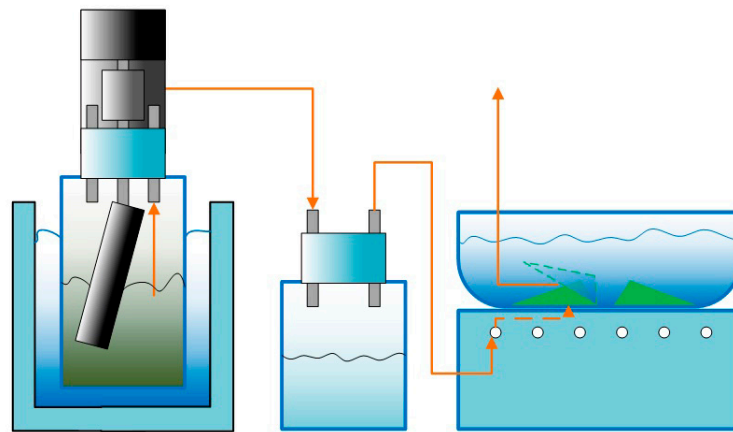# Development of characterization methods for lignocellulosic biogas substrates

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Development of characterization methods for lignocellulosic biogas substrates

by

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Preface

When starting my education at Lund University over five years ago, I thought I would use my future title as an Engineer in Biotechnology to grow artificial organs, solve crime scenes or possibly cure cancer. Now I know that the work of a bioprocess engineer involves so many other fields, equally as important. After finding a new interest in environmental sustainability research, I can now proudly present a master thesis that have given me an introduction to the many challenges and struggles such researchers have to battle. Biogas might not be the only solution to the environmental issues that today's society faces, but it can be a piece of the puzzle.

This master thesis has been carried out at the Department of Chemical Engineering in Lund, Sweden. Thank you to Åsa – for endless guidance throughout this project and for reminding me to focus on one thing at the time; to Ola – for the time you have spent on discussing everything from minor ideas to bigger problems with me; to Birgitta for performing the BET-analysis for me; to Hamse, Balazs, Fredrik and Johan – for always be willing to help out when I have been lost in the labs and to everyone else at the department who have been supportive, positive and made this experience a great one.

Abstract

As the need of environmentally friendly alternatives to fossil fuels is growing bigger, so is the need for knowledge and research around possible new raw materials that could serve that purpose. One discussed product is biogas. Since biogas can be produced from not only food waste and wastewater residue, but from any substrate that contains fats, proteins and/or carbohydrates, with the help of degrading microorganisms, there is a new interest for using lignocellulosic biomass such as straw as a raw material. The biggest issue with using lignocellulose is its low bioaccessibility to enzymes because of its crystalline structure, where cellulose polymers are stabilized by hemicellulose and lignin polymers. Furthermore, straw contains a lot of trapped air which causes it to float on the liquid surface in the fermenters, minimizing the contact surface area towards the degrading enzymes. Different mechanical pre-treatment methods for the straw aim to solve that problem. In order to facilitate the optimization of these pre-treatment methods, there is a need for a more time efficient characterization method than the biochemical methane potential tests that are usually run for 30-50 days, to determine the methane yield. This master thesis investigated whether enzymatic hydrolysis for 3 days of different straw samples would show the same tendency in how much cellulose and hemicellulose that were degraded to soluble sugars, as in how high the methane productivity of those samples would be. Wheat, rye, barley and canola straw as well as granulated wheat straw (achieved by extrusion) were analysed. The results showed a correlation between the methane yield and the xylan/xylose digestibility indicating that an increase in density, particle size and total surface area also generated an increase in cumulative methane yield and digestibility of xylan to xylose. To investigate this characterization method further, extensive statistical analysis should be performed on more samples.

Sammanfattning

I takt med att behovet av miljövänliga alternativ till fossila bränslen blir allt större, växer även behovet av kunskap och forskning kring nya råvaror som kan omvandlas till biobränslen. En omdiskuterad produkt är biogas. För att framställa biogas krävs ett råmaterial innehållande antingen fett, protein och/eller kolhydrater som metaboliseras av tillsatta mikroorganismer under syrefria förhållanden. Vid biogasframställning får inte framtagandet av råvaran konkurrera med livsmedel varvid den största delen biogas produceras från matavfall och avloppsrester. Nu undersöks även halm som en potentiell energikälla på grund av dess höga kolhydrathalt där den största andelen är bundet i kristallina polymerkomplex; lignocellulosa. Det största problemet med att använda lignocellulosa är dess låga biotillgänglighet. På grund av sin kristallstruktur, där cellulosapolymerer stabiliseras av hemicellulosa- och ligninpolymerer, förhindras enzymerna, vilka endast klyver de icke-kristallina delarna av lignocellulosan, från att bryta ned polysackariderna till mindre sockerarter. Vidare innehåller halm mycket luft som får den att flyta upp till vätskeytan inuti fermentorerna, vilket minimerar dess kontaktyta till de nedbrytande enzymerna. Det finns olika mekaniska förbehandlingar för halmråvara som syftar till att lösa det problemet. För att underlätta optimeringen av dessa förbehandlingsmetoder finns det ett behov av en mer tidseffektivt karaktäriseringsmetod av råvaran. Vanligtvis utförs biokemiska metanpotentialtester som körs i 30-50 dagar för att bedöma kvalitén av en råvara. Detta examensarbete har undersökt huruvida enzymatiska hydrolystester (som tar 3 dagar att köra) skulle kunna vara en alternativ karaktäriseingsmetod. Detta gjordes genom att nedbrytbarheten av cellulosa och hemicellulosa i olika halmprov (uppmätt via enzymatisk hydrolys) jämfördes med metanpotentialen (uppmätt via biokemiska metanpotentialtest) från samma halmprov. Om likartade tendenser (ökningar/minskningar) av dessa två parametrar kunde detekteras mellan olika halmprover, så skulle det påvisa en möjlig korrelation. Vete-, råg-, korn- och rapshalm samt granulerad vetehalm (vilket uppnåddes via extrudering) analyserades. Resultaten påvisade en korrelation mellan metanutbytet och xylan/xylos-nedbrytbarheten, dvs. hur väl hemicellulosan i provet hade brutits ned. Detta samband indikerade att en ökning av densitet, partikelstorlek och total yta av halmen också genererade en ökning av metanutbytet och nedbrytbarheten av xylan till xylos. För att stärka och undersöka denna karaktäriseringsmetod ytterligare, bör omfattande statistiska analyser utföras på ännu fler prover.

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1 Introduction

The search for environmentally friendly fuels to replace fossil ones is an ongoing challenge. Nowadays there are plenty of alternatives: biodiesel, bioethanol, biogas etc. Despite carrying a promise of having a much reduced impact on global warming, it is not yet enough to compete with energy potential in fossil fuels. In order to reduce the production costs, the biofuel production processes have to be optimized and further possible reagents and substrates have to be investigated. In the case of biogas, the substrate can be anything that contain carbohydrates, proteins or fats. Recently, straw has been investigated as a possible substrate for such a process. As straw is a by-product from the agricultural industry, it does not compete with the food industry. Straw is also investigated for bioethanol production but for that process to be feasible, the productivity has to be rather high. If the straw was instead/also used for biogas production, the process could be placed locally which would decrease the transportation costs.

1.1 Short project background

A research group at the Department of Chemical Engineering, at Lund University, was recently introduced to a project initiated by TK Energi. The project is called *"Increased biogas yield from straw, straw bedding, and other agricultural waste"* (referred to as the BioYield project) and is funded by the Energy Technology Development Programme (EUDP) by the Danish Energy Agency. The project aims to investigate a new mechanical pre-treatment method for straw raw material in biogas production processes. This new method will supposedly be less energy demanding and produce a fine powder out of the straw. When the powder is later led into the biogas fermenter, the smaller straw particles will mix more easily with water, hence increase their bioavailability for the digesting enzymes. In order to optimize this pre-treatment method, the affected parameters have to be studied in regard of how much they are correlated to the cumulative bio-methane potential, sugar yield being the main parameter.

1.2 Aim

The main objective with this master thesis was to investigate the following hypothesis:

> A higher sugar yield from enzymatic hydrolysis of straw indicate that a higher methane yield from anaerobic digestion of the same straw could be expected.

Consequently, the main aim was to confirm or reject this hypothesis. The general objective of this master thesis was to further investigate the correlation between different properties in straw, under various conditions, and the amount of biogas that can be produced out of that raw material. Thereby, the general aim was to propose time efficient characterization methods for lignocellulosic biomass.

1.3 Scope

This master thesis included a literature review on lignocellulosic biomass, with the focus on straw, as well as practical experiments in lab scale, in order to confirm or reject the poised hypothesis. The practical experiments consisted of batch biochemical methane potential (BMP) tests, enzymatic hydrolysis, fibre analysis (NREL), buoyancy tests and pore size measurements. All experiments were conducted on straw. To revise abbreviations that have been used in this report, see Appendix 1: List over utilized abbreviations. A popular science summary of the project can be found in Appendix 7: Popular science summary (Swedish).

2 Literature review

2.1 From biomass to biogas

Biogas is a mixture of mainly methane (CH_4) and carbon dioxide (CO_2) gas. The higher the methane concentration, the higher energy potential of the biogas (since carbon dioxide is not combustible). Combustion of methane is an exothermal reaction which is why biogas can be used as fuel for engines or heating (Adelekan, 2012).

In order to produce biogas, some kind of organic substrate is incubated in a fermenter together with a mixture of different suitable microorganisms. This substrate is usually a waste product from an industry such as sludge from wastewater treatment plants, expired aliments from food industries and agribusinesses (Anon., u.d.). Production of biogas can therefore be regarded as a recycling process. During the incubation, the carbohydrates, proteins and fats in the substrate are degraded into biogas by the microorganisms at anaerobic conditions; so called anaerobic digestion (AD) (Björnsson, et al., 2014), (Adelekan, 2012). The mix of microorganisms and substrate is usually referred to as biomass.

The biogas production process can be designed in multiple ways and operated under many different conditions, depending on what type of substrate that is digested. First, the substrate raw material is usually pre-treated with the purpose to increase its bioaccessibility for the digesting enzymes produced by the microorganisms. This is often achieved by altering the particle size of the raw material, diluting it in water to a certain total solids content (TS) and/or increase the solubility of the polysaccharides by applying high temperatures (Hendriks & Zeeman, 2009). If the total solids content (TS) of the biomass is higher than 15% the process is regarded as a solid-state anaerobic digestion (SS-AD) process (Yang, et al., 2015).

Then, the pre-treated raw material is led into the fermenter together with a suitable inoculum. The fermentation is mostly run at mesophilic temperatures (20-45°C). Methane production from biomass happens via four different reaction pathways, namely hydrolysis, acidogenesis, acetogenesis and finally methanogenesis. Different organisms are more active during different reaction steps such as methanogens are responsible for the methanogenesis. It is therefore crucial to the biogas production that the inoculum provides the right kind of microorganisms. The microorganisms excrete enzymes that catalyses the reactions (Montgomery & Bochmann, 2014).

When producing biogas, there are two outlet streams from the fermenter. One contains biogas which can be further purified to natural gas standards and analysed via gas chromatography (Garcia-Peña, et al., 2012). The solid state is called the digestate and contains, besides microorganisms, indigestible compounds, such as lignin and minerals, but also substrate that has not been degraded. Digestate can therefore be utilized as for example fertilizer because of its high nutrient content (Makádi, et al., 2012).

In full scale industries, the biogas production is usually continuous whilst laboratory experiments mostly are executed as batch tests. During continuous biogas production, the system operates at steady-state under optimized conditions. The retention time is regulated by the flow rate. A batch system however, has a differentiating methane productivity meaning that because the amount of substrate is not kept constant, the methane production rate varies (Anon., u.d.).

2.2 Lignocellulosic biomass

Lignocellulose is a polysaccharide complex stored in the cell wall of plant cells and form long fibre tubes. One fibre consists of multiple cellulose molecules that bind together in crystalline structures with hydrogen bonds and van der Waals forces. Multiple tubes are held together by lignin and hemicellulose, see Figure 1 (Rubin, 2008).
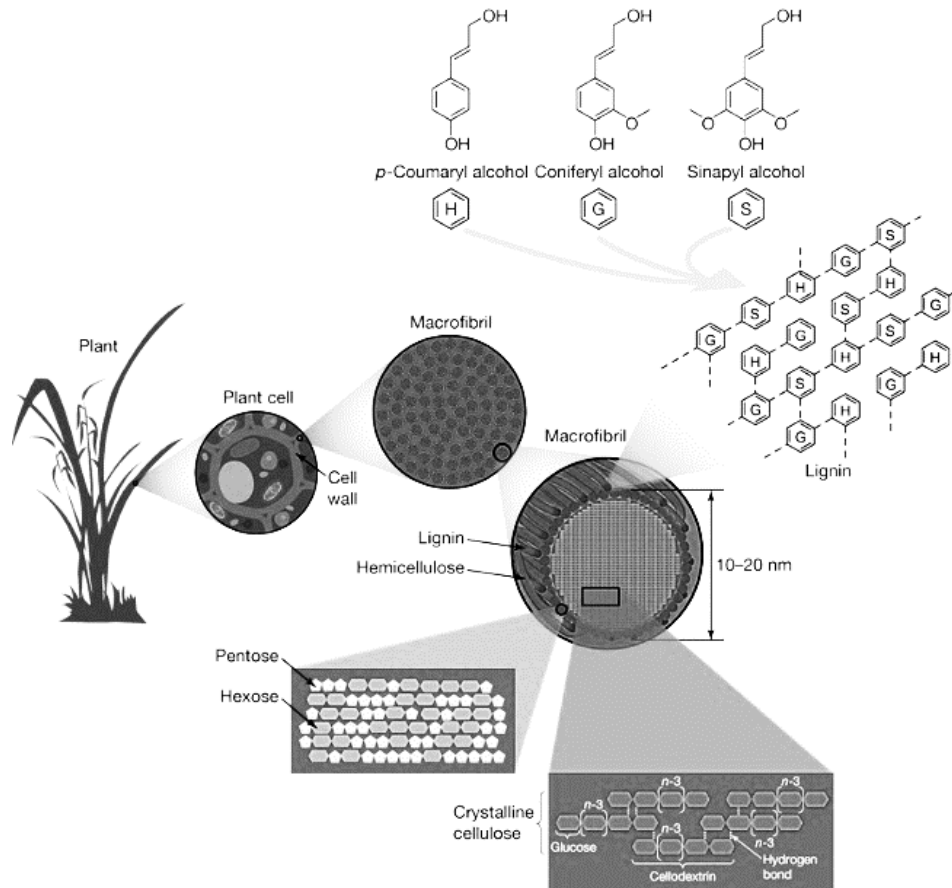
Figure 1. How cellulose, lignin and hemicellulose are structured in a plant cell wall (Rubin, 2008). Published with kind permission from Nature Publishing Group.

Cellulose is a polysaccharide that only contains glucose molecules. The glucose molecules are bound to each other via β(1-4) linkages, creating very long and straight polymers also called β-glucan. It is because of this linkage that the different cellulose polymers can be layered so closely together inside the plant. Starch, which is also a glucose based polysaccharide, does not have the same linear shape since its monomers bind together via α(1-4) creating a more spiral shaped form. Because of this difference in molecular structure, the cellulose is stronger and acts as the plants backbone (stem) while the starch is less stable and acts as the plants energy storage (grains) (Anon., 2011).

The hemicellulose is usually amorphous (non-crystalline) with branches of various polysaccharides, in contrary to the cellulose which is mostly crystalline. Lignocellulose can be found in all plants and is therefore an easily accessible substrate for ethanol and biogas production. Its high concentration of polysaccharides makes it especially interesting. But, in order to make the energy potential more available for conversion into for example methane, the polysaccharides

have to be cleaved into smaller sugars. Only the amorphous parts of the polymers can be hydrolysed. However, the integrated lignin prevents most of the cellulose and even hemicellulose to be degraded since their compact structure is a big steric hinder for the degrading enzymes (Yang, et al., 2015). Therefore, the lignocellulose first has to be broken down into smaller pieces, increasing the bioavailability. This can be achieved by utilizing different pre-treatment methods on the raw material. However, some pre-treatment methods will also increase the formation of inhibitory compounds (Berglund Odhner, et al., 2012).

The third main compound in lignocellulose is lignin. Lignin is a water insoluble polymer made up of different aromatic alcohol groups, see Figure 1 . Because of its cross-linkages between the different alcohol groups, the lignin forms a fibrous matrix making it resistant to digestion into methane by microorganisms (Anon., u.d.). These properties make lignin a possible inhibitory factor of methane production when lignocellulose is used as a raw material (Hendriks & Zeeman, 2009).

2.3 Straw

Straw is the stem of for example wheat or maize crops and can be classified as a waste product from the agricultural industry. As it is now, straw is mainly burned and transformed to electricity and/or heat in thermal plants. In Sweden and Denmark, the annual amount of straw utilized for this purpose measure approximately 100,000 tons and 1.5 million tons, respectively. However, Sweden could increase that amount with a factor ten (Anon., 2005).

Straw is mostly composed out of lignocellulose; cellulose, hemicellulose and lignin. Apart from those compounds it also contains some proteins, sugars, organic acids, ash, wax and little to no lipids (Björnsson, et al., 2014). Because the crops grow under multiple unique conditions, it results in a wide diversity when it comes to the quantitative composition of straw. Wheat straw that is cultivated at one place does not necessarily have the same chemical composition as wheat straw cultivated at a different location. The composition also depends on what time of year that the crop has been harvested, which fertilizers that have been used, how much sun the crops have been exposed to etc.

2.4 Metabolic pathway for microbial fermentation of lignocellulose

The digestion of lignocellulose, through anaerobic fermentation can be divided into four different steps; the hydrolysis, acidogenesis, acetogenesis and methanogenesis. The hydrolysis is an extracellular reaction catalysed by excreted enzymes (cellulases) from microorganisms present in the inoculum, whilst the other reaction steps are intracellular. The hydrolysis and acidogenesis have a pH optimum around 4-6 meanwhile the methanogenesis is favoured at a higher pH level around 6.5-8 (Montgomery & Bochmann, 2014).

2.4.1 Hydrolysis

During the hydrolysis, the longer polymers of protein, hemicellulose/cellulose and fat are cleaved into smaller amino acids, sugars and fatty acids, respectively, by the addition of water. The reactions are catalysed by hydrolysing enzymes. Lignocellulosic materials provide hemicellulose and cellulose as substrates for these reactions. As mentioned earlier, the lignin is not hydrolysed and degraded be the present microorganisms and can therefore not be regarded as a substrate. Because of the complex structure of the lignocellulose, the hydrolysis is often regarded as the limiting step in the methane production process (Montgomery & Bochmann, 2014).

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2.4.2 Acidogenesis and acetogenesis

During the acidogenesis, the monomers produced during the hydrolysis are further converted to volatile fatty acids (VFA) such as butyric, acetic, propionic, valeric, caproic and lactic acid. These acids lower the pH value, creating an acid environment. An important side product to the acidogenesis is gaseous hydrogen. Measurements of the hydrogen yield from the acidogenesis can indicate which reactions have been favoured (Motte, et al., 2013).

The acetic and butyric acids are most easily degraded and later converted to methane during the acetogenesis and methanogenesis. A molar butyric/acetic acid ratio of 1.5 is often desired when optimizing the microbial conditions in a biogas production process (Motte, et al., 2013). If the longer VFAs are dominantly produced, it could instead indicate and/or lead to inhibited methane production.

2.4.3 Methanogenesis

The last step in the biogas synthesis process is the methanogenesis. In this reaction, the acetate generated in previous steps is converted to methane and carbon dioxide. The carbon dioxide can in turn also be converted to methane, in the presence of hydrogen, but the acetate gives the higher and dominating methane yield. The stoichiometric reactions are showed below (Seifert, et al., 2012):

$$CO_2 + 4\,H_2 \rightarrow CH_4 + 2H_2O$$

$$CH_3COOH \rightarrow CH_4 + CO_2$$
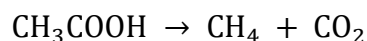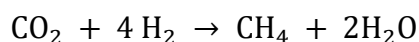
Lignocellulosic biomass does not usually contain any microorganisms that can perform the methanogenesis; so called methanogens. It is therefore crucial to the methane production process to utilize an inoculum with a high concentration of such organisms (Yang, et al., 2015).

2.5 Enzymatic hydrolysis of lignocellulose

Instead of obtaining hydrolysis of the substrate by the addition of microorganisms, pure enzymes of known character can also be added as a catalyst. The biogas process is very similar to an ethanol production process. The main difference is that ethanol can only be produced from glucose and xylose and not from proteins or fats. During ethanol production, the lignocellulose is first hydrolysed by the addition of a mix of enzymes. Therefore, the type of hydrolysing enzymes needed for ethanol production is quite singular. These enzymes can be purchased directly from a manufacturer. After the hydrolysis, the glucose is then led into a fermenter where yeast cells convert it into ethanol. To hydrolyse fats and proteins, a bigger variety of enzymes are needed which is why the enzymatic hydrolysis method is not used as a supplement to microorganisms in the biogas industries, but occasionally as a complement.

When designing biogas processes, many biomass characteristics and their effect on the methane yield, retention time and energy demand are investigated. However, these kind of experiments are very time consuming since the fermentation often take more than 30 days to perform. Enzymatic hydrolysis on the other hand, can often be achieved within 48-72 hours. If the sugar yield, produced by enzymatic hydrolysis of a lignocellulosic biomass, could be linked to the same parameters and show similar correlations, it would make the investigations much more time efficient.

In a study where spent wheat straw (SWS) and wheat straw (WS) were compared as potential biomasses for biogas production, the sugar yield had also been measured via enzymatic conversion. The study showed that SWS had the highest sugar yield (glucose and xylose), cellulose and hemicellulose conversion rates as well as the highest cumulative methane yield. The biogas production tests were carried out in 1-L batch reactors at 37°C for 30 days (Cui, et al., 2011). This study suggests that the stated hypothesis for this master thesis (paragraph 1.2 Aim) should be confirmed.

2.6 Pre-treatment methods for lignocellulose

It is becoming more and more common to integrate a pre-treatment step of the raw material prior to the fermentation process for biogas production. A pre-treatment method aims to optimize the process, but depending on which parameter that is optimized the methods can differ a lot. The process can be optimized in regards of reaction rates, retention times, methane yield or productivity, capacity, energy consumption, production costs etc. The optimization also varies a lot with the kind of raw material that is used since the disadvantages can be very substrate specific. Straw, for example, is very hard to dissolve in water which is why many pre-treatment methods for straw aim to decrease its floating ability.

2.6.1 Thermal pre-treatment

Thermal pre-treatment is, as the notion suggests, a method where the raw material is exposed to high temperatures under high pressure. This added energy breaks the hydrogen bonds in the crystalline cellulose and lignocellulose structures. Since there is an added risk for formation of inhibiting by-products at higher temperatures, the thermal pre-treatment usually has an optimum below a specific temperature. During for example thermal hydrolysis, the substrate is diluted to 15 – 20% dry matter content, then heated to 170 – 200°C under a pressure of 20 – 30 bar for about 20 minutes. A lot of the thermal energy can be recovered from the pre-treated raw material in contrast to when steam explosion is used instead. During steam explosion the substrate is also heated to around 160 – 220°C under increased pressure for 5-60 minutes. The substrate is then flashed which creates a so called steam explosion that destroys the cells and the cell wall structures. It is harder to recover thermal energy from this process (Montgomery & Bochmann, 2014).

2.6.2 Chemical pre-treatment

Chemical pre-treatment methods aim to break down the lignocellulose and/or make it more available for the hydrolysis. Alkaline chemicals, such as NaOH, attack the lignin and breaks it down through delignification, thus exposing the hemicellulose and cellulose. Acidic chemicals on the other hand breaks down the hemicellulose bound to lignin. Finally, a third option would be oxidative chemicals that also break down the lignin in lignocellulose. What all three chemicals have in common is that they seek to disrupt the crystalline fixed structures of lignocellulose, however to a high cost (Montgomery & Bochmann, 2014).

2.6.3 Biological pre-treatment

As the different metabolic pathways for anaerobic digestion of lignocellulose have different pH level optima, biological pre-treatment divides the process into two steps and reactors. In the first reactor, the pH level is kept around 4 – 6 which is the optimum for the hydrolysis and acidogenesis. It is in the next reactor, around a pH level of 6.5 – 8 that the methanogenesis takes place. This kind of biological pre-treatment is called pre-acidification. There are also other methods that include the addition of various microorganisms such as for example fungus, the addition of hydrolysing enzymes (Montgomery & Bochmann, 2014).

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2.6.4 Mechanical pre-treatment

Mechanical pre-treatment methods aim to decrease the particle size, thus increase the accessible surface area, of the straw. Hammer and ball mills grind the straw into flatter fibres and destroys the cells by pressing the material together. The opened cells expose the cellulose and hemicellulose within the cell walls, making them more available to enzymes. Knife mills and shredders on the other hand cuts the straw into smaller pieces which also destroys the cells. A common problem for mechanical pre-treatments are stones and metal pieces that may be mixed with the feedstock. Especially knife millers are torn down rapidly by these kind of impurities. Therefore, the operation costs may increase due to reparations. Hammer mills are not as sensible to those materials but on the other hand has a 2 to 5 times higher energy demand (Montgomery & Bochmann, 2014).

2.6.5 Extrusion of straw

Many pre-treatments methods are not exclusively mechanical, chemical, thermal or biological but a mix of two or more. An example of such a method is extrusion. An extruder looks like a large screw that presses for example the straw through a sieve which cuts it into smaller pieces. Heated water can simultaneously be added to the inlet. The screw creates high pressures up to 300 bar and temperatures from $60 - 300°C$ (partially due to the friction but mostly by heat exchangers) which is why this method is often referred to as thermo-mechanical, some would even argue chemical because of the water. Extrusion is very energy demanding and the screws as well as the sieves has to be replaced continuously, increasing the materials costs for this method (Montgomery & Bochmann, 2014).

Lignocellulosic materials can be extruded under dry or wet conditions; so called dry and wet extrusion. In the first case, the substrate is added at a moisture content of around 15% meanwhile wet extrusion indicates a moisture content of about 75% (Odhner, et al., 2015). Although studies show very little difference in methane yield or substrate digestibility based on dry versus wet extrusion, the moisture content of the substrate does affect the energy demand for the extruder. Dry disintegration generally has three times as high energy demand as wet disintegration (Lehmann & Eberhard, 2012). Additionally, dry extrusion cause higher material damage, thus increasing those costs.

2.7 Physical characterization parameters

Biomass physical characterization parameters include accessible biomass surface area, cellulose crystallinity, degree of polymerization (DP), pore volume and biomass particle size. Knowledge about the correlation between these parameters and the effect they have on the hydrolysis, hence the methane yield, could facilitate the optimization of a mechanical pre-treatment method.

It is known that a reduced particle size can lead to an increased accessible biomass surface area. Consequently, the degree of polymerization and the crystallinity of the cellulose are both reduced. Furthermore, a reduced particle size increases the hydrolysis yield and the reaction rate with 5-25 and 23-59%, respectively (Hendriks & Zeeman, 2009). Since variations in the particle size affect many other parameters simultaneously, it is hard to determine whether it is the DP, the surface are or the crystallinity that in turn affects the methane yield the most.

2.7.1 Particle size and surface area

The particle size of lignocellulosic biomass is usually measured through sieving which always generates a distribution curve. When the hydrolysis of the lignocellulose begins, active enzymes

binds to its surface and starts cleaving the fibres into smaller fragments. Therefore, the hydrolysis reaction rate is dependent on the available surface area of the biomass. The surface area can be divided into two types; external and internal. The external surface is always exposed meanwhile the internal surface area is what makes up the small voids inside the crystalline structure of the lignocellulose. These pores are often so small that the probability for any enzymes to enter and hydrolyse the cellulose is extremely low. Thus, the cellulose there is inaccessible for enzymes.

In order to increase the hydrolysis reaction rate and thereby possibly the methane productivity, the external surface area can be increased. This is normally achieved by reducing the particle size of the biomass. Various mechanical pre-treatment methods can be used for that purpose. Even though smaller particles provide a larger enzyme accessible surface area, they should not be too small. Too small particles have showed to have an inhibiting effect on the hydrolysis because of the risk of cellulose, or mostly hemicellulose, degradation to weak acids (Kyong Ko, et al., 2014). As explained in the acidogenesis paragraph above, these acids result in a lowered pH value which creates an unfavourable environment for the methanogenesis to take place (Motte, et al., 2014).

2.7.2 Porosity
Porosity is a ratio between internal void volume of a particle and its total volume (Kuva, et al., 2014). The porosity is often related to the ratio between internal and external surface area at constant particle size. A way to increase the biogas yield would be to increase the internal surface area at a constant external surface area, thus increasing the porosity. Expansion of the internal surface area could make it more available for water, thus allowing hydrolysis to take place. In this case it is important that the wetted area of the internal surface increases (Yang, et al., 2015). However, if the internal surface area largely exceeds the external one, the hydrolysing enzymes can get trapped inside of the crystalline structure, thus inhibiting the reaction (Hendriks & Zeeman, 2009). In addition, porous particles do not necessarily generate a high methane yield. For the large internal surface area to be available to the enzymes, the pore diameter has to exceed the size of the enzymes; around 5 nm in diameter (Driemeier, et al., 2016). Some research also indicates that the hydrolysing enzymes may sometimes be attached on the surface of the microorganisms (Angelidake & Sanders, 2004). In that case, the pore diameter would have to be greater than the size of the microorganisms; 0.5 – 5.0 µm (Kubitschek, 1990).

2.7.3 Degree of cellulose polymerization (DP) and crystallinity
The degree of polymerization (DP) is a value that defines how many monomeric units a polymer is made out of. In the case of cellulose, the DP value signifies the number of glucose molecules per polymer chain (Sanchez-Vazquez, et al., 2013). Since the hydrolysis of cellulose aims to break the polymers into smaller fragments, the DP value could be a way of measuring the efficiency of that reaction and/or the hydrolysing enzyme(s). Shorter cellulose polymers (low DP) provide a higher number of chain ends to which the hydrolysing enzyme can bind (Hallac & Ragauskas, 2011).

Crystalline cellulose is composed out of numerous cellulose polymers that bind together via hydrogen bonds. The shorter the polymers are, the weaker the bonds are between them. Therefore, crystalline cellulose is more easily convertible to amorphous cellulose if the polymers are shorter since less energy is required to break the bonds (Hallac & Ragauskas, 2011).

2.7.4 Matted scum layer

One of the biggest problems when processing fibrous lignocellulosic biomass is the fact that it mixes poorly with the water phase. Instead, the material floats up to the surface, creating a matted scum layer. This happens due to the fact that the biomass, in this case straw, contains a lot of trapped air. Although cellulose has a higher molecular weight than water, the air causes the straw to float (Björnsson, et al., 2014). A poor mixing capability leads to a small wetted surface, thus a small enzyme accessible surface area. Consequently, the reaction rate of the hydrolysis becomes very slow which increases the production costs of the biogas. In order to find a fitting pre-treatment method that can solve the floating issue, the phenomena should be studied further.

2.7.5 Total solids content (TS)

The ratio between water and total solids content (TS) has also proven to affect the methane productivity. Anaerobic digestion of lignocellulosic biomass has a low methane productivity if the solid content is high. This is because the mass transfer between the different mediums becomes slow, and therefore also the methane production rate. Another reason to why the yield decreases when the TS increases might be that carbon dioxide and hydrogen gas become trapped inside the solid structures, preventing them from mixing with the liquid phase. That kind of accumulation will inhibit the hydrogen consuming reactions in the methanogenesis. Also, if the carbon dioxide and gaseous methane produced in the methanogenesis do not become eliminated from the liquid medium, the reaction will find an equilibrium, thus stagnate the methane production (Yang, et al., 2015).

As mentioned in previous paragraphs, the molar ratio of butyric/acetic acid can be used as an indicator to whether the process operates under optimal conditions or not. A value of 1.5 is desired. Studies have shown that this molar ratio is affected by the TS content. Motte et al. (2013) presented data from a study of wheat straw fermentation where the methanogenesis had been intentionally inhibited. The TS content was varied from 10 – 33%. Results showed that even though the substrate conversion rate measured higher at 10% TS content, the optimal molar ratio butyric/acetic acid was not achieved until a TS content of 28%. In conclusion, the TS content might affect which metabolic reactions that are favoured and by that the degradation rate of the lignocellulose (Motte, et al., 2013). These results also prove that even a TS content of 28% is optimal from a theoretical point of view, it might increase the mixing difficulties in the tank resulting in that another TS content is chosen as a setting point.

3 Materials and methods

3.1 Straw samples with diverse properties

The aim of this project was to find a relatively quick characterization method for lignocellulosic biomass intended for methane production. In order to do so, it was important to find different lignocellulose samples to be analysed that would probably vary a lot in maximum methane production. Such samples would hopefully also generate varying results from the enzymatic hydrolysis analysis. Thus, wheat straw was mechanically pre-treated under different conditions, to create samples with diverse physical properties. In addition, analyses on different kinds of straws such as rye straw, barley straw and canola straw were also run. The straw samples with variations in physical and chemical properties were named Straw Group A and Straw Group B, respectively. A short overview of what tests that were run on what straw group is shown in Table 1.

Table 1. A descriptive summary over what tests that were run on what straw group.

Analysis	Measured parameter	Straw Group A	Straw Group B
BMP test	The bio-methane potential	X	X
BET-analysis	Surface area, pore volume, pore diameter	X	
Bouyancy	Floating tendency and scum formation	X	
NREL analysis	Extractives, lignin and carbohydrate content		X
Enzymatic hydrolysis	Carbohydrate degradability	X	X

3.2 Preparation of straw samples with varying physical properties (A)

First, it was decided to pre-treat wheat straw in an extruder and vary the settings so that extruded straw samples with different properties would be obtained. The extruder was a twin-screw extruder, see Figure 2 (Extruder MSZK 15, LEHMANN Maschinenbau, Jocketa, DE).

Figure 2. The twin screw extruder used in this project where the input is on top of the machine and the outlet opening can be seen to the right in the picture.

Parameters that could be varied with this method were: the frequency, meaning how fast the screws would rotate; the TS content of the straw by the addition of water; the outlet flux which could be varied by increasing or decreasing the outlet opening. In this case, only the outlet opening was varied from half open to fully open at a constant rotation speed. This was done in the hopes that it would affect the pressure inside the extruder and thereby generate straw with varying physical properties. Dry extrusion was the method of choice since it meant that no time had to be spent to moisture the straw and control its TS content. To control that the alteration of the outlet opening truly affected the extrusion process, the load tendency was documented for every setting. The load tendency functions as a power indicator for the extruder; the higher the load tendency, the higher the energy demand. The extrusion process resulted in four different straw samples; Straw1, Straw2, Straw3 and Straw4.

3.3   Preparation of straw samples with varying chemical properties (B)

As mentioned in the previous paragraph, the need for straw samples of diverse properties was crucial for this project. Besides altering the physical properties of straw, the chemical diversity that comes with different straw species was also investigated. Four different kinds of straw were included in the analysis: wheat, rye, barley and canola. All of them were shipped to the lab from Denmark. The different straw kinds were milled to smaller particles in order to facilitate the handling of them. However, the particles were not nearly as small as those of the extruded straw.

3.4   Automatic Methane Potential Test System (AMPTS)

An automatic BMP test system, called AMPTS II (AMPTS II, Bioprocess Control, Lund, SE), was utilized during this project. Figure 3 shows a simple illustration of the AMPTS set-up, containing three different units. Unit A is a 500 mL glass bottle placed in a heated water bath and equipped with an agitator. This is where the fermentation takes place. The gas (arrows) then travels to unit B where the CO_2 and H_2S gas react with NaOH and are dissolved in the liquid phase. As a result, only methane gas continues to Unit C which is a measuring device that registers every time one of the caps flips open. The caps have known volumes and flip open

when filled with a specific amount of methane. Unit C registers and saves all measured information by itself but when connected to a network, this information can be gathered from a website. During start-up, the impeller speed is also regulated from this website.
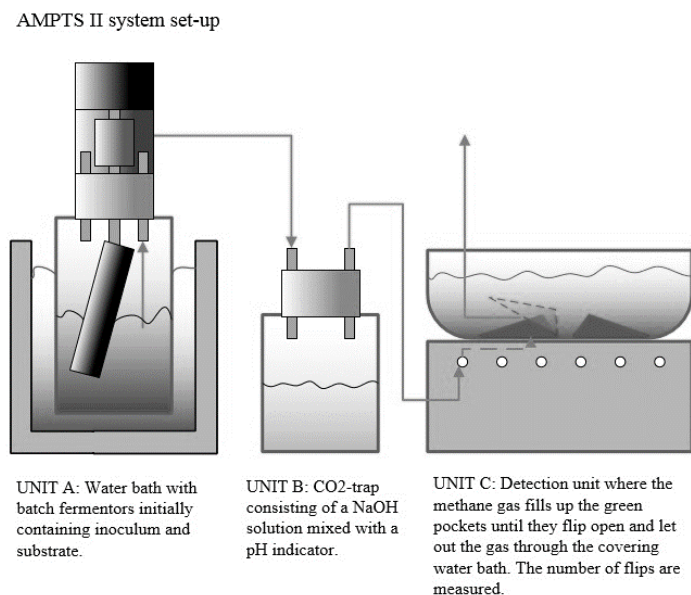
AMPTS II system set-up

UNIT A: Water bath with batch fermentors initially containing inoculum and substrate.

UNIT B: CO2-trap consisting of a NaOH solution mixed with a pH indicator.

UNIT C: Detection unit where the methane gas fills up the green pockets until they flip open and let out the gas through the covering water bath. The number of flips are measured.

Figure 3 An illustration over the AMPTS II set-up with three different units; A, B and C. The arrows represent the gas flow through Tygon® tubes.

Two AMPTS system set-ups, BPC01 and BPC02, were used during this project where each set-up held 15 bottles. The two systems were run twice each, à 30 days. The first experiment will be referred to as Experiment A and the second as Experiment B, in coherence with what straw group that was analysed; Straw Group A and Straw Group B, respectively.

3.4.1 Experiment A and B
Both Experiment A and B were designed according to the same principles and assumptions. To study the performed calculations, see Appendix 2: Set-up of BMP test, Experiment A and Appendix 3: Set-up of BMP test, Experiment B. The BMP test for the straw samples was run at 37°C for 30 days at an impeller speed of 75% of the maximum speed. To allow gas production in the bottles, the headspace was set to 200 mL, leaving the remaining 300 mL to be filled with inoculum, straw and water. In order to calculate the necessary amounts of each substance, it was decided that the total volatiles solids (VS) load would be limited to 1 g VS/100 mL water. The VS ratio between inoculum and substrate was set to 2:1. The total TS value in the bottles containing straw, measured approximately 10%.

Before starting the measurements, the system was flushed with pure nitrogen gas to provide an anaerobic environment for the microorganisms in the inoculum. The inoculum was collected from a wastewater treatment plant in Lund, Sweden. By choosing an inoculum from a source with very diverse substrates (sludge), it increases the chances of having microorganisms that could survive on lignocellulosic biomass. Two different control samples were run: one containing only inoculum and one with cellulose powder as a substrate. Table 2 shows what straw samples that were run during Experiment A and B.

Table 2. The constitution of Experiment A and B.

Experiment A	Experiment B
Inoculum	Inoculum
Cellulose powder	Cellulose powder
Untreated straw	Wheat straw
Straw1	Rye straw
Straw2	Barley straw
Straw3	Canola straw
Straw4	

The amount of utilized substrate, here straw, was very small when running the AMPTS set up which posed difficulties in obtaining straw samples that were representative. Therefore, all samples were tested in triplicates. The yield from these replicates differed markedly which is why the standard deviation (SD) was calculated. If the SD from three replicates overlapped the standard deviation from three other replicates, the two different straw samples were regarded as statistically indifferent. Finally, as both experiments contained straw types with different chemical composition, the cumulative methane yield generated by the BMP tests were put in relation to the carbohydrate content in the straw. It was assumed that 1 g carbohydrates could maximally be converted into 415 NmL CH_4 gas, where the term *NmL* indicates that the volume has been normalized to 0°C at 1 atm (Angelidake & Sanders, 2004).

3.5 BET-analysis

To investigate whether the extrusion of the straw had had any effect on its physical structure, a Brunauer-Emmett-Teller (BET) analysis was performed, however by another person at the Department of Chemical Engineering. Cellulose, the untreated straw and the extruded straw samples (Straw Group A) were put through a BET-analysis. The BET-analysis is a way of measuring the pore characteristics of a sample. By letting nitrogen gas condense on the surface of the straw particles and thereby finally filling up the pores, the total surface area, the pore diameter and the pore volumes can be obtained. The procedure is carried out under an increasing partial pressure starting at near vacuum. Prior to the BET-analysis, the samples were put under vacuum so that any solvents that might occupy any pores could be flushed out. The degasification occurred at 80°C for 5 days meanwhile the BET-analysis took approximately 2 days to finish. None of the samples passed the leakage test meaning that there were still some solvents, such as water, left in the samples when the addition of nitrogen began. This means that the measured pore volumes might have been underestimated.

3.6 Buoyancy tests

In this minor experiment, the straw from Straw Group A was mixed with water in a measuring cylinder and the appearing layers were studied. The straw had only sedimented for about 10 minutes before they were studied. The aim of this experiment was to investigate whether the pretreated straw and the untreated straw would show any difference in floating layers.

3.7 NREL analysis

To characterize the chemical composition of the straw and quantify its compounds, a number of different analysis were carried out according to the laboratory analytical procedures (LAP)

formulated by the National Renewable Energy Laboratory; hence, NREL analysis. These methods give qualitative and quantitative information regarding the components listed below:

- Water
- Dry matter/Total solids (Sluiter, et al., 2008)
    - Ashes (Sluiter, et al., 2005)
    - Organic material
        - Raw fat
        - Raw protein
        - Fibres
            - Nitrogen free extractives (Sluiter, et al., 2005)
            - Raw fibres (Sluiter, et al., 2008)
                - Lignin
                - Starch
                - Glucose
                - Mannose
                - Galactose
                - Xylose
                - Arabinose

Even though the analysis performed during this project were based on the LAPs, the in house methods of the laboratories at Department of Chemical Engineering sometimes deviated from them. Since straw does not contain a significant amount of protein or fat (Björnsson, et al., 2014), those analysis were left out. Also, the extractives (here nitrogen free extractives) were not quantified since the most important components for this specific project was the carbohydrates. Therefore, only the dry matter content, the ash content and the raw fibres analysis were carried out.

3.7.1 The total solids analysis (TS)

To determine the water content, or more interestingly the total solids content (TS), the samples were dried in an oven (Oven E28, BINDER, Tuttlingen, DE) overnight at 105°C. By subtracting the final sample weight after drying from its initial weight, the total water content can be calculated (assuming that no organic volatiles evaporate at this temperature). The dried straw was considered to contain 0% water and constitutes thereby the dry matter content, i.e. the total solids fraction (TS). The water content and the TS value were calculated accordingly:

$$\text{Water content} \, (\%) = \frac{(m_{before} - m_{after})}{m_{before}} \qquad \text{(Eq. 1)}$$

$$\text{TS} \, (\%) = \frac{m_{after}}{m_{before}} \qquad \text{(Eq. 2)}$$

The total solids content (TS) can also be referred to as dry matter content (DM). In this report, concentrations are often given as % (db.) or % of ODW, meaning that the concentration has been calculated on a dry basis (db.) or as a percentage of the oven dry weight (ODW). All four different ways of writing describe the same concentration.

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3.7.2 The ash and volatile solids analysis (VS)

The dried sample, free of water, were put into an ignition oven (4800 furnace, THERMOLYNE, Iowa, USA) at 575±25°C for 3 hours. The decrease in total sample weight that occurs is due to the evaporation of the volatile solids (VS) in the sample. Only the ashes are not combusted during this analysis. The VS content was calculated as:

$$VS\,(\%) = \frac{(m_{ODW} - m_{after})}{m_{ODW}}$$
(Eq. 3)

where m_{ODW} is the sample oven dry weight and m_{after} the sample weight after burning.

3.7.3 The nitrogen free extractives analysis

Quantification of present nitrogen free extractives in the straw was done by extracting the sample with water followed by ethanol. This kind of analysis is rather time consuming as the extraction process takes about four days per sample. If the analysis is not performed, the lignin fraction will be overestimated in following steps. Since the lignin cannot be digested in the BMP tests, its concentration in a substrate is an interesting factor to study. However, if the extractives fraction show to be rather small, it might be possible to neglect. To investigate this reasoning, both non-extracted canola and extracted canola straw were put through the fiber analysis. Canola straw was suspected to contain the highest amount of extractives and was therefore chosen for the extraction analysis.

The extraction process was performed with a Soxhlet apparatus (EZ 100, BEHR Labor Tachnik, Düsseldorf, DE), Figure 4. Approximately 5 g of canola straw was put into the thimble and placed inside the glassware. The receiving flask, at the bottom of the set-up, was filled with 190 mL distilled water. When all three soxhlet apparatus had been assembled, the heating was turned on and kept on for 24 hours. The process functions so that the solvent (water or ethanol) in the receiving flask evaporates when heated. At the top of the soxhlet tube there is a cooling mantle that condensates the solvent which will then gather inside and around the thimble. Since the thimble is made of cellulose paper, all extractives are able to pass through and thus separate from the rest of the straw.

Figure 4. The extraction set-up with Soxhlet apparatus where the sample is put into the thimbles (white, filter paper tubes) and extracted into the receiving flasks at the bottom.

When the water extraction was finished, the receiving flasks (containing water and water extractives) were emptied into tared glass flasks and then filled up with 190 mL 96% ethanol, upon which the ethanol extraction process began. The two solvents were later separated from the extractives through evaporation (Multivapor P-6, BÜCHI, Flawil, CH) under low pressure. For the water evaporation, the procedure was carried out at 60°C, 50 mbar and for the ethanol evaporation the temperature was set to 55°C, 115 mbar. The solids remaining in the glass flasks after evaporation were the nitrogen free extractives.

The wet thimble containing the extracted canola straw was removed from the soxhlet apparatus and with the help of approximately 100 mL 96% ethanol, the straw was washed onto a cellulose filter paper. By vacuum filtration, the ethanol was quickly removed and the straw solids were scraped off the cellulose paper and into cups which were put in the oven at 45°C for later use.

3.7.4   The carbohydrate and lignin analysis

The raw fibre analysis was performed in order to determine what and how much carbohydrates and lignin that the straw is constituted of. An illustration of the laboratory procedure is shown in Figure 5 below.
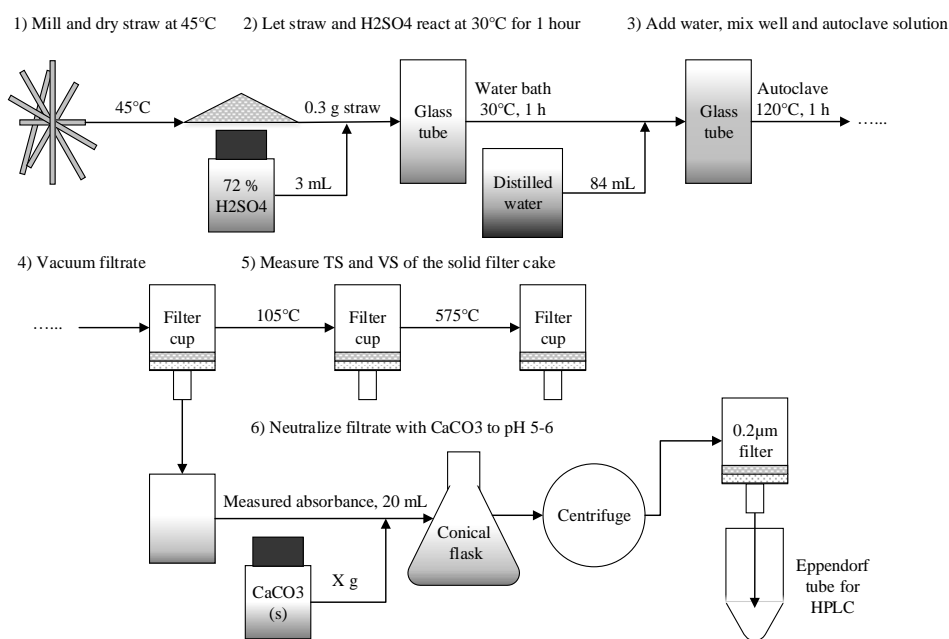
Figure 5 Flow sheet over the procedure of a raw fiber analysis (only the straw sample is included, not the standard sugar solution).

First, the untreated straw was prepared for analysis by milling it prior to drying it at 45°C overnight. At the same time, the filter cups needed for the analysis were dried at 575°C for 4 hours. To prevent the glass from breaking, a ramping program was utilized. Then, approximately 0.3 g milled dried straw sample was mixed with 3 mL 72% sulfuric acid and put on a water bath at 30°C for 1 hour in glass tubes (NREL tubes). After the water bath, 84 mL of distilled water was added to the tubes. A control sample consisting of 4 mL standard sugar solution (SRS) mixed with 80 mL distilled water was transferred to a similar tube followed by the addition of 3 mL 72% sulfuric acid. The preparation of the SRS is presented in Appendix 4: Sugar recovery

standard (SRS). Both tubes were well mixed and put into the autoclave (Systec DX-150, Microbiology International, Frederick, USA) for 1 hour at 120°C. When the tubes had cooled to room temperature, the samples were vacuum filtrated using the burnt filter cups. The filter cups were then dried at 105°C overnight meanwhile the filtrate was analysed in a spectrophotometer (UV-160, SHIMADZU, Singapore, SG). Then, the filtrate was neutralized to a pH of 5-6 by the addition of calcium carbonate prior to centrifugation (Heraeu Labofuge 200 Centrifuge, Thermo Fisher Scientific Inc., Osterode am Harz, DE) and filtration with 0.21 μm filters into Eppendorf tubes. The Eppendorf tubes were stored in a freezer until the HPLC analysis could be performed. Finally, the dried filter cups were incarcerated at 575°C (again, using a ramping program) which generated the ash content values for the solids.

3.7.5 Acid soluble lignin content (ASL)

When measuring the acid soluble lignin content (ASL), by spectrophotometric analysis, the absorbance was assumed to relate to the concentration according to the following equation (Sluiter, et al., 2012):

$$ASL\ (\%) = \frac{A_{sample} \times V_{sample} \times Dilution}{\varepsilon \times ODW_{sample} \times Pathlength_{cuvette}} \times 100 \tag{Eq. 4}$$

Where A_{sample} is the measured absorbance at 320 nm, V_{sample} is the total filtrate liquid volume in which the straw was initially dissolved, $Dilution$ is the dilution factor used to make sure that the sample landed within an acceptable absorption range, ε is the absorptivity at 320 nm, ODW_{sample} is the added straw sample on dry basis (g) and finally $Pathlength_{cuvette}$ is the width of the cuvette through which the light travels in the spectrophotometer. In this experiment, the absorptivity was 30 L/g*cm at 320 nm based on NREL recommendations. The cuvette was 1 cm wide and the volume V_{sample} measured 86.73 mL. The dilution was made by adding distilled water to the filtrate so that the measured absorbance lay within the interval 0.6 – 1.

The filtrate from the first three samples (wheat1, wheat2 and rye1) were accidentally pre-diluted by addition of ca. 70 mL distilled water to the 87 mL sample solutions before being diluted further in preparation for the spectrophotometry. Therefore, the results may not be totally accurate, for those samples. However, since no extraction analysis was performed on the straw (except for canola straw), the lignin content was generally a bit overestimated.

3.7.6 Insoluble lignin content

The acid insoluble lignin content (AIL) can be calculated based on the following equation:

$$AIL\ (\%) = \frac{m_{cruc.+TS} - m_{cruc.+ash}}{ODW_{sample}} \times 100 \tag{Eq. 5}$$

Where $m_{cruc.+TS}$ is the weight of the filter cup and total solids (after drying at 105°C), $m_{cruc.+ash}$ is the weight of the filter cup and ashes (after incarceration at 575°C) and ODW_{sample} is the oven dry weight of the initial sample (before acid hydrolysis).

3.7.7 Carbohydrate content

The monomeric sugar content in the filtrated and neutralized samples were measured via an HPLC (ICS-3000, Dionex Corp., Sunnyvale, USA). In this case, the allowed sugar concentrations ranged between 0.01 – 0.1 mg/mL. Based on the known sugar concentrations in the SRS,

the dilution factor was approximated to 20; hence 19 parts of distilled water was added to one part sample. To convert the monomeric sugar concentrations in the liquid solution to polysaccharide content in the straw samples, further calculations were made, see Appendix 5: Carbohydrate determination.

3.8   Enzymatic hydrolysis

All different types of straw that has been analysed for their biochemical methane potential through BMP tests, were also analysed for their degradability through enzymatic hydrolysis. Approximately 1 g straw was added to a tube along with 30 mL 0.1 M, pH 5.5 sodium acetate buffer and 55 µL enzyme cocktail. After incubation (Hybridization Incubator combi-H12, FINEPCR, Seoul, KR) at 50°C, pH 5.0-5.5 for 72 hours, the samples were filtrated and analysed for their sugar content. The pH level was measured with a calibrated pH meter (HI-8424 pH Meter, HANNA Instruments, Bedfordshire, UK). Control samples containing only buffer and enzymes and control samples containing only buffer and straw, were also incubated and analysed so that any errors could be detected. By comparing the monomeric sugar concentrations detected in the solution, with the initial polymeric sugar content in the straw, the digestibility could be calculated, see Appendix 6: Enzymatic hydrolysis. Only the xylan (polymeric xylose) and glucan (polymeric glucose) were investigated and estimated as a representation of the total hemicellulose and cellulose content, respectively.

The enzyme cocktail used in this experiment was called Cellic® CTec3 and had an activity of 187 FPU/g solution, where FPU stands for filter paper unit, and the desired enzyme concentration was set to 10 FPU/g sample (db.) (Resch, et al., 2015). However, this enzyme concentration was recommended for biomass loadings not larger than 10 mg per mL reaction slurry. As the straw loading in this experiment measured approximately 30 mg per mL reaction slurry, the enzyme load should probably have been more than 55 µL; a fault that probably decreased the enzyme activity in the flasks.

4 Results and discussion

The results have been divided into three sections: Part A, Part B and Part C. This was done to facilitate the discussion around the results and to clearly identify correlations between the different analyses.

4.1 Part A: Physical characterization

4.1.1 Dry extrusion
The wheat straw was extruded under four different operating conditions. To be certain that a decrease of the outlet opening had any effect on the extrusion process itself, the power consumption i.e. the tendency load of the extruder was documented, see Table 3. The outlet cross area is presented as a percentage where 100% means that the outlet was fully open and 0% means that is was completely closed.

Table 3. The results show how the size of the outlet opening (100% meaning fully open and 0% meaning fully closed) affect the power consumption of the extruder.

	Straw1	Straw2	Straw3	Straw4
Outlet opening	100%	83%	67%	50%
Tendency load	8-9	30-35	45-50	55-60

Based on these results, a clear correlation can be drawn between the outlet opening and the power consumption; the smaller the opening, the higher the power consumption. This phenomena was probably due to an increased pressure that build up inside the extruder as its rotation speed was kept constant. When the power input/tendency load reached a level of 60, the extruder failed to push the straw forward. As a result, the extruder had to be run backwards and then forward again to be able to overcome the increased pressure. Therefore, the fourth batch of extruded wheat straw (Straw4) was processed a bit differently from the other three.

4.1.2 BET-analysis
The extruded wheat straw samples Straw1, Straw2, Straw3 and Straw4 were put through a BET-analysis together with untreated straw and cellulose powder. The apparatus used to perform this analysis could only ensure statistically reliable results if the sample had a larger surface area than around 5 m^2/g. However, all straw samples showed to have such a low density that their analysed total surface areas did not fulfil this criteria. Consequently, the results may not be correct. As the analysis was made in triplicates, the accuracy of the results may be enough to compare the samples between each other. The results are shown in Table 4.

Table 4. The summarized results achieved from the performed BET-analysis that were executed on cellulose, untreated straw and 4 different extruded straw samples.

	Cellulose	Untreated	Straw1	Straw2	Straw3	Straw4
Total surface area (m²/g)	1.14	0.394	0.846	0.922	1.07	1.15
Average pore diameter (Å)	148	140	121	126	136	158
Total pore volume (µL/g)	4.21	1.38	2.57	2.92	3.66	4.54
Pressure (P/Po)	0.994	0.995	0.994	0.994	0.994	0.995

As can be seen in Table 4 above, the BET-analysis produced results in terms of surface area and total pore volume as well as average pore diameter for all the different samples. The cellulose and the Straw4 sample have very similar BET surface areas of 1.14 and 1.15 m²/g, respectively. In addition, the total pore volume measured 4.21 and 4.54 µL/g for the cellulose and the Straw4 sample, respectively. A reason to why the two mentioned samples seem to differ only in average pore diameter (147 Å for cellulose and 158 Å for Straw4) could be that the Straw4 sample might have fewer but bigger pores and might also differ in particle size and density which affects the porosity.

When comparing the straw samples, a general pattern could be detected. The total surface area increases with the pressure that was applied to the straw during the extrusion (Straw1 rating the lowest and Straw4 the highest). The total pore volume increases in the same way as well as the average pore diameter (except for the untreated straw). This means that the increase in pressure under the extrusion process had a consistent effect on the extruded straw, thus indicating that there could be a correlation between the extrusion pressure and the surface area, pore diameter and pore volume.

The untreated straw did not have a big total surface area, which coincides with the fact that the particles were very large. Also the pore volume was very small compared to the other straw samples. As the untreated straw is probably more fibrous and the cellulose polymers more crystalline in their structure, the results from the BET-analysis could indicate that the untreated straw has very few and shallow pores with wide pore diameters. Meanwhile, the pores of the extruded straw might be more and sometimes narrower. If these pores were created by breaking the crystalline straw structures, by the steam explosion effect or by folding of the particle surface is not possible to determine from only these results.

All measured pore diameters largely exceeded 5 nm, which is the average size of a cellulose enzyme (1 Å = 0.1 nm). These results suggest that any present pores should be wide enough to enable enzymes to enter, during the enzymatic hydrolysis. During the BMP tests however, the enzymes might be attached to the microorganisms, which are bigger than the measured pore diameters (namely 0.5-5.0 µm) (Angelidake & Sanders, 2004). Thus, the possible contact surface area between the enzymes and the straw might be bigger during the enzymatic hydrolysis experiments than during the BMP tests.

4.1.3 Buoyancy
When letting the different straw samples mix with water in measuring cylinders and set for about 10 minutes, the phenomena illustrated in Figure 6 was observed.
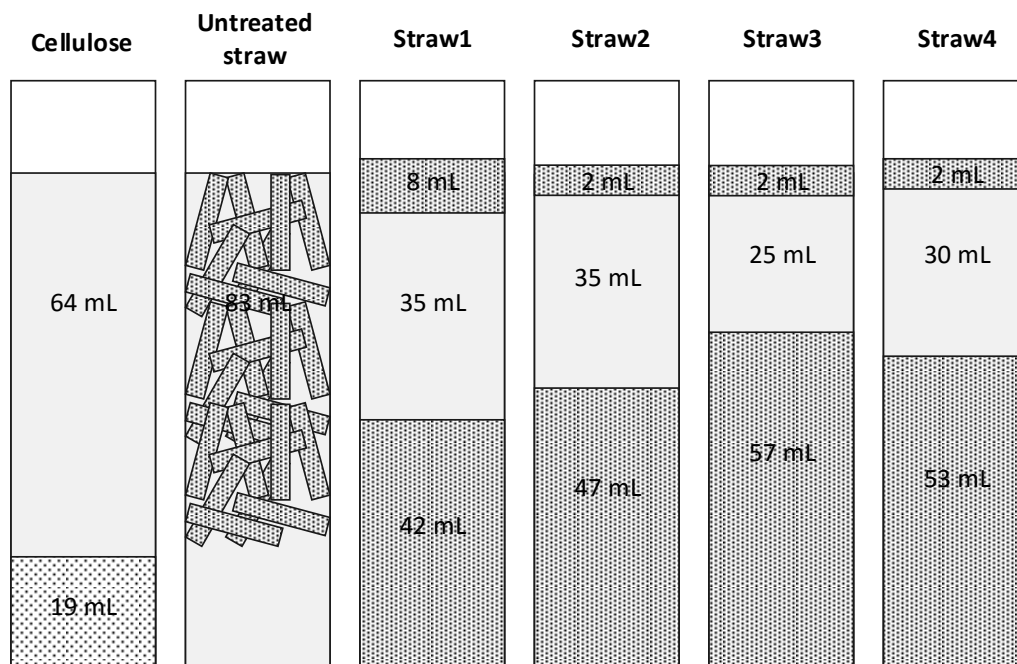
Figure 6. An illustration of how the different straw samples as well as cellulose behaved when put into water where the solid colour represents water and the dotted represents cellulose/straw. Straw1, Straw2, Straw3 and Straw4 show the average results from duplicate samples.

In the case of cellulose, all of the added powder sank to the bottom of the cylinder and did not create a scum layer on the surface. Regarding the untreated straw, all of the bigger straw strands were striving towards the surface. Even though this sample created a floating straw layer, no scum was detected after mixing. Finally, the extruded straw samples (Straw1 – Straw4) all had two layers of material in the cylinder; one bottom and one top layer. The top layer consisted of bigger straw particles and of a scum layer of very small particles. The bottom layer consisted of bigger particles nearest the bottom and smaller ones higher up in the column.

It is difficult to determine whether the floating capacity of the straw that was studied in these laboratorial tests, would lead to the same results if performed in scale-up biogas tanks. In an industrial biogas production process, there is a continuous flow through the reactor as well as mixing that could affect the foaming phenomena. However, the untreated straw would most probably float up to the surface in a bigger tank as well.

4.1.4   BMP tests

The BMP test was run at 37°C for 30 days in two different set-ups; BPC01 and BPC02. The results generated from both set-ups have been summarized and are shown in Figure 7. The graph shows the cumulative methane yield (in NmL CH_4/g VS) plotted against the number of days. Every curve represents the average of three bottles except for the cellulose and the untreated straw curves, which are both the average of six bottles (three from BPC01 and three from BPC02). As three bottles per system were filled with only the inoculum, it was possible to calculate how much that inoculum contributed to the total methane production. The calculated amount was then subtracted from the cumulative methane yield for each sample. In summary, all curves in the graph below have been regulated accordingly.
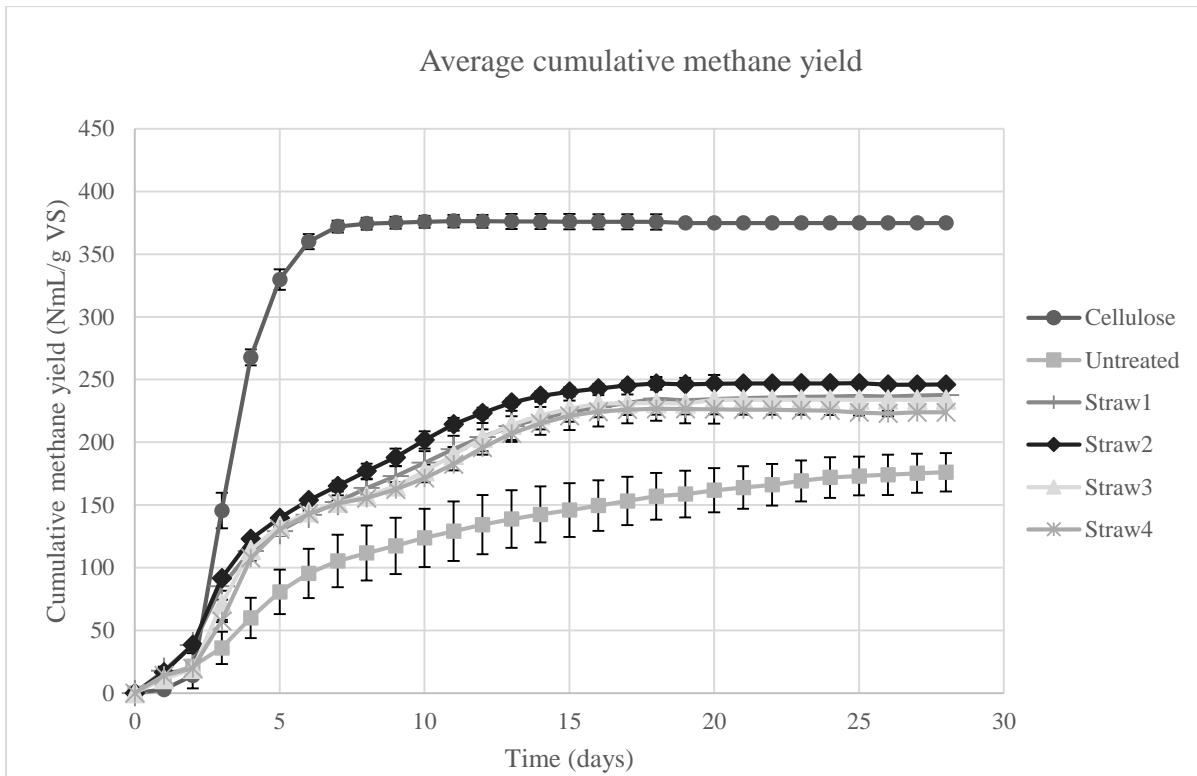
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Figure 7. The average cumulative methane yield, based on three to six replicates, produced from the substrate only (not the inoculum). The graph also shows standard deviations.

The cellulose was digested very quickly, compared to the different straw samples, and reached its methane maximum at 380 NmL/g VS, after around eight days. The extruded straw samples (Straw1 – Straw4) however, took much longer time to reach their maximum methane levels (220 – 260 NmL CH_4/g VS) and did not differ significantly from each other as their standard deviations sometimes overlap.

Cellulose powder does not contain any lignin which is why it is more water soluble than ligno-cellulose and does not have such a rigorous structure. It is therefore to be expected that the hydrolysis reaction rate of cellulose powder is higher compared to straw. As explained earlier (paragraph 2.4.1 Hydrolysis), the hydrolysis is the limiting reaction step for the methane production. Consequently, the cellulose powder samples were digested more quickly explaining why those samples reached their methane maximum faster the straw samples, and also why the methane yield was significantly higher.

Only Straw2 and Straw4 seem to be comparable at the end of the BMP test. As Straw4 was extruded under a higher pressure, it was expected that this sample would have a smaller particle size and therefore a larger bioaccessibility, compared to Straw2. A possible reason to why Straw2 show a higher cumulative methane production than Straw4 could be that inhibitory by-products might have formed in the Straw4 bottles. Another possibility is that because the extruder was run backwards and forward multiple times during the extrusion of Straw4, larger pressure drops might have occurred. These pressure drops could have caused the lignin to re-condense on the surface of the straw particles and thereby clogging smaller pores, preventing the hydrolysing enzymes from entering those pores. The results from the BET-analysis showed that Straw4 had wide and probably shallow pores compared to other straw samples. If this was what happened, it could explain why Straw4 generated less methane than expected.

Finally, the untreated straw generated the lowest methane yield (170 NmL CH$_4$/g VS) and the replicates differed a lot from each other, hence the large standard deviation. The low methane yield was expected since untreated straw had a small total surface area and also a low density which caused it to float on the liquid surface, thereby mixing poorly with the inoculum. Studying the graph in Figure 7, the methane production rate seems to show a lag phase the first 2-3 days of the experiment. This suggests that the degradation rate of polysaccharides into acetate was slow which could be a result of the microorganisms having to adapt to a new environment. The more probable reason though, is that the mass transfer rate between the solid straw and the liquid containing the inoculum was slow due to the fact that the untreated straw formed a layer on the surface.

In conclusion, extrusion of wheat straw increased the methane yield by about 30% compared to the untreated straw. This yield increase is in great accordance of results generated by *Swedish University of Agricultural Sciences* who also report a methane yield increase of 30% for untreated wheat straw compared to extruded wheat straw (Odhner, et al., 2015). However, the methane yield from the extruded straw does not seem to be very dependent on the different settings used on the extruder.

4.2   Part B: Chemical characterization

4.2.1   NREL analysis

The chemical composition of wheat, rye, barley and canola straw was analysed in duplicates and the results are presented in Table 5. The concentrations are given as a percentage of the oven dry weight (ODW) of the samples.

Table 5. The composition of wheat straw, rye straw, barley straw and canola straw. Both the results for the extracted canola straw (Ext. Canola) and the non-extracted canola straw (Canola) are presented. All concentrations are given as % ODW.

% of ODW	Wheat	Rye	Barley	Canola	Ext. Canola
Ash	3.2	6.0	3.2	8.0	8.0
Lignin	21	23	21	21	16
- ASL	1.3	1.7	1.5	1.2	0.83
- AIL	20	21	19	20	15
Cellulose	22	33	36	27	26
Hemicellulose	14	20	20	12	12
Other carbohydrates	5.0	6.4	6.3	6.2	5.0
Extractives	-	-	-	-	16
- Water ext.	-	-	-	-	12
- EtOH ext.	-	-	-	-	4.2
Other compounds	35	12	16	26	17

When studying the composition of canola straw and compare it to the extracted canola straw, a great difference in lignin content can be detected, especially the acid insoluble lignin (AIL). Apart from lignin, the AILs may also include fractioned proteins. If no extraction has been performed on the straw prior to the AIL determination, some of the protein derivatives will end up in that category along with other nitrogen containing compounds. The point of comparing the lignin content measurements between extracted and non-extracted canola was to investigate whether any difference would show. As the difference was quite big, it becomes difficult to

argue that the measured lignin content in the other straw types (wheat, rye and barley) represents their actual lignin content. Looking at the numbers, rye seem to contain more lignin than wheat. However, if rye contains much more extractives than wheat, the result might have been the reverse after an extraction procedure. This makes it difficult to discuss the impact the lignin content had on the methane potential and the sugar yield in subsequent tests. The carbohydrate content in the non-extracted canola, on the other hand, did barely deviate from the carbohydrate content in the extracted canola. In regard of that, the sugar concentrations in the other straw samples will therefore be assumed to have been correctly measured.

The more detailed results from the sugar determination are shown in Table 6. All concentrations are given as a percentage of the total dry weight (ODW) of the straw.

Table 6. The carbohydrate content in wheat, rye, barley and canola straw. Both extracted canola straw (Canola) and non-extracted canola straw (Ext. Canola) have been analysed.

% of ODW	Arabinan	Galactan	Glucan	Xylan	Mannan	TOT
Wheat	1.8	1.3	22.2	13.8	1.6	41
Rye	2.2	1.3	33.3	20.3	2.5	60
Barley	2.5	1.5	35.6	20.1	2.5	62
Canola	0.9	2.0	26.6	12.2	3.3	45
Ext. Canola	0.6	1.4	25.7	12.3	3.0	43

4.2.2 BMP tests

The BMP test performed on the straw samples with a variation in chemical composition (Straw Group B) resulted in the graph shown in Figure 8.
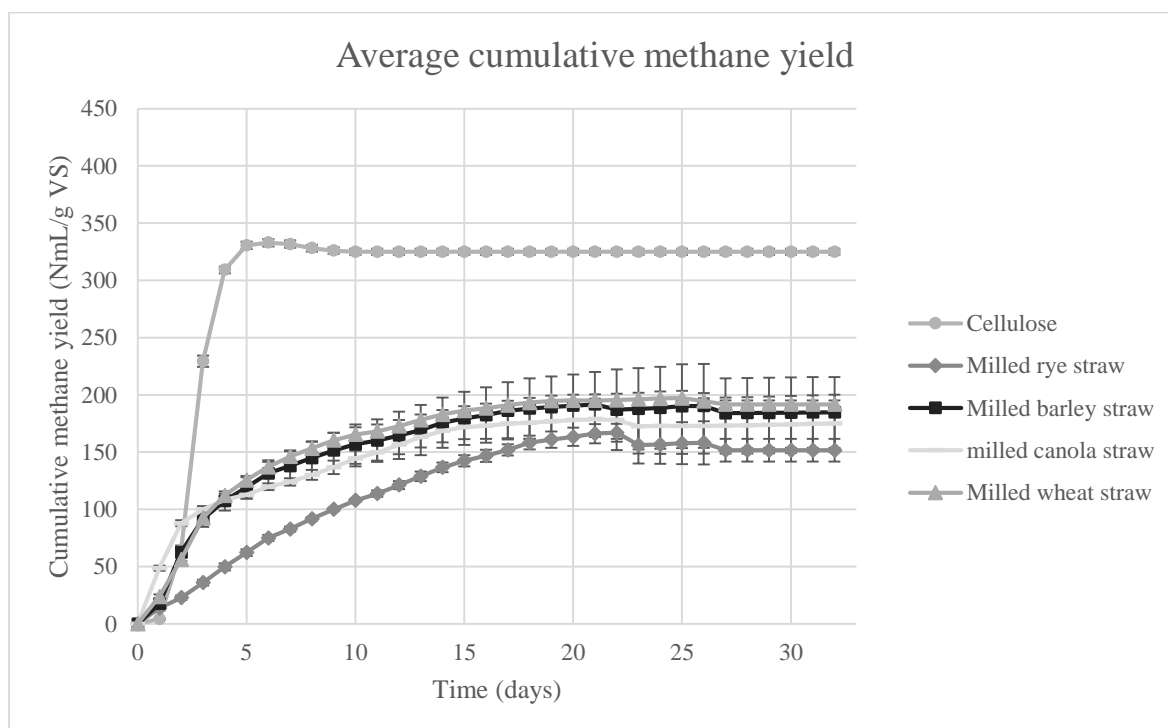

Figure 8. The cumulative methane yield generated by the BMP test, over 30 days.

Just like the first BMP test (Experiment A), the cellulose reached its maximum methane potential very quickly. As for the four straw samples, the cumulative methane yield lie between 150-200 NmL CH$_4$/g VS in the following order: Wheat > Barley > Canola > Rye. The biggest difference between the different straw types can be noticed in the beginning of the fermentation where canola straw started generating methane gas before any other substrate. The rye straw was clearly the biomass with the slowest methane production rate.

According to the chemical composition analysis (see Table 5), rye straw had the highest lignin content which could be an inhibiting factor to the methane production. However, the lignin content in rye only exceeded the lignin content in barley with 2 percentage points (pp.). A bigger difference can be noticed in the *other compound* content which measured 16, 26 and 35% for barley, canola and wheat straw, respectively, meanwhile the same fraction only measured 12% for rye straw. Since this fraction was only a result of a mass balance, it includes most of the compounds that have not been quantified, such as proteins and fats. Hence, the greater methane yield could be because of possible fat and/or protein contents. As mentioned earlier, the methane potential of fats is significantly bigger than the one of carbohydrates.

4.3 Part C: Enzymatic hydrolysis

The hydrolysis was run for 72 hours at 50°C. It was very difficult to filter the samples, even after centrifugation which might indicate that the enzymes did not degrade the polymers that well. The pH level measured 5.30 in the tubes after the hydrolysis. Since that level is within the buffer interval for the 0.1 M sodium acetate, it will not be regarded as an inhibiting factor. The sugar composition analysis on the samples after the enzymatic hydrolysis, are presented in Table 7 below. These sugars were the ones that had dissolved in the water phase through degradation by the added enzymes. Therefore, the sugar content should be expressed in monomeric sugars. However, to facilitate the comparison between the results in Table 7 and the results in Table 6, concentrations are given as polymeric sugar content.

Table 7. The polymeric sugar content in the straw samples after enzymatic hydrolysis.

% of ODW	Arabinan	Galactan	Glucan	Xylan	Mannan
UnWheat	0.007	0.04	0.018	0.11	-
Wheat	0.006	0.03	-	0.24	-
ExWheat	0.12	0.11	2.2	0.95	0.25
Barley	0.0034	0.03	-	0.24	-
Enz	0.34	-	0.83	-	-

The quantification of the sugar content in the straw samples that had undergone enzymatic hydrolysis showed to be rather problematic. Even after altering the dilution factor of the samples when preparing them for the HPLC analysis, some of the sugar concentrations were still too low to be detected. The rye straw and the canola straw did not generate any results at all which is why those samples have been disregarded. However, since the amount of enzymes added to each vial (55 µL) was so low compared to the total volume (30 mL), which affects the probability of a substrate to bind to the enzyme, the enzymatic hydrolysis reaction rate was not expected to be very high. The reason to why the xylan (xylose) content showed to represent the highest sugar content, could be that hemicellulose is more amorphous than cellulose and thereby more easily available to the enzymes. Initially, the amount of water soluble sugars in the straw

samples were assumed to be negligible. However, it is very uncertain how much that fraction would have contributed to the concentrations in Table 7.

Table 8 shows the calculated total digestibility of xylan (hemicellulose) in the straw samples (both Straw Group A and B) compared to the measured methane yield achieved from the same samples. The methane yield is given as both *NmL CH₄/g VS* and *% of maximal BMP*. The latter term is based on the measured total carbohydrate content in each type of straw, where the carbohydrates were all assumed to have a maximum BMP of 415 NmL CH₄/g carbohydrate. Since many results from the HPLC measurements were inconclusive, only the digestibility of xylan to xylose has been calculated and is presented in the table.

Table 8. The measured methane yield in comparison to the enzymatic digestibility of all prepared straw samples.

	Methane yield (NmL CH₄/g VS)	Methane yield (% of max. BMP)	Digestibility (% of xylan)
Untreated wheat straw	170	97	0.80
Milled wheat straw	200	114	1.7
Extruded wheat straw (Straw4)	220	125	6.9
Milled Rye straw	160	61	-
Milled Barley straw	190	72	1.2
Milled Canola straw	180	90	-

When comparing the methane yield (in NmL/g VS) to the digestibility (of xylan to xylose), there is a noticeable coherence between the different straw samples. If arranging the straw samples according to the measured cumulative methane yield (in NmL/g VS) it would results in: Extruded wheat straw > Milled wheat straw > Milled barley straw > Untreated wheat straw. If arranging the same samples according to the calculated digestibility it would result in: Extruded wheat straw > Milled wheat straw > Milled barley straw > Untreated wheat straw. This is the same order in which the samples were ranked for their methane yield. Cui et al. (2011) investigated the correlation between the same parameters (methane yield vs. digestibility) presented results where an achieved cumulative methane yield of 150 NmL/g VS generated a corresponding xylan digestibility of 9%, and a methane yield of 100 NmL/g VS generated a corresponding xylan digestibility of 11% . The BMP tests were performed at 37°C for 30 days and the enzymatic hydrolysis was performed at 50°C for 72 h (Cui, et al., 2011). The substrate in this case was spent wheat straw and wheat straw, respectively. In this project, the difference in xylan digestibility has been higher (6 pp.) when the increase in cumulative methane yield was 50 NmL/g VS (extruded straw compared to untreated straw).

Studying the methane yield expressed as % of maximum BMP of the results generated by the wheat straw, it can be observed that the yield has been calculated to above 100% for the milled and extruded wheat straw. This is of course impossible which indicates that an error has been made when measuring and/or calculating the carbohydrate content in wheat straw. As explained earlier, the filtrates of the wheat straw samples were accidentally diluted during the fibre analysis, hence the actual carbohydrate content is probably higher. A higher carbohydrate content would decrease the methane yield to more reasonable numbers (<100%). However, the tendency amongst the three different wheat straw sample is still the same when comparing to the

methane yield given as NmL CH_4/g VS and to the digestibility; Extruded wheat straw > Milled wheat straw > Untreated wheat straw. The most important reason to the observed tendency is probably the increase in density, the increase in particle surface area and the carbohydrate/lignin content in the raw material.

In conclusion, the results suggest that the stated hypothesis (paragraph 1.2 Aim) can be confirmed. However, the statistical certainty of the analysis leading up to this conclusion is very vague and the repeatability should be tested further in order to determine if this characterization method is reliable.

5 Conclusion

Based on the analysis that have been carried out throughout this project, it can be concluded that differences in physical properties as well as chemical properties in straw can have an influence on the methane yield during a BMP test. An increased density and thereby a decreased floating capacity, achieved by e.g. mechanical pre-treatment such as extrusion, will generate a higher cumulative methane yield. The chemical properties of the straw, such as protein and fat content, have to be further investigated in order to strengthen any theory to why the straw types generated different cumulative methane yields.

The similar tendencies in cumulative methane yield versus the xylan digestibility suggest that enzymatic hydrolysis of lignocellulosic biomass could be a potential characterization method for biogas raw materials. An increase in cumulative methane yield amongst the physically altered straw samples also generated an increase in xylan digestibility.

Since further statistical analysis are needed in order to strengthen this theory, the stated hypothesis "A higher sugar yield from enzymatic hydrolysis of straw indicate that a higher methane yield from anaerobic digestion of the same straw could be expected" could be neither rejected nor confirmed.

6 Future work

This project has mainly been about identifying parameters for properties in straw that can be measured and correlated to its bio-methane-potential. One of the biggest challenges was to create straw samples with different properties. Since a BMP test took 30 days to carry out, it was first after a significant amount of time that it was possible to determine whether the alteration of a chosen parameter mattered for the straw methane production. If no difference could be detected, the enzymatic hydrolysis test on those straw samples were of no use. Having analysed the choice that was made regarding in which order the experiments were executed, one conclusion can be drawn; a lot of time would have been saved if the enzymatic analysis were run on the straw samples prior to the BMP tests. As the hypothesis was about detecting a correlation between those experiments, the change of strategy should not affect the results negatively. For further investigation of the stated hypothesis, it is recommended to execute the enzymatic hydrolysis experiment prior to the BMP test.

Also, the straw itself was very difficult to run tests on. Since only about 1g of straw per BMP bottle was used, there was no guarantee that the chosen samples were representative for the straw type; especially when it came to the untreated one. When running the enzymatic hydrolysis tests, only 1 g straw was used per bottle as well. The reason in this case was that the bottles were literally too small to fit any more. A small amount of straw also meant that the amount of enzymes added to the bottles was minimal which increased the risk of human errors and inconclusive results. This error factor should be studied further, possibly with larger volumes that allow a larger amount of sample. It is also important for the method that the glucan digestibility is determined since cellulose is the most rigid compound in lignocellulose.

Mechanical pre-treatment of lignocellulosic biomass was the backbone to this project. Therefore it was desirable to, at first hand investigate physical properties and variations in straw. The hypothesis could have been tested with different kind of lignocellulosic biomass, instead of only straw, which might have resulted in bigger differences in methane and sugar yield. However, the developed characterization method was meant as a tool when optimizing mechanical pre-treatment methods. This thought of mechanical pre-treatment would probably be optimized on the same substrate trying to affect and alter its properties. Developing a characterization method that only can be applied when major changes are made in the pre-treatment step was therefore judged to not be enough. That is why the experiments were run on straw only.

To continue this project and investigate further identifiable parameters, the following analyses are recommended:

- Extensive statistical analysis to determine the accuracy of the characterization method
- Measurements of the straw particle size
- Measurements of the actual straw density
- Optical studies of the pore characteristics on the straw particles
- Characterization of the inoculum to enable comparison between different BMP tests
- Develop a characterization method that not only predicts the increase/ decrease of the cumulative methane yield, but also the increase/decrease of the reaction rate
- Execute scale-up experiments as well as continuous
- Perform statistical analysis to determine which chemical or physical characteristic of lignocellulosic biomass that affects the methane yield the most

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8 Appendix 1: List over utilized abbreviations

Abbreviation	Description
AD	Anaerobic digestion
AMPTS	Automatic Methane Potential Test System
AIL	Acid Insoluble Lignin
ASL	Acid Soluble Lignin
BET	Brunauer-Emmett-Teller
BMP	Biochemical Methane Potential
DM	Dry Matter
DP	Degree of Polymerization
FPU	Filter Paper Unit
HPLC	High-Performance Liquid Chromatography
NmL	Normalized mL
NREL	National Renewable Energy Laboratory
ODW	Oven Dry Weight
SD	Standard Deviation
SS-AD	Solid State Anaerobic Digestion
TS	Total Solids
VFA	Volatile Fatty Acids
VS	Volatile Solids

9 Appendix 2: Set-up of BMP test, Experiment A

To determine how much inoculum, substrate and water that was needed in order to set-up the first BMP test (Experiment A), the TS and VS contents were measured, see Table 9. The calculated amounts of inoculum, substrate and water needed for the first BMP test, Experiment A, are also presented in the table.

Table 9. The calculacted initial amounts of inoculum, straw and water that were added to the AMPTS bottles for Experiment A and their corresponding TS and VS content.

BPC01/02	TS (% wb.)	VS (% db.)	Inoculum (g)	Substrate (g)	Water (g)
Inoculum	4.5	67	66.7	-	236
Cellulose	95	100	66.7	1.05	236
Untreated	90	95	66.7	1.16	236
Straw1	93	95	66.7	1.13	236
Straw2	95	96	66.7	1.10	236
Straw3	98	96	66.7	1.13	236
Straw4	98	96	66.7	1.10	236

The needed amounts of inoculum, substrate and water were calculated with the help of the equations stated below, with the constraint to only fill the bottles with 1 g VS/100 mL water. As the headspace in each bottle was set to 200 mL, the total volume of water became 300 mL/bottle which was approximated to 300g/bottle. Additionally, the VS mass ratio between the inoculum and the substrate was set to 2:1.

$$2 \times VS_{subs.} \times TS_{subs.} \times m_{subs.} = VS_{inoc.} \times TS_{inoc.} \times m_{inoc.} \qquad \text{(Eq. 6)}$$

Where the product on the left hand side, which equals the weight of the VS in a sample, was set to 1 g in accordance to the reasoning above. The added water was calculated as shown in the equation below.

$$m_{water} = 300g - (1 - TS_{subs.}) \times m_{subs.} - (1 - TS_{inoc.}) \times m_{inoc.} \qquad \text{(Eq. 7)}$$

10 Appendix 3: Set-up of BMP test, Experiment B

The preparations for the second BMP test, Experiment B, were done in the same way as the previous test; Experiment A (Appendix 2: Set-up of BMP test, Experiment A). The calculated amounts of inoculum, substrate and water are presented in Table 10. The measured TS and VS content for each substance is also shown.

Table 10. The set-up for the second BMP tests of Straw Group B.

BPC01/02	TS (% wb.)	VS (% db.)	Inoculum (g)	Substrate (g)	Water (g)
Inoculum	4.6	67	66.7	-	236
Cellulose	95	100	66.7	1.05	236
Wheat	92	97	66.7	1.12	236
Rye	92	95	66.7	1.14	236
Barley	92	97	66.7	1.12	236
Canola	91	93	66.7	1.18	236

11 Appendix 4: Sugar recovery standard (SRS)

As a positive control for the fibre analysis, a sugar recovery standard (SRS) was prepared. The standard contains a number of different monomeric sugars, listed in Table 11, of known concentrations. The sugar concentrations were decided so that they would lay within the interval of the chemical composition of all kind of straws used (canola, barley, rye and wheat) (Pronyk & Mazza, 2012).

Table 11. The composition of the prepared sugar recovery standard (SRS) used for carbohydrate determination.

	SRS theoretical conc. (mg/mL)	Weighed amount (g) in 100 mL water	SRS conc. (mg/mL)	Type of sugar	Anhydro correction factor
D-(+) glucose	26.25	2.630	26.30	C-6	0.90
D-(+) xylose	13.5	1.3521	13.52	C-5	0.88
D-(+) galactose	1.125	0.1122	1.122	C-6	0.90
-L(+) arabinose	1.125	0.1128	1.128	C-5	0.88
D-(+) mannose	1.050	0.1027	1.027	C-6	0.90

The theoretical concentrations signify the sugar content wanted in the SRS solution, and not the corresponding amount in straw. The theoretical concentration was calculated so that 4 mL standard would be the necessary amount to add to the NREL tubes with a total of 87 mL liquid. The following equation was utilized:

$$C_{monomer} = \frac{m_{straw} \times \% Sugar}{V_{srs}}$$
(Eq. 8)

Where $C_{monomer}$ is the desired monomeric sugar concentration in the SRS solution, m_{straw} is the sample weight in the NREL tubes (=0.3g), *%Sugar* is the theoretical polymeric sugar mass fraction in straw and V_{SRS} is the needed SRS volume to be added to the NREL tube (=4 mL). As the resulting sugar concentrations was also measured in the HPLC, the calculations only had to be approximate.

12 Appendix 5: Carbohydrate determination

The HPLC measured the monomeric sugar concentration in the liquid solution achieved from hydrolyzing straw with sulfuric acid. The SRS samples provided information about how much sugar had been degraded further to smaller organic compounds. The quotient called recovery average, $\% R_{avg.sugar}$, between the recovered sugar concentration $C_{SRS,HPLC}$ (detected by HPLC) and the initial sugar concentration $C_{SRS,Before}$ (prior to hydrolysis) was calculated as:

$$\% R_{avg.sugar} = \frac{C_{SRS,HPLC}}{C_{SRS,Before}} \times 100 \qquad \text{(Eq. 10)}$$

This quotient was calculated for each monomeric sugar type. Then, the recovery average was applied to the measured sugar concentrations in the samples with unknown concentrations (straw samples). Taking both the dilution factor, *D*, and the correct anhydro correction factor, *Af*, into account, the polymeric sugar concentrations $C_{Straw,Polymer}$ was calculated as:

$$C_{straw,polymer} = \frac{C_{straw,HPLC} \times D \times Af}{\% R_{avg.sugar} / 100} \qquad \text{(Eq. 11)}$$

Where the concentrations were given as mg/mL. Finally, the polymeric sugar content, *% Sugar,* in the straw was calculated as:

$$\% Sugar = \frac{C_{straw,polymer} \times V}{ODW_{straw}} \times 100 \qquad \text{(Eq. 12)}$$

Where *V* is the volume of the filtrate (=86.72 mL) and ODW_{straw} is the oven dry weight of the straw samples that were added to the NREL tubes.

The anhydro correction factors for every monomeric sugar is presented in Table 12.

Table 12. The anhydro correction factors for every type of sugar.

	Type of sugar	Anhydro correction factor
D-(+) glucose	C-6	0.90
D-(+) xylose	C-5	0.88
D-(+) galactose	C-6	0.90
-L(+) arabinose	C-5	0.88
D-(+) mannose	C-6	0.90

13 Appendix 6: Enzymatic hydrolysis

The total volume of the slurry in each tube was set to 30 mL out of 50 mL and the amount of straw sample was set to 1 g (db.). The enzyme cocktail used in this experiment was called Cellic® CTec3 and had an activity of 187 FPU/g solution, where FPU stands for filter paper unit. As the desired enzyme concentration was set to 10 FPU/g sample (db.) the amount of added enzymes could be calculated as:

$$m_{enzyme} = m_{sample(db.)} \times \frac{10 \, \text{FPU/g sample (db.)}}{187 \, \text{FPU/g enzyme}} \qquad \text{(Eq. 13)}$$

Where m_{enzyme} is the amount of enzyme solution and $m_{sample \, (db.)}$ is the amount of sample on dry basis. The enzyme solution as well as the buffer solution were assumed to have the same density as water; 1.00 g/mL. With this assumption, the total buffer volume could be calculated as:

$$V_{buffer} = V_{tot} - \frac{m_{enzyme}}{\rho_{water}} - (1 - TS) \times \frac{m_{sample(wb.)}}{\rho_{water}} \qquad \text{(Eq. 14)}$$

Where V_{buffer} is the added volume of buffer per tube, V_{tot} the total slurry volume in a tube (30 mL), ρ_{water} the density of water, TS the total solids content in the sample and $m_{sample \, (wb.)}$ is the amount of sample in a tube (wb.). The enzyme concentration is usually set to 10 FPU/g WIS, where WIS means water insoluble solids, but in the case of very dry substrates, such as straw, it can be approximated to the oven dry weight (ODW).

To know how well the enzymes have succeeded in degrading the polysaccharides to their corresponding monomers, the digestibility was calculated accordingly:

$$Digestibility(\%) = \frac{m_{monomer}}{Af \times m_{polymer}} \qquad \text{(Eq. 15)}$$

$m_{monomer}$ is the amount of monomeric sugar derived from the straw sample through enzymatic hydrolysis, Af the anhydro correction factor and $m_{polymer}$ the initial amount of polymeric sugar in the straw sample.

14 Appendix 7: Popular science summary (Swedish)

Populärvetenskaplig sammanfattning av examensarbetet *Utveckling av karaktäriserings-metoder för lignocellulosarika biogassubstrat.*

Smältande isar, extrema naturkatastrofer och rekordhöga temperaturer; det rapporteras dagligen om nya händelser som påstås ha orsakats av den globala uppvärmningen. De flesta svenskar är numer familjära med uttrycket och miljömedvetenhet är något som blir mer och mer uppmuntrat. Den mest välkända påverkande faktorn är användningen av fossila bränslen. Biogas är ett av flera miljövänligare alternativ som det i dagsläget bedrivs mycket forskning kring.

Biogas består till största del av koldioxid och metan, där metangasen är den enda brännbara beståndsdelen. Gasblandningen görs oftast från matrester från livsmedelsindustrin eller från avloppsslam vid vattenreningsverk. Det enda kravet på råvaran är att den ska innehålla fett, protein eller kolhydrater, för att då kunna brytas ned till metan av tillsatta mikroorganismer. Denna speciella sorts mikroorganismer lever under syrefria förhållanden vilket tillåter metanbildning. Eftersom halm är en stor restprodukt från jordbruksindustrin, undersöks nu möjligheten att framställa biogas från den.

Halm innehåller en stor del kolhydrater i form av lignocellulosa. Lignocellulosa består av tre olika ämnen; lignin, cellulosa och hemicellulosa. Medan cellulosan och hemicellulosan kan beskrivas som långa kedjor av sammanbundna sockermolekyler, är ligninet uppbyggt av ringformade alkoholer. Tillsammans bildar de väldigt stabila fibrer i cellväggen på växter och utgör deras motsvarighet till människans skelett. Dessa fibrer är därför också väldigt svåra för mikroorganismerna att bryta ned till mindre sockerarter, såsom glukos och xylos, för att vidare kunna omvandla dessa sockerarter till metan. Dessutom är ligninet icke nedbrytbart och utgör ett stort hinder. Förutom problematiken kring lignocellulosans stabila strukturer och relativt höga ligninhalt så innehåller halm också mycket luft. Den innestängda luften resulterar i att halmen lätt flyter upp till ytan inuti en fermentortank. Detta fenomen minskar kontaktytan mellan mikroorganismerna, som befinner sig i vätskefasen, och halmen. På så sätt försvåras biogastillverkningen och produktionstiden förlängs markant. I en produktionsprocess innebär en förlängd produktionstid också ett krav på större tankar vilket ju påökar materialkostnaderna samt energibehovet för uppvärmning för en sådan produktion.

För att lösa dessa problem finns det olika sorts förbehandlingsmetoder vars syften oftast är att pulverisera halmen till mindre beståndsdelar så att dess densitet höjs och att den därigenom sjunker. Optimeringen av dessa förbehandlingsmetoder kräver att halmpulvret ska kunna utvärderasmed hjälp av en karaktäriseringsmetod. Idag utförs oftast s.k. biokemiska metanpotentialtester där man i labbskala testar hur mycket metan som kan fås ut från ett halmprov. Dessa tester tar dock 30-50 dagar och det finns en önskan om en mer tidseffektiv karaktäriseringsmetod. Via ett examensarbete har nu enzymatisk hydrolys, där man tillsätter enzymer som bryter ned halmen till mindre sockerarter varvid nedbrytbarheten av halmen kan mätas, undersökts som en potentiell karaktäriseringsmetod. Resultaten visade på en korrelation mellan hemicellulosans nedbrytbarhet och dess metanpotential, samt dess egenskaper såsom halmens totala yta, densitet och kemiska sammansättning. För att stärka karaktäriseringsmetoden ytterligare krävs däremot fortsatta studier och omfattande statistiska analyser.