180.7	4.4	NA
S0:100-T	214.8	11.7	18.9
S0:100-CT	223.6	18.3	23.8
S0:100-CCT	234.4	4.2	29.7
S80:20-8M	311.4	10.1	NA
S80:20-T	327.7	24.0	5.2
S80:20-CT	299.1	5.3	-3.9
S80:20-CCT	327.5	1.4	5.2

The effect for S0:100 ranges from 18.9 to 29.7 %. To see the effect of pretreatment, the results must be compared to a reference. Since there was no non-treated straw used as a standard in this part of the project, the reference for the straw samples are the milled straw. Since all the samples are mechanical pretreated, the sample that was only milled were the least treated sample, and is used as a reference.
The effect for S80:20 ranges from -3.9 to 5.2 %. For the ensiled samples, the reference sample is S80:20-8M, which was used in Part I. S80:20-8M was not pretreated any other way than co-ensiling, and therefore is used as a reference for the ensiled pretreated samples. S80:20-8M did use inoculum, which were collected at a different time, which could have a small impact on the results. Another reason for using S80:20-8M as reference, is to see if the co-ensiling is enough pretreatment or if additional pretreatment will increase the BMP.

Based on the BMP visualisations in Fig. 16, the effects are as expected.

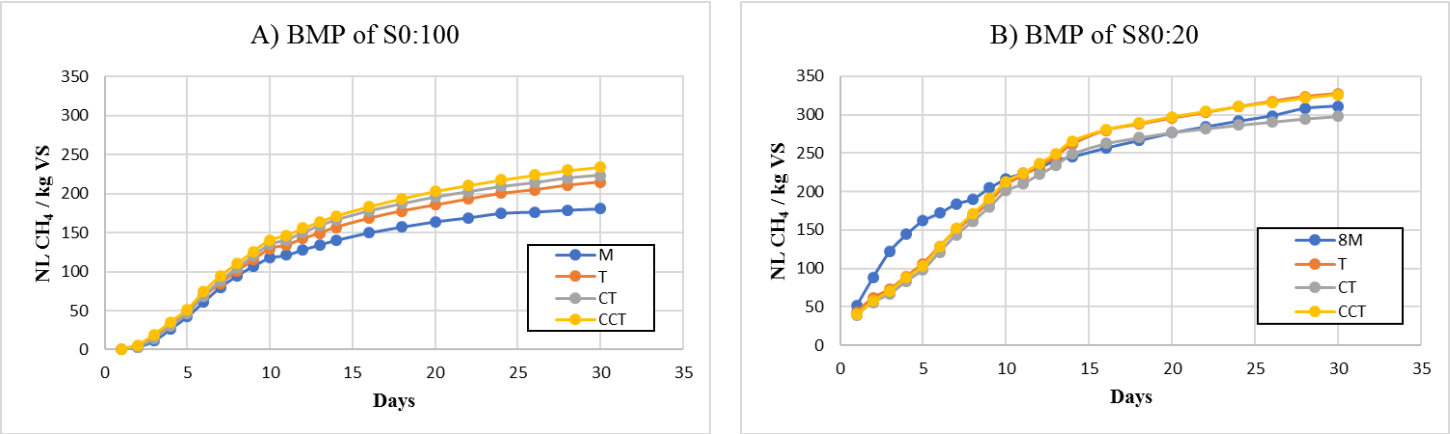

Fig. 16 – Cumulative methane production of (A) Straw; (B) Co-ensiled samples. (M) Milled; (T) Hydrothermal; (CT) Chemical before Hydrothermal; (CCT) Combined Chemical and Hydrothermal.

Fig. 16A, where it is straw, shows the effect of other pretreatments on the milled straw is very effective. An increase of 30% in BMP, is very high.

For the part of co-ensiled samples (Fig. 16B), the effect is indefinite. From -3.9% to 5.2% effect, is not a clear result – which corresponds to the visualisation where they all are alike in BMP.

This concludes that further pretreatment, other than milling, on straw gives a high improvement in BMP, whereas co-ensiled samples, do not need further pretreatment to increase BMP.

4.2.4. First-order kinetics analysis results of the further pretreated samples.

The kinetics for Part II, are conducted the same way as for Part I – using the same formula, but the BMP for pretreatment instead. Table 22 shows the results for kinetics.

Table 22 - First-order kinetics hydrolysis constant for Part II.

Sample Name	k day^{-1}	RMSE $\left(\frac{NL\ CH_4}{kg\ VS}\right)$
S0:100-2M*	0.035	0.282
S0:100-M	0.064	2.498
S0:100-T	0.055	2.462
S0:100-CT	0.055	2.832
S0:100-CCT	0.056	2.622
S80:20-T	0.081	1.749
S80:20-CT	0.088	1.945
S80:20-CCT	0.083	1.946
Inoculum	0.068	0.256
Inoculum w. WW	0.059	0.254

*: Results from S0:100-2M, used in Part I.

The constant ranges from 0.055 to 0.064 day $^{-1}$ for S100:0, and from 0.081 to 0.083 day^{-1} for S80:20. As seen in Table 22, S0:100-M to S0:100-CCT and S80:20-T to S80:20-CCT have nearly identical constants and RMSE. When seen the BMP results in section 4.2.1., this is expected, since the results do not vary a lot, but have the same visual shape. The RMSE ranges from 0.254 $\frac{NL\ CH_4}{kg\ VS}$ to 2.832 $\frac{NL\ CH_4}{kg\ VS}$ which is acceptable but still a bit high.

When comparing S0:100-2M with a constant of 0.035 day^{-1} with S0:100-M which have 0.064 day^{-1}, the milling has an impact on the hydrolysis speed. After milling the hydrolysis is almost twice as fast.

In Fig. 17, a visualisation of the kinetics model, with the measured and the estimated BMP for S0:100-CCT, is shown.

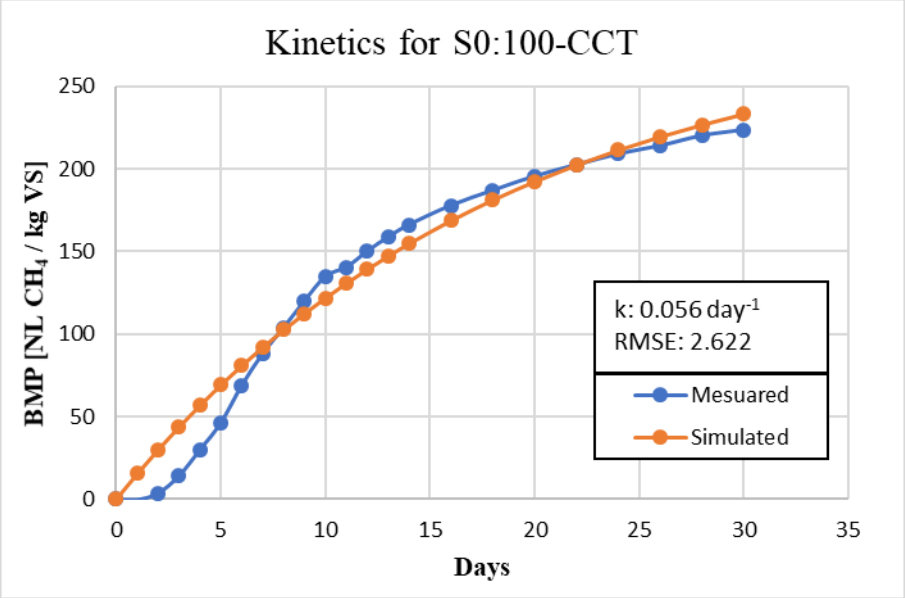

Fig. 17 - Visualiasation of RMSE and k-value for S0:100-CCT. Mesuared is values obtained from the BMP; Simulated is values from first-order kinetics.

As seen in Fig. 17 the estimated BMP show a fine first-order cumulative curve, the measured one show a slight sigmoidal form. This is due to the lag phase in the beginning (first 2-3 days), which is normal for slowly degradable biomass.

Comparing the two samples which were combined pretreated, the constants were 0.056 day^{-1} for S0:100-CCT and 0.083 day^{-1} for S80:20-CCT. The constants are both low, but there is still a difference between these. For S0:100-CCT it is lower than the ensiled sample, which were expected, due to it only being straw, which is pretreated. The ensiled sample does contain some sugar beet, which is easier degraded than straw, which will increase the constant.

When comparing both constants to S0:100-2M, which were used in Part I, the difference is quite noticeable. S0:100-2M have a constant of 0.035 day^{-1} which is very low compared to both S0:100-CCT and S80:20-CCT. The combined pretreatment have influenced the constant. An increase from 0.035 to 0.056 day^{-1}, indicates that pretreatment does affect the constant in a positive way.

4.2.5. Biodegradability for further pretreated samples

Other way to see the effect of further pretreatments, is to see if the biodegradability has increased, indicating more biomass have been degraded.

Since S0:100 is assumed to be alike the S0:100-2M that was used in Part I, and S80:20-8M is used, the TBMPs are already known. The results can be seen below in Table 23.

Table 23 – The biodegradability results of further pretreatments.

Sample Name	BMP $\left[\frac{NL\ CH_4}{kg\ VS}\right]$	TBMP $\left[\frac{NL\ CH_4}{kg\ VS}\right]$	BD [%]
S0:100-M	180.7	660.44	27.36
S0:100-T	214.8	660.44	32.52
S0:100-CT	223.6	660.44	33.86
S0:100-CCT	234.4	660.44	35.49
S80:20-8M	298.6	640.27	46.63
S80:20-T	327.7	640.27	51.18
S80:20-CT	299.1	640.27	46.71
S80:20-CCT	327.5	640.27	51.15

The BD for S0:100 samples ranges from 27% to 35%, while sample S80:20 ranges from 47% to 51%. It can be concluded that for ensiled samples there is no tendency throughout the pretreatment methods. And since they are overlapping with BD where they should increase throughout the methods, it concludes that there is no clear difference in BD for ensiled samples.

For S0:100 samples, there is a tendency for the BD to increase, throughout the different pretreatment methods. From only milled treatment to combined treatment, there is an increase of 8%. This is a clear difference, which also is reflected in the BMP difference that is 30%. Therefore, the further pretreatment has a high effect on the BD on S0:100 samples.

4.3. SEM topographic images

SEM topographic images was observed to see the effect of co-ensiling, chemical and hydrothermal pre-treatment on the surface structure of wheat straw and sugar beet. The pretreatment of wheat straw and sugar beet should cause structural changes in surface accordingly to the theory. The surface structure of lignocellulosic biomass, as wheat straw, will have a tight and ordered structure, compared to the surface structure of biomass as sugar beet, which structure will be random and unordered.

The obtained SEM images of untreated and pretreated straw can be seen in Fig. 18.

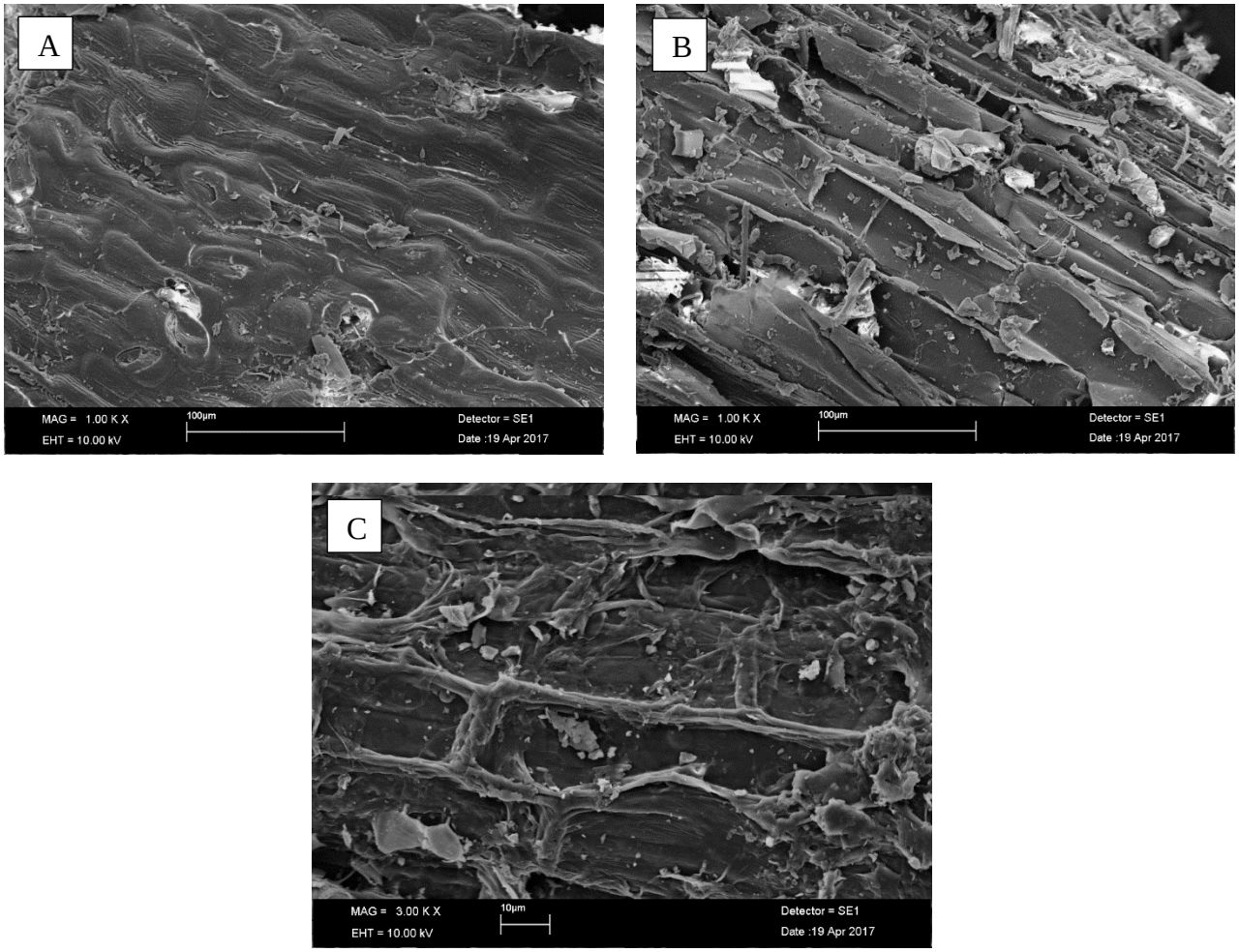

Fig. 18: SEM of (A) mechanical pretreated wheat straw; (B) ensiled wheat straw (6 months), which have been mechanically pretreated; (C) wheat straw pretreated with hydrothermal pretreatment at 121°C /1.2 bar for 30 min and chemical pretreated with tannery waste water in 24 h at 37°C.

The difference between the lignocellulosic straw and the sugar beet can be seen in Fig. 18A as the straw and Fig. 18A as the sugar beet. The sugar beet structure, which are more random and unordered, may be the reason why the biodegradability is higher for sugar beet compared to lignocellulosic biomasses as straw. The biodegradability is related to the tightness of structure, accessible surface area of the biomasses and the structural differences between sugar beet and straw, which are observed with the SEM images. This indicates that sugar beet is randomly structured and the accessible surface area is high for sugar beet.

The individual plant cells are visible on the SEM images of the wheat straw in Fig. 18, which can have the size of 10 to 100 µm (Blue, 2017). Furthermore, the cell walls can be observed, which have a size that varies from 0.1 to 10 µm (Islam, 2013). The cell wall can be observed on all the SEM images in Fig. 18, but especially in Fig. 18C, the cell walls can be observed, since this SEM image is more zoomed in.

The mechanical pretreated wheat straw in Fig. 18A got a tight and ordered structure. In contrast to the mechanical pretreated straw in Fig. 18A, ensiled (6 months) and mechanical pretreated straw in Fig. 18B have a loosened and fractured surface structure. Also at the mechanical, hydrothermal and chemically pretreated straw in Fig. 18C, the structural changes at the surface significant, and the cell walls are becoming fractured and destroyed. There is a thin film layer on the mechanical pretreated straw in Fig. 18A, which other studies (e.g. Yao and Chen, 2016) expect to be a wax layer. The ensilage and the combined chemical and hydrothermal pretreatment both destroy the waxy surface, which may increase the biodegradability of the biomasses.

There is therefore a significant effect on the surface structure at lignocellulosic biomass, as straw, when it gets either ensiled-, alkaline chemical- and hydrothermal pretreated.

The obtained SEM images of untreated and ensiled sugar beet can be seen in Fig. 19.

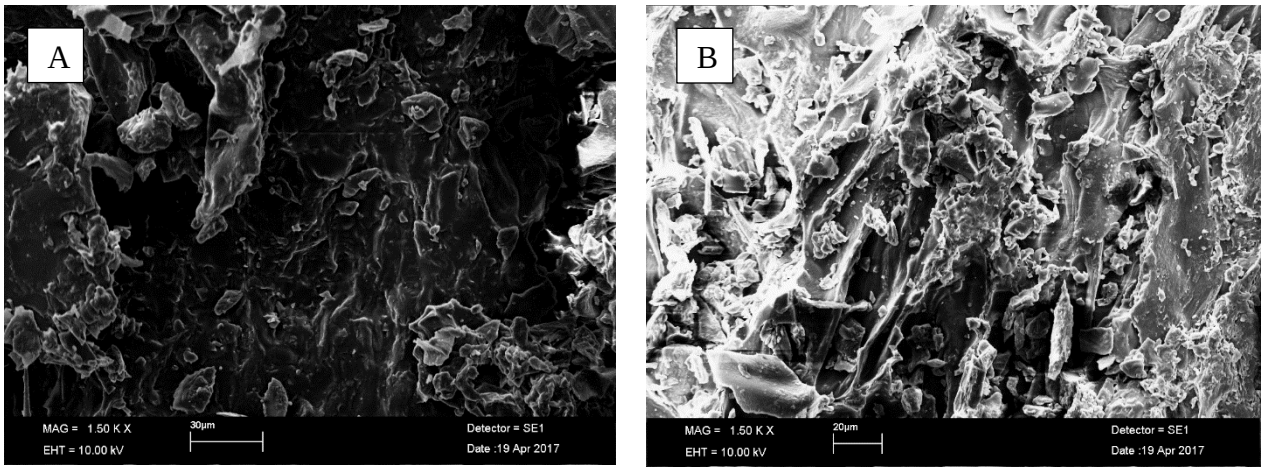

Fig. 19: SEM of (A) S100:0-6M, which has been mechanical pretreated; (B) S100:0-6M, which have been mechanical pretreated.

The sugar beet biomass has a high biodegradability, and it is therefore not desired to hydrothermal- or chemical pretreat it. The ensilage is however not energy or material requiring, so it may be applied to sugar beets. The effect of ensilage on the surface structure has therefore also been analysed with a scanning electron microscope. The random and unordered structure of sugar beet can be observed in Fig. 19A. A similar random and unordered structure can be observed in Fig. 19B, but the level of disorder is higher after the ensilage. This may indicate that there is a lesser ensilage effect on the structure, and would thereby result in a higher degradability (Lima *et al.*, 2013).

4.4. Results of FTIR-PAS spectroscopy analysis

The chemical changes were examined by using FTIR spectroscopy.

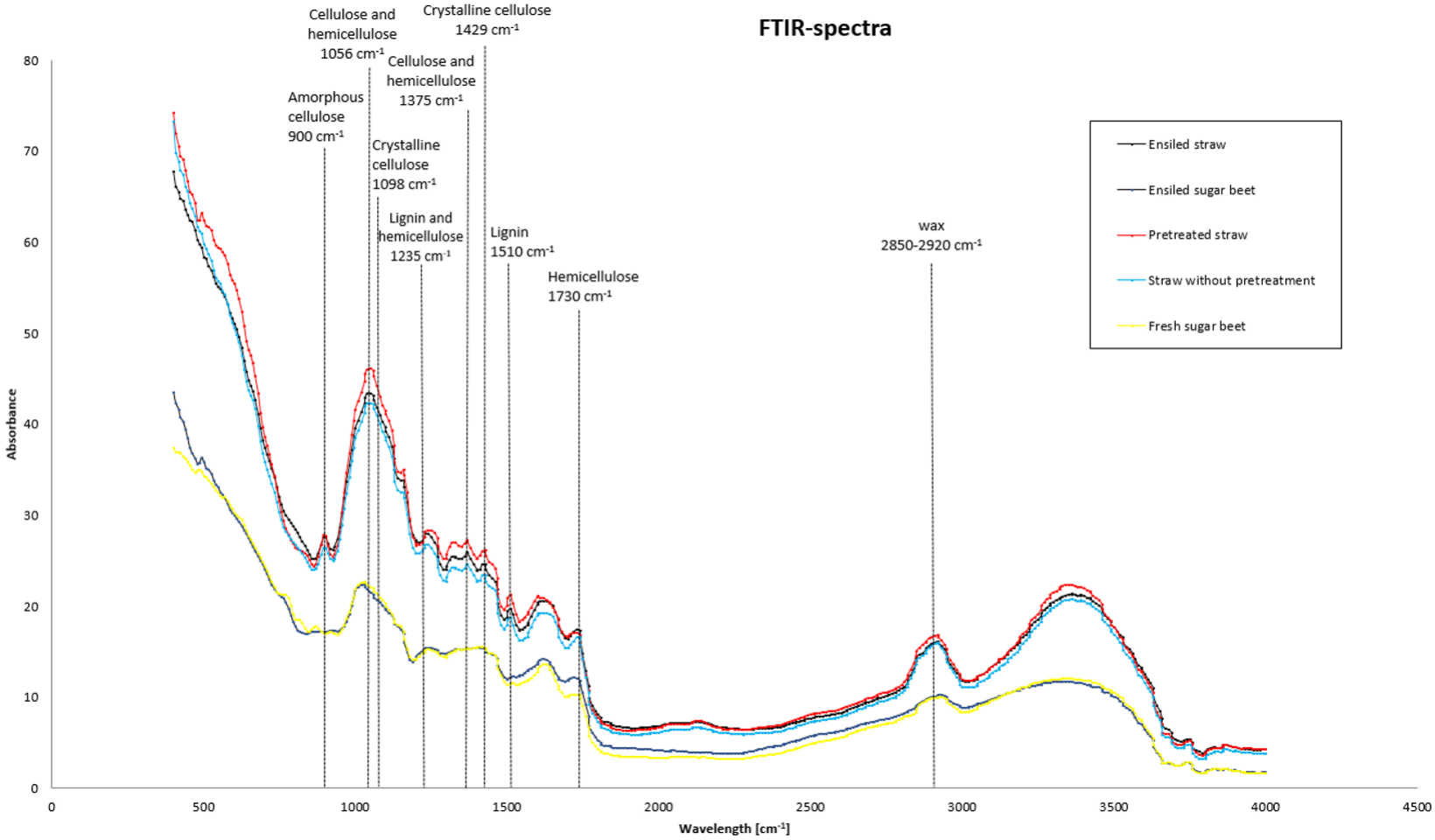

Fig. 20 – FTIR spectra of samples, for comparison of effect from pretreatment.

The FTIR spectra (Fig. 20) only indicated minor changes between the different pretreatments applied. A reason for the minor changes may be that the pretreated biomasses was not washed after the pretreatment was conducted. The lignin and hemicellulose would therefore not be removed and the chemical changes can therefore not be determined. The focus is therefore between the two biomasses; sugar beets and straw.

The peak at 1098 cm^{-1} and 1429 cm^{-1} refers to the crystalline cellulose bands, which is are larger for lignocellulosic straw than for sugar beet (Kristensen *et al.*, 2008). The amorphous cellulose had also been examined with the peak at 900 cm^{-1}. The FTIR spectra for sugar beets has no peak at 900 cm^{-1}, which indicates that the sugar beet contains no amorphous cellulose. This follows the theory about lignocellulosic having a larger crystallinity of cellulose, which decreases the biodegradability compared to sugar beets.

The peak at 1730 cm^{-1} (un-conjugated C=O stretch vibrations in hemicellulose), at 1056 cm^{-1} (C-O stretch for cellulose and hemicellulose) and at 1375 cm^{-1} (C-H deformation in cellulose and hemicellulose) are higher for straw than for sugar beet. The peaks in that interval are broader for sugar beet, which may indicate the easily degradable cellulose (non-crystalline) are more available in sugar beet, than in straw (Kristensen *et al.*, 2008).

The peak at 1235 cm^{-1} refers to the C-O stretch in lignin and hemicellulose. By comparing the sugar beet and straw, it can be observed that straw contains more lignin and hemicellulose than sugar beet, and thereby making straw less biodegradable. The peak at 1510 cm^{-1} refers to the aromatic skeletal from lignin, which can be observed at the FTIR spectra of straw, but not for sugar beet. The lignin content is one of the major reasons for the low degradability of straw compared to sugar beet, so the observed and increased lignin content for straw underlines the determined BMP changes between sugar beet and straw. The content of lignin can also be observed at the peak at 1329 cm^{-1}, which refers to the syringyl and guaiacyl condensed lignin (Li *et al.*, 2010).

Another factor which can decrease the biodegradability is the content of wax on the surface of the biomass. The broad peak at 2850 to 2920 cm^{-1} corresponds to CH_2- stretching from wax, which are higher for straw than sugar beet and therefore contributes to the lower biodegradability (Kristensen *et al.*, 2008).

5. Further discussion

5.1. Part I – Co-ensiling

Sample S94:6-6M

The TS and VS results shows that sample, S94:6-6M, with a concentration of VS of 178 g/kg is deviating from S94:6-2M and S94:6-8M, which both have 247 g/kg and 241 g/kg. This low VS compared to the other samples, are the same for the total easily degradable VS, which S94:6-6M have 11 g/L, where S94:6-2M and S94:6-8M have 19 g/L and 23 g/L. This indicates that S94:6-6M is an unreliable sample, because the lower VS will increase the BMP based on the VS, and thereby making an overestimating. BMP after 30 days for S94:6-6M is 411 $\frac{NL\ CH_4}{kg\ VS}$ which is much higher than both 305 $\frac{NL\ CH_4}{kg\ VS}$ and 354 $\frac{NL\ CH_4}{kg\ VS}$ for respectively S94:6-2M and S94:6-8M. This is due to the low VS, and it underlines that the sample is unreliable.

The synergistic effect for S94:6-6M was determined to 16%, but was not included in table 18, because it was considered unreliable. It was still used as the example in the data analysis calculations, so the deviation can be seen. By comparing the synergistic effect of S96:4-2M and S94:6-8M, which were -7% and -4% respectively, then the synergistic effect of 16% for S96:4-6M indicates that the sample is unreliable. The negative synergistic effect is due to experimental errors, which causes variation of the estimated BMP to the measured BMP.

5.2. Part II – Further Pretreatments

The microorganism lag phase

From the visualisation of BMP in the results of Part II, there is a lag phase for the microorganisms in the beginning of the BMP measurements, when comparing with the BMP results obtained in Part I. The reason that this lag phase occurs is due to the degassing of inoculum. When degassing the inoculum, the anaerobic digestion, which is caused by the microorganisms, is temporary stopped because there is no feedstock available and there are not absolute anaerobic conditions. Therefore, when adding a new feedstock to the microorganisms, then it takes time for the microorganisms to acclimate to the new conditions and continue the anaerobic digestion.

The first-order kinetic models do not take account for the lag phase either, which provides a more unreliable estimation that results in a lower hydrolysis constant. This can be observed at the RMSE values, which are high, because of the deviation of the lag phase.

Tannery waste water inhibition

The application of tannery waste water for the chemical pretreatment brings some problems along. It has to be examined if the pretreatment of the biomass with the tannery waste water inhibits the anaerobic digestion. The biomass had the tannery waste water removed from the biomass after the pretreatment, but the biomass was not washed before the anaerobic digestion began. The BMP of inoculum with and without tannery wastewater were measured to be 63.5 $\frac{NL\ CH_4}{kg\ VS}$ and 63.9 $\frac{NL\ CH_4}{kg\ VS}$ respectively. This excluded that tannery waste water could inhibit the microorganisms during the anaerobic digestion.

pH of the tannery waste water

The tannery WW used in this project was supposed to contain alkaline chemicals as e.g. lime. Further studies have reported a pH of 12 (Vazifehkhoran, 2016). The pH was measured to 9, which were far less alkalinity than expected. The expected pH was 12, which would most likely show a higher effect on the BMP and biodegradability, because the alkaline chemicals would enhance the altering of the lignocellulosic structure.

6. Perspectives

One of the advantages of using degassed manure and biomass for biogas production is that the digestated manure from the anaerobic digestion has lower methane emission, which is a major greenhouse gas, during storage. However, if the biomass is chemically pretreated with industrial waste water e.g. tannery waste water, it would contain diverse undesirable components, which could causes negative environmental effect. For example, heavy metals in tannery waste water would not be degraded biologically into non-toxic products. The use of digestate as fertiliser would therefore risk accumulating of heavy metals on the fields and thereby create a toxic environment.

The Danish legislation of the digestate is included in the livestock declaration (Retsinformation, 2015) under §3.9, which treats degassed manure that are mixed with vegetarian biomass and waste from biogas productions. The only limit values in this legislation are for phosphor and nitrogen, but not for heavy metals. This means that there is no legal restriction in using the digestate degassed together with industrial wastewater as fertiliser, but most likely not for long. On the 1st of April, it was proposed to the Danish Parliament that there should be implemented restrictions and limit values for heavy metals (Poll *et al.*, 2016) in manure and degassed biomass from biogas plants. A solution for removing the heavy metals must also be developed to utilise the digestate as fertiliser.

A solution to the problem of using the chemical pretreated digestate as fertiliser could be to wash off the tannery waste water after the pretreatment process and before the biomass is used in the anaerobic digestion. This would also allow to recycle the tannery waste water and use it for different batches of biomass to some extent. It would have been relevant to wash off the tannery waste water after the chemical pretreatment in the BMP experiments, so the BMP results would demonstrate the effect of this method and if the washing of the biomass had any effect on the BMP for the biomass. It must be expected that the biomasses, which have been ensiled contains alcohols and VFA, which could be washed out of the biomass with the tannery waste water. The application of the chemical pretreatment with tannery waste water should therefore be discussed before it is implemented.

Another problem that can occur when using agricultural biomass is if the biomasses have been sprayed with pesticides. The pesticides would be accumulated in the digestate, which either would create a toxic environment for the microorganisms in the anaerobic digestion, which would inhibit the production of biogas. Furthermore, would the digestate, which containing pesticides, be used as a fertilizer and thereby send the pesticides back to the fields, which also would create a negative effect.

7. Conclusion

The utilization of 2G biomasses for biogas is very desired due to its sustainability. Denmark produces large amounts of cereal, which are the source to the waste product; straw. The straw has a good potential for biogas production, but because of its lignocellulosic structure is it hard to degrade into biogas. The aim of the thesis was therefore to find innovative pretreatments of lignocellulosic biomass, and the conclusions of the thesis are:

- The mass and energy loss from the ensiling storage and pretreatment method was highest for mono-ensiling of sugar beet, whereas increasing the co-ensiling with sugar beet and straw would result in lower VS losses, because of the higher dry matter concentration.
- BMP increased during the ensilage period, which indicated that 8 months of ensilage gives a higher BMP than 2 month of ensilage. This was both seen for mono- and co-ensiling samples.
- The thesis shows that the lignocellulose had a great impact on the hydrolysis constant and biodegradability factor. Sugar beet, which was mono-ensiled for 2 months, had a hydrolysis constant of 0.235 day^{-1} and a biodegradability of 84.0%. Straw, which was mono-ensiled for 2 months, had a hydrolysis constant of 0.035 day^{-1} and a biodegradability of 28.3%.
- The highest synergistic effect of co-ensiling samples, were observed for S80:20-8M which was 28.0%.
- Further pretreatment on straw samples, showed a great effect in using combined hydrothermal and chemical pretreatment, compared to only mechanical pretreatment. This can be seen, with an increase in BMP of 29.7% and an increase in biodegradability to 35.5%.
- The hydrolysis constant of further pretreatment on straw is improved compared to the mono-ensiled straw. Mono-ensiled straw had 0.035 day^{-1} and mechanical pretreated straw had 0.064 day^{-1}, which is almost twice the hydrolysis constant. The other pretreatments on straw, had nearly the same hydrolysis constant as mechanical, indicating only milling influenced the constant.
- Further pretreatment on co-ensiled samples showed no clear effect. With an experimental error of 10%, the effect of these pretreatments compared to co-ensiling alone, gave unclear results. This indicates that the co-ensiling alters the structure like the further pretreatments, and therefore are no clear difference observed in the BMP. The altering of the structure was therefore analysed with SEM.

- SEM showed that the structure was destroyed and more fractured after co-ensiling or combined hydrothermal and chemical pretreatment for lignocellulosic straw, but no physic changes could be observed for the sugar beet. Therefore, the effect of pretreatments is more distinct on straw than sugar beet.

This BSc project highlights that co-ensiling has a great potential to increase biogas production of lignocellulosic biomass such as cereal straw, and it confirms a potential of industrial wastewater as alternative pretreatment media replacing costly chemicals.

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Appendix I

Overall Data

This is an excel file, which contains an overview of all the important data.

BMP

For calculating BMP, there has been 2 approaches.

1. Calculating Biogas
2. Calculating Methane

This account for both Part I and Part II.

Biogas		
Sheet No.	**Sheet Name**	**Description**
1	"Gas ML"	The raw measured gas.
2	"Total Gas"	The cumulative measured gas.
3	"Dry Gas (Normal State)"	Biogas converted to normal standards, accumulated.
4	"Dry Gas (Non Accu.)	Biogas converted to normal standards, not accumulated.
5	"Visualization"	Shows the accumulated normalised biogas measured.
6	"Gas wo. Ino"	The measured biogas, without inoculum.
7	"g → kg + VS"	Data of samples (e.g. VS, amount sample measured)
8	"NL gas ~ kg w.w."	Biogas calculated per kg w.w..
9	"NL gas ~ kg VS"	Biogas calculated per kg VS.

Methane		
Sheet No.	**Sheet Name**	**Description**
1	"Gas ML"	The raw measured gas.
2	"Dry Gas (Normal State)"	Biogas converted to normal standards, accumulated.
3	"Dry Gas (Non Accu.)"	Biogas converted to normal standards, not accumulated.
4	"GC"	The methane composition
5	"Methane"	Non-accumulated biogas timed the methane concentration.
6	"Total Methane"	Accumulated "Methane" sheet.
7	"Visualization"	Shows the accumulated normalised biogas measured.
8	"Gas wo. Ino"	The measured biogas, without inoculum.
9	"g → kg + VS"	Data of samples (e.g. VS, amount sample measured)
10	"NL CH_4 ~ kg w.w."	Biogas calculated per kg w.w..
11	"NL CH_4 ~ kg VS"	Biogas calculated per kg VS.
12	"STD Methane kg VS"	Calculation of Standard Deviation for each sample.

Physiochemical

This excel account for all data regarding physiochemical data.

Physiochemical Analysis		
Sheet No.	**Sheet Name**	**Description**
1	"TS & VS"	Calculations of corrected TS & VS + VS Loss.
2	"Elementary Analysis"	Calculation of TBMP.
3	"Alcohol & Sugar"	Alcohol and sugar results from HPLC.
4	"VFA"	VFA results from GC.
5	"VFA Standard curve"	Standard curve based on the VFA results.