Final report

1.1 Project details

Project title	EUDP-10-Silage pretreatment of green crops for 2nd generation bioethanol production
Project identification (program abbrev. and file)	EUDP-10 Journalnr.: 64010-0005
Name of the programme which has funded the project	Energiteknologisk Udviklings- og Demonstrations Program - EUDP
Project managing company/institution (name and address)	Technical University of Denmark (DTU) Technical University of Denmark Dept. of Chemical and Biochemical Engineering Building 229, DTU, 2800 Lyngby (Project was initiated at "DTU Risøe", but transferred due to Department mergers 01.01.2012)
Project partners	DTU Novozymes A/S (Company) DLF Trifolium (Company)
CVR (central business register)	CVR/VAT number 30060946
Date for submission	Aug 31. 2015

1.2 Short description of project objective and results

Dansk: Nye metoder til forbehandling og omdannelse af plantebiomasse til energiformål er en afgørende forudsætning for det biobaserede samfund, idet nuværende teknologier er energikrævende og dyre. I dette projekt var tesen, at ensilering, dvs. anaerob våd solid state fermentering med mælkesyrebakterier, kunne være en forbehandlingsmetode for græs til bioenergiformål. Projektet var et samarbejde mellem DLF Trifolium, Novozymes A/S og DTU Kemiteknik, og viste: 1) At ensilering forbedrer cellulose-nedbrydning af græs-biomasse, men ikke kan stå alene som forbehandlingsmetode til at forbedre enzymatisk cellulosenedbrydning af græs biomasse 2) at ensilering u-undgåeligt medfører tab af kulhydrat i biomassen, men 3) at ensilering kan være en indledende forbehandling før varmebehandling af biomassen (hydrotermisk forbehandling) da temperaturen for varmebehandlingen kan sænkes. For grønt græs viste arbejdet, at effekten af ensilering varierer med høsttidspunktet for græsset, primært fordi græs-biomassens sammensætning varier hen over sæsonen og med græssets modenhed. Den samlede konklusion er, at ensilering kan være en nyttig teknologi integreret med opbevaring af biomasse til videre forarbejdning til energiformål.

English: New methods of pretreatment and conversion of plant biomass for energy is a prerequisite for the bio-based society, as current technologies are energy-intensive and expensive. This project examined whether ensiling treatment, ie anaerobic wet solid state fermentation with lactic acid bacteria, could be a pretreatment method for grass for bioenergy purposes. The project was a collaboration between DLF Trifolium, Novozymes A/S and Department of Chemical Engineering DTU, and showed: 1) silage improves cellulose degradation of grassland biomass, but cannot stand alone as a pretreatment method for improving enzymatic cellulose degradation of grass biomass 2) that ensiling un-avoidably causes loss of carbohydrates in biomass, but 3) that ensiling can function as an initial pretreatment before heat treatment of the biomass (hydrothermal pretreatment) as the temperature of the heat treatment can be lowered. For green grass the work showed that the effect of silage varies with harvest time of grass, mainly because grass biomass composition varies over the season and with the grass maturity. The overall conclusion is that silage can be a useful technology integrated with storage of biomass for further processing for energy purposes.

1.3 Executive summary

In this project, ensiling was evaluated as a method of pretreatment for subsequent enzymatic saccharification of cellulose and hemicellulose in grassland biomass, by using the temperate grass *Festulolium* Hykor. Assessments were also made on wheat straw biomass. The method was additionally combined with hydrothermal treatment, in order to decrease the required severity of an industrially applied pretreatment method.

The first part of the project was devoted to method development for controlled grass ensiling at lab scale. A simple and flexible standard method for laboratory ensiling with a high reproducibility was developed, which is well suited for high-throughput experiments.

A comprehensive study on important parameters in ensiling was conducted to identify optimal ensiling conditions providing the best possible pretreatment effect. The parameters were biomass composition, four different seasonal cuts of grass, different dry matter (DM) content at ensiling, and an addition of different lactic acid bacteria species for the ensiling. The study confirmed that ensiling does work as a partial pretreatment and can improve the enzymatic cellulose convertibility of grass. Low dry matter ensiling was found to improve the effects of pretreatment due to a higher production of organic acids in the silage. The effect of applied lactic acid bacteria species was, however, insignificant. Cellulose conversion was noted to be largely determined by the stage of maturity of the four different cuts of grass. Less mature grass had high convertibility but less cellulose and vice versa. It was concluded that an optimal maturity of grass exists, which gives an optimal glucose release. However, limitations of the method were also noted. The ensiling of grass came with a considerable loss of water soluble carbohydrates (WSC), which was higher than the improved glucose release. Furthermore, the amount of released glucose was not adequate to support an efficient production of ethanol. Lastly, the conversion of xylan was extremely low in both grass and grass silage.

Optimization of the enzymatic saccharification of grass was attempted through improvement of the hemicellulase content in the enzyme blend. However, neither additional xylanases (Cellic HTec2® and ß-xylosidase) nor hemicellulose degrading esterases (acetyl xylan esterase and ferulic acid esterase) showed any improvements of xylan or glucan convertibility. It was noted that the hemicellulose structure of *Festulolium* Hykor appeared unexpectedly resistant to enzymatic degradation. Due to the low conversion results on *Festulolium* Hykor, it was concluded that ensiling does not provide sufficient pretreatment effect to be a stand-alone pretreatment method for grassland biomass. Ensiling was therefore combined with hydrothermal treatment (HTT), and the pretreatment combination was applied to both grass (*Festulolium* Hykor) and wheat straw (*Triticum aestivum*), in order to compare the effect upon two categorically different biomasses. For wheat straw, ensiling in combination with HTT helped increase the HTT effect and facilitated a reduction in optimum HTT temperature of 10 to 20 °C. This could, however, not be proven for green grass (*Festulolium* Hykor), since the overall release of mono- and oligosaccharides for the combined pretreatment of grass did not exceed HTT of grass alone. This was due to a combination of high loss of water soluble carbohydrate (WSC) during silage storage of grass and only minor improvements of HTT induced by ensiling. In comparison, the ensiling of wheat straw improved cellulose convertibility by a maximum factor of 1.9 at 170 °C, where the ensiling of grass only improved cellulose convertibility by a maximum factor of 1.3. The reason for the inaccessible xylan in grass is believed to be found in a high complexity of branching and cross linkages creating a heterogeneous and resistant grass hemicellulose. However, further studies are necessary.

Utilisation: The study concludes that ensiling may provide a pretreatment effect, depending on the silage conditions and the recalcitrance of the biomass. However, ensiling will always be at the expense of an amount of WSC; and the significance of the gain from the pretreatment effect versus the loss of WSC will again depend on the silage conditions and the nature of the biomass. Ensiling was proven not to be a stand-alone pretreatment of *Festulolium* Hykor but should be considered as a sound method for biomass storage with possible benefits to biomass conversion. On the other hand, ensiling provided significant improvements to a combined pretreatment of ensiling and HTT. Ensiling is not merely a pretreatment method, but an integrated storage and pretreatment method with positive effects on agricultural management, biomass feedstock logistics, and biomass conversion.

1.4 Project objectives

The overall aim of the study was to investigate and evaluate ensiling as a pretreatment method for grass biomass to help utilize plant biomass glucose from cellulose for energy purposes. The measure for pretreatment effect primarily involved the degree of enzymatic saccharification of lignocellulose. The study was mainly conducted using temperate grass with the double aim then of also assessing the bioenergy potential of green grass for ethanol and biogas production. However, investigations were also carried out on wheat straw, which is the typical biomass concerning cellulosic bioethanol production in Denmark and elsewhere in Europe and partly in China. Because of the applied bioenergy technology context of the project the project was also devoted to assessing ensiling in combination with hydrothermal treatment. Ethanol fermentation and anaerobic digestion was conducted in lab scale to assess further processing of pretreated grass and grass silage into energy carriers The original project objective was exclusively to assess ensiling on grassland, temperate grass ("green grass"), but during the project it was decided to examine ensiling also on wheat straw, and to examine the enzymatic saccharification in response to ensiling in combination with hydrothermal treatment on both green grass (*Festulolium* Hykor) and wheat straw.

Project evolution and risks:
Ensiling is the classical method of forage crop preservation optimized throughout the past two centuries to provide nutrient rich animal feed all year round. Ensiling encompasses moist solid state anaerobic fermentation by lactic acid bacteria (LAB). The ensiling involves production of organic acids and a decrease in pH that consequently prevents growth of fungi, yeasts and bacteria which may otherwise decompose the carbohydrate structure in the biomass. Three main factors influence the outcome of ensiling: (i) Biomass composition; (ii) biomass DM at ensiling, and (iii) the microbial community responsible for the fermentation.

Although ensiling is thus a classic approach for forage crop preservation, its effect on grass as pretreatment was unclear when the project was initiated: Prior to the present project, ensiling as a biological pretreatment had reported very few results (four studies only) of cellulose conversion through enzymatic hydrolysis, all with the aim of producing energy carriers of either ethanol or biogas. The studies reported improved enzymatic saccharification for the ensiled biomass, but in the studies, the biomass and the conditions of ensiling varied considerably, making it difficult to derive consistent rules for optimal ensiling for lignocellulose pretreatment and for deriving conclusions of the effect: The trade-off is that for the ensiling treatment to function, some sugar must be converted by the ensiling bacteria; this use will represent a "loss" unless the ensiling effect of predigestion and lowering pH aiding further cellulose degradation, will improve the glucose yields beyond the inevitable "loss" from the ensiling. Due to the significant explorative and basic knowledge provision requirement of the project, the project was carried out as a PhD project at DTU with extensive internal collaboration and technical and academic involvement at DTU, and with both Novozymes A/S (world leading industrial enzyme supplier with a vision to produce enzymes for cellulosic energy purposes) and DLF Trifolium (major Danish Seed and Seed Science Company, the world's largest producer of clover and grass seeds) participating very actively in the project throughout, supplying supervision as well as materials in the form of enzymes and grass biomass.

The main risk factors of the project were that the idea that ensiling could enhance cellulosic biomass degradation did not hold, secondly, that green grass would not be suitable for bioenergy production after ensiling of the grass biomass. These two risk factors were known from the onset, and that was the reason for the project examining these core ideas very thoroughly and systematically in a bioenergy goal-oriented research project. The set-up with a close interaction, frequent and regular communication among the three partners, as well as the reporting to EUDP, allowed continuous adjustments and optimal project management to accomplish the project efficiently. For example, it was originally envisaged that the project would end up with a pilot scale test for energy production from grass (60 kg biomass bales), however, as it became evident that the ensiling pretreatment did not work efficiently enough to present a relevant stand-alone pretreatment, it was dec ided (and approved by the EUDP grant giver) to not conduct the pilot scale testing. Instead, further work was done to optimize the enzymatic conversion of the green grass by use of a fan of experimental, accessory enzymes in the interest of both the companies and the project. Lab-scae experimetns for bioethanol and biogas production was then conducted very systematically in response to the results obtained from the deeper examinations of pretreatment and enzymatic conversion.

1.5 Project results and dissemination of results

Ensiling refers to the process of making silage, and comprises basically of an anaerobic containment of moist agricultural biomass, wherefrom the biomass undergo acidic fermentation resulting in production of silage**;** The purpose of ensiling is to preserve agricultural crops and prevent them from rotting. Crops with a moisture content higher than 20% (<80% DM) are naturally subjected to spontaneous degradation by aerobic bacteria, yeasts, and fungi, e.g. known from the process of composting. In contrast ensiling preserves the biomass through an anaerobic solid state fermentation by lactic acid bacteria (LAB), which produce organic acids and decrease pH (Fig 1). The acidification inhibits growth of microorganisms and prevents biomass degradation. Biomass can therefore be stored as silage for an extended amount of time until use.

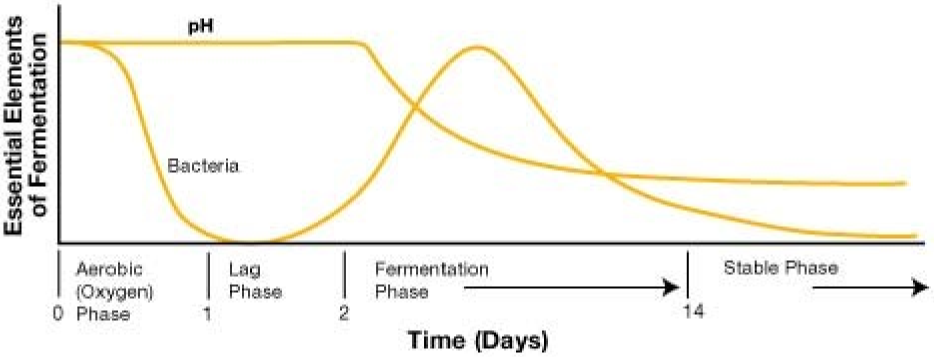

Figure 1. Sketch Evolution pH and bacterial growth as a function of days after ensiling.

Source: Nutritional Ecology of the Ruminant, P. Van Soest, Cornell University Press, 1994, p. 217.

Even though ensiling is a solid state fermentation, it still takes place in the liquid state of the biomass, thus biological, and chemical processes are highly affected by the availability of water. Notably, increased amounts of water increase the mobility of soluble compounds. For the fermenting microorganisms water is a determining factor for both growth rate and minimum pH tolerance. At low DM the growth rate are higher, and the pH tolerance lower. This means that lactic acid production is faster and terminal pH is lower, at lower DM. DM content is also a controlling factor for the growth of the competing microorganisms, which pose a risk to a successful silage fermentation. Low DM ensiling has an increased risk of clostridia contamination due to the higher water activity, where both LAB and clostridia thrive. At higher DM clostridia is efficiently inhibited, but here the risk of yeast and fungi contamination increases due to their higher tolerance to low water activity.

Biomass; the idea of ensiling as biomass pretreatment: The microbial activity and acidic conditions during the ensiling and long-term storage of silage are known to degrade the structure of the biomass to a certain degree. These changes in the lignocellulosic structure suggest that ensiling can be applied as a biological pretreatment method for further conversion of lignocellulosic biomass. The type of biomass reacts considerably different to the silage treatments, some proves almost unaffected, and some undergo large structural changes resulting in high pretreatment effect. It is also an important issue for ensiling as pretreatment method for biological conversion that it is based on sugar fermentation. The goal of the pretreatment for bioenergy is to facilitate a higher release of fermentable monomers, however in order to ensile the biomass the silage fermentation require a certain amount of soluble sugars, which are irreversibly lost to organic acids. It is therefore of significant importance that a total sugar balance is considered.

Several pretreatment methods developed for cellulosic bioethanol, such as hydrothermal treatment, steam explosion, and weak acid hydrolysis, has degradation of hemicellulose as their main pretreatment effect. However these methods involves high temperatures and/or addition of chemicals, thus a high energy input, and require corrosion and pressure resistant equipment, resulting in high capital cost of the pretreatment. Pretreatment has therefore also been estimated to be one of the most costly operations in cellulosic ethanol production. Compared to such pretreatment methods, ensiling require less energy and involves lower capital costs due to the ambient temperature and pressure, all at the expense of longer reaction time, which is 'free' as long as it is incorporated into the overall logistics. Additionally, the combination of storage and pretreatment could generate new scenarios of decentralized pretreatments, which would benefit the return for the farmer, and ease conversion at the biorefinery. For this project it was chosen to work with a forage grass type biomass, which was believed to be less recalcitrant compared to mature dry agricultural residues with a high degree of lignification

It is obvious that the processed biomass is a highly determining factor for the fermentation. It is however important to know the general effects different biomasses have on the silage fermentation, in order to control the process accordingly. While it is straight forward to control the DM and what inocula is used for ensiling, it is far more complex and not fully possible to indirectly control biomass and its composition. Many ecological and biologcal biomass factors have significant effect on the silage fermentation, such as the amount and specific composition of WSC, buffering capacity of the biomass and the epiphytic microflora of unknown magnitude and composition. The traditional feed crops for ensiling contain all more or less sufficient WSC for the silage fermentation, temperate grasses contain sucrose and fructans. The significant difference between ensiling of green crops for forages and ensiling of lignocellulosic residues is, that lignocellulosic residues do not have sufficient readily available carbohydrates to facilitate the rapid and necessary lactic acid fermentation that is required for preservation at low DM. Different strategies have been applied to overcome this. Organic acids can be added directly instead of LAB fermentation, lignocellulytic enzymes can be added to releases fermentable carbohydrates from the lignocellulose or sugars can be added as substrate for LAB fermentation.

Project results compilation (all data are outlined in detail in the published papers and the PhD Thesis, see later):

Lab-scale ensiling: An initial part of the project was to establish and standardize a functional laboratory method for the ensiling of biomass.

Several laboratory techniques of experimental silage fermentation have been developed through research and development in forage preservation and silage quality for animal feed to allow multiple treatments and replications in the study. The most common approach of all methods is to keep biomass in a container (silo) thereby securing a minimum supply of oxygen for an extended time of storage. The main difference between the approaches is to use different types of silos allowing for a different amount of parameter control. A general rule of thumb in the laboratory is, however, that the more parameters we can monitor and control, the more complex the laboratory unit gets, and the less treatments and replications are possible. Due to the large deviation in practices and conditions of ensiling in the available studies, in this project, it became a goal in itself to be able to examine multiple silage conditions in relation to further biomass conversion, in order to provide specific knowledge of different silage conditions. Subsequently, it was necessary to have a method that could support high-throughput experiments in order to study the effect of multiple silage conditions at the same time. As a result the method could not be too complex and each treatment preparation too time consuming, and instead compromising a high degree of parameter monitoring and control. Based on a literature view a method using a food packaging vacuum machine and the method was tested against air tight glass jars, and found good correlations between them. However, the vacuum packaging method had the advantage of higher reproducibility and reduced labor intensity and it was more applicable for high throughput experiments It was therefore decided to use the vacuum based ensiling method in this project; The quality of the silage was evaluated based on measurements of pH and concentration of organic acids (lactic-, acetic-, propionic-, formic-, and butyric acid) as well as monosaccharides (glucose, xylose, arabinose, galactose and fructose) in water extracts from a known amount of silage. The initial work resulted in the development of a simple and flexible standard method for laboratory ensiling with a high reproducibility, and which was well suited for high-throughput experiments (Fig 2).

Figure 2: Vacuum packaging (Variovac EK10) and two silage bags with *Festulolium* Hykor

Ensiling of grass

The first study on ensiling as a biological pretreatment method was conducted in order to find conditions for ensiling which had an optimal effect on pretreatment. It is well known that biomass composition, initial DM and addition of LAB inocula, are among the parameters that have most significant effect on silage fermentation. The objective of the study was therefore to investigate the relations of these three important factors upon enzymatic saccharification of cellulose after ensiling. Four cuts of *Festulolium* Hykor (DLF TRIFOLIUM, Denmark) over the growing season from 01.06.2011 to 01.11.2011, were ensiled at three different DM concentrations (low, 21-24%; medium, 28-35%; high, 41-50%) and with three different inocula treatments, a heterofermentative (CCM), a homofermentative (GP) and a blank (Water). The biomass composition was naturally varied by seasonal change and plant maturity at harvest. See material and methods Paper I for more details.

Composition and silage quality analysis of pH and organic acids after ensiling (Fig 3 and 4) showed large deviations in-between the four cuts, which points to the fact that the silage fermentation was largely affected by biomass composition. The difference in silage fermentation of the four cuts can, however, additionally be due to differences in the amount and origin of epiphytic microorganisms, which inevitably will vary in-between the four cuts. The organic acid concentration was generally higher for the low DM treatments, and the highest concentration of around 10 (w/w)% was observed for the two less mature grass cuts, 2nd and 4th, which correlates for 2nd cut with a higher concentration of WSC (Paper I), but not for 4th cut which had a lower amount of WSC, however, this cut was significantly more immature, and thus DM digestibility would be low suggesting that substrates for silage fermentation could have come from the structural fiber e.g. hemicellulose.

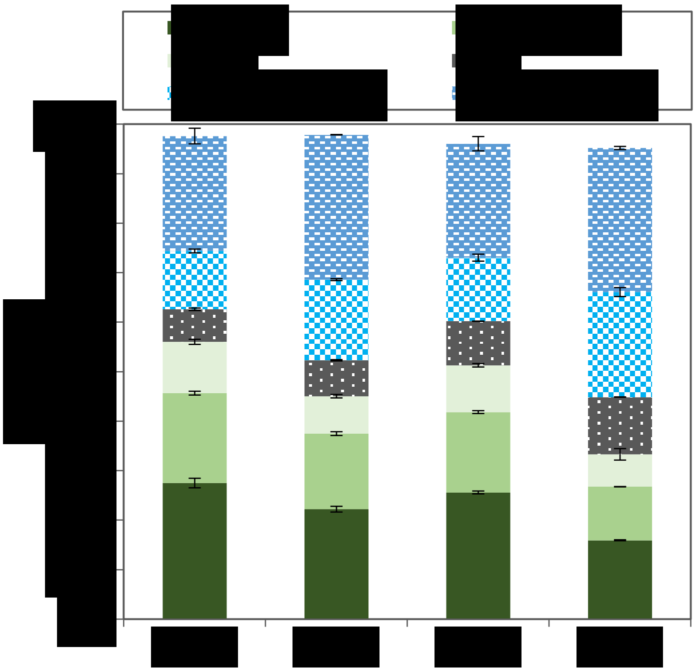

Figure 3: Composition of four cuts of *Festulolium* Hykor 2011

The organic acid concentration was generally higher for the low DM ensiling. Plotting DM against the production of different organic acids shows that lactic acid in fact increased linearly with decreasing DM ($p<0.05$), as opposed to acetic- and propionic acid. Lactic acid which was the dominating acid in all treatments and the main contribution to the trend of high organic acid concentration at low DM (Fig 5). However, plotting DM against the total organic acid production for the four different cuts reveals once again the large deviations in silage fermentation between the four cuts (Fig 5). The organic acid production decreased, however, for all four cuts at a higher DM ensiling, and the slope of the decrease was steeper for the two less mature grasses (2nd and 4th cut).

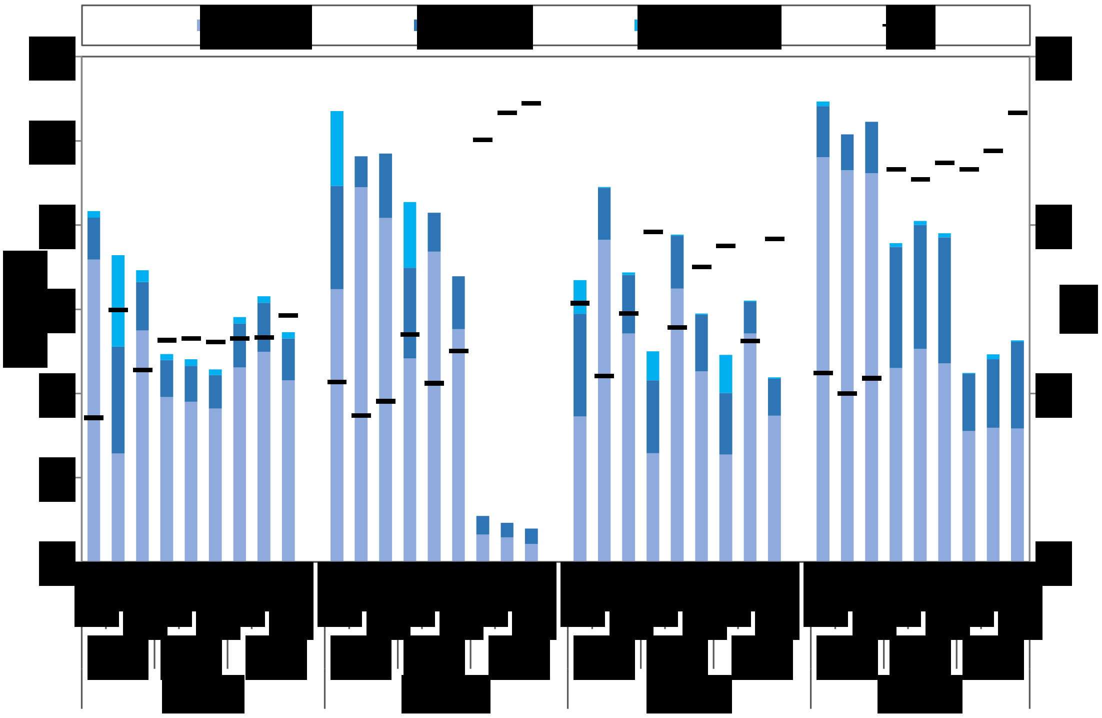

Figure 4: Organic acids and pH after ensiling; Analyzed in water extractions of silage grass. Four cuts of grass ensiled at three levels of DM (in percentage) and three inocula treatments Inocula: CCM: LACTISIL CCM (containing *Lactobacillus Buchneri*), GP: LACTISIL GP (containing *Pediococcus pentosaceus* and *Lactobacillus plantarum*), Water: No addition of LAB.

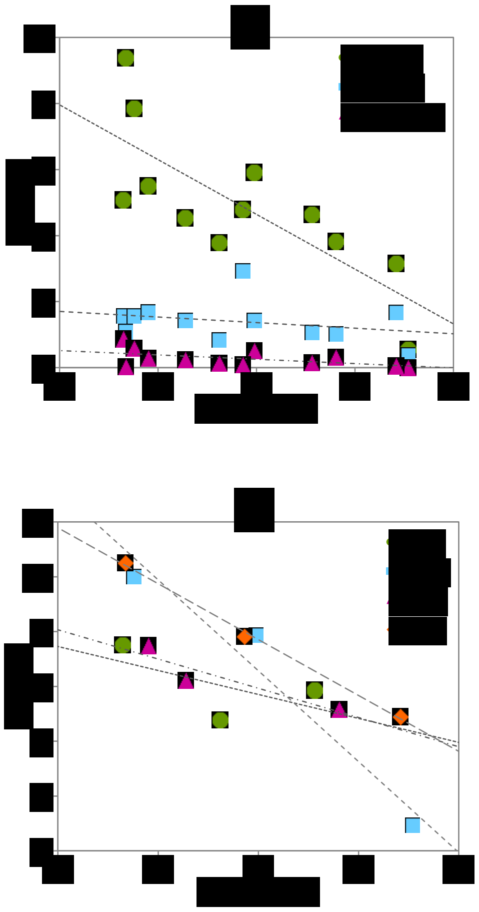

Figure 5: **A**. Main organic acids vs. DM;
B. Total organic acids vs. DM,

Combining ensiling and hydrothermal treatment
Wheat straw (Paper II) & grass (Paper III)

HTT is a well-established pretreatment method based on high temperatures (170 – 220°C) and pressure without addition of additives, which have proved advantageous to scale-up, due to its simple approach. Furthermore it is most likely that HTT could benefit from ensiling due to the organic acids brought by the silage. Pretreatment severity are largely affected by pH, and it is well known that addition of acids and lowering of pH in pretreatments at elevated temperatures, increases the severity of the pretreatment.

The combination of ensiling and HTT has previously been studied but the supplementary effect of ensiling as opposed to dry storage, was not addressed previously.

In the current project, two studies were conducted combining ensiling and HTT; one using wheat straw and one using grass (*Festulolium* Hykor). The studies were written into separate manuscripts and detailed results are presented in Paper II and Paper III, respectively. The objective of both studies was to investigate the effect of ensiling on the outcome of the HTT, as compared to dry storage at different operating HTT temperatures. The choice of applying two biomasses was to find consistencies and differences of the new approach, when using two considerably different biomasses. Wheat straw and grass distinguish each other by, wheat straw being an agricultural residue with no available WSC and harvested at high DM, and grass being a non-food energy crop (in the context of bioenergy) with plenty of WSC, and harvested at low DM. Both WSC content and DM is important to the silage fermentation and are therefore important to the process as a whole.

Hypothesis

- Organic acids in the silage produced during ensiling would induce increased pretreatment effect of the HTT, measured as solubilisation of hemicellulose and convertibility of cellulose in subsequent enzyme saccharification.
- Additionally ensiling in itself provide an effect on pretreatment, which will add to the overall efficiency of the combined pretreatment.
- The improved pretreatment effect give rise to a significant decrease in operating HTT temperature, which potentially can reduce energy consumption of the pretreatment.

Experiment

Wheat straw: Wheat straw does not contain significant amounts of WSC, it was therefore necessary to facilitate silage fermentation by other means. It was thus decided to add 7 (w/w)% WSC in the form of xylose. The high DM of wheat straw at harvest imply that also water is added to the wheat straw before ensiling. The wheat straw in this study were stored in dry bales and rehydrated before ensiling to a DM of 35%, which matches the reported operational DM in HTT at the Inbicon demonstration plant. Wheat straw was thus ensiled by addition of 7 (w/w)% xylose, addition of a LAB inoculum consisting of pure heterofermentative *Lactobacillus buchneri*, at 35% DM, and stored for 4 weeks.

Grass: Ensiling of grass was based on the results in the first experiments (above) and in Paper I. Fresh grass was ensiled at low DM (26%) and addition of the homofermentative LACTISIL GP inocula, containing *Pediococcus pentosaceus* and *Lactobacillus plantarum*. The grass silage was also stored for 4 weeks.

Hydrothermal pretreatment of ensiled and dried biomasses were performed on a pilot scale pretreatment unit 'Mini IBUS' (DTU, Denmark) at three different temperatures (170 °C, 180 °C, and 190 °C) for 10 min. at a biomass loading of 1kg DM biomass per pretreatment. Solid and liquid fractions were separated and subjected to compositional analysis. The effect of pretreatment was tested against glucose and xylose release in a following enzyme hydrolysis of solid fractions and overall release of sugars based on the analysis and mass balance. Details of materials and methods for wheat straw and grass can be seen in Paper II and Paper III, respectively

Results and discussion

The silage fermentation of both biomasses were successful, lowering pH to just below 4. Ensiling of wheat straw produced acetic acid and lactic acid concentrations of 2.8 (w/w)% and 2.4 (w/w)%, respectively, and it was observed that over 1 (w/w)% of the added xylose was recovered, suggesting that efficient silage fermentation of wheat straw could be achieved with even less amounts of xylose. Ensiling of grass produced less acetic acid (1.7 (w/w)%), but significantly more lactic acid (6.5 (w/w)%), which corresponded to the results of acid production in Paper I of 1st cut and low DM. The difference in silage fermentation is a consequence of different inocula and different substrate, which in grass promoted lactic acid production and in wheat straw promoted acetic acid.

The compositional analysis showed no significant changes in the amounts of lignocellulosic components after ensiling for any of the two biomasses, which was also observed for the grass (1st cut) in Paper I. Obviously wheat straw and grass differs largely in their composition. Wheat straw contain much more secondary cell wall (cellulose hemicellulose and lignin), here adding-up to 84% of the total DM, than grass, adding merely up to 50%of total DM. Wheat straw have 15 (w/w)% more cellulose, 10 (w/w)% more hemicellulose and 9 (w/w)% more lignin, but the ratio between the components is nevertheless similar, approx. one half cellulose, one third hemicellulose, and one fifth lignin (5:3:2). This might suggest that the different amounts of lignocellulose are equally recalcitrant The quantity of cellulose and hemicellulose have great importance to ethanol production as it comprise the potential fermentable sugars. In this regard, wheat straw have a clear advantage over grass for ethanol production. The grass contained high amounts of extractives, both as water extractives and ethanol extractives (Table 1).

Table 1: Composition biomasses before HTT. The numbers are presented in weight percentages of biomass DM, followed by standard deviations.

Biomass	**Glucan**	**Xylan**	**Arabinan**	**Lignin**	**Ash**	**EtOH extractives**	**H_2O extractives**
Wheat straw	40.2 ±0.2	22.3 ±0.1	3.3 ±0.0	18.6 ±1.1	5.2 ±0.2	6.3 ±0.2*	
Wheat straw silage	39.7 ±0.0	24.1 ±0.4	2.6 ±0.0	17.5 ±1.2	3.1 ±1.1	2.4 ±0.8	4.6 ±0.1
Grass	25.2 ±0.9	14.1 ±0.5	2.1 ±0.1	9.3 ±0.1	8.0 ±0.5	11.9 ±0.1	21.1 ±1.1
Grass silage	24.2 ±0.3	14.5 ±0.2	2.4 ±0.1	9.3 ±0.1	5.3 ±0.0	10.5 ±0.4	25.0 ±1.5

*only ethanol extraction

Water extractives include WSC (monomers and short chain oligomers), organic acids, and a large fraction of total crude proteins, but will also include minerals and silica on surface of the biomass all adding to the extracted mass. Ethanol extraction removes the hydrophobic waxy layer surrounding the cell wall, together with most cell content and the colour pigment chlorophyll, which is clearly seen as decolouring of the extracted fibers, leaving almost pure secondary cell wall.

It is important to consider the non-structural carbohydrates in grass which comprise a significant amount of the biomass, as a potential source for ethanol fermentation, due to the relatively lower amounts of cellulose and hemicellulose. Severe pretreatment could very likely result in high degradation of these carbohydrates, reduce ethanol yield and create significant amounts of inhibitory compounds such as HMF, furfural, levulinic- and formic acid. It was therefore expected that pretreatment of grass would be relatively more vulnerable to the high HTT temperatures than wheat straw. The HTT pretreatment caused solubilisation of biomass DM, which increased with the temperature.

This can be seen by a decreasing DM recovery in the solid fraction. The recovery of wheat straw DM is similar to that of wheat straw silage, but ensiling of grass led to a more pronounced decrease in DM recovery in solid fraction. The expected solubilisation of hemicellulose due to the autohydrolysis mechanism, can be observed by three means, (I) a decreasing content of xylan and arabinan in the solid fraction (

*Table*2), (II) an increasing concentration of xylose and arabinose in the liquid fraction (

*Table*3), and (III) a calculated recovery of hemicellulose based on the two former and the mass balance. The latter gives additionally also the degree of hemicellulose degradation during HTT.

Table 2: Composition of solid fraction after HTT. The numbers are presented in weight percentages of DM in solid fraction, followed by standard deviations. DM recovery in solid fraction is the percentage of DM left as solid fraction after HTT pretreatment, based on mass balance over HTT pretreatment.

Biomass	**Temp. °C**	**Glucan**	**Xylan**	**Arabinan**	**Lignin**	**Ash**	**EtOH extractives**	**DM recovery in solid fraction***
Wheat straw	**170**	40.3 ±2.4	24.8 ±0.8	2.3 ±0.1	21.3 ±0.1	4.8 ±0.3		88.7
	180	45.1 ±1.5	25.2 ±0.2	2.0 ±0.0	21.6 ±0.3	4.0 ±0.2		83.4
	190	50.5 ±0.2	22.4 ±0.4	1.5 ±0.2	23.0 ±0.2	5.0 ±0.2		77.1
Wheat straw silage	**170**	40.2 ±1.0	20.1 ±1.3	1.3 ±0.2	23.0 ±0.4	4.2 ±0.0		92.3
	180	43.2 ±1.0	18.5 ±1.2	1.6 ±0.1	24.5 ±0.4	4.2 ±0.3		85.7 ±3.2
	190	54.3 ±0.6	11.8 ±0.6	0.4 ±0.0	25.9 ±0.6	4.0 ±0.1		76.5
Grass	**170**	31.2 ±0.5	18.7 ±0.4	2.7 ±0.0	18.8 ±0.6	7.4 ±0.5	11.8 ±0.4	75.3
	180	33.0 ±0.2	17.1 ±0.1	1.8 ±0.1	19.0 ±0.7	8.2 ±0.2	16.4 ±0.5	69.6
	190	35.0 ±0.4	10.3 ±0.1	1.0 ±0.0	17.0 ±0.6	8.4 ±0.1	22.8 ±0.9	61.6
Grass silage	**170**	36.9 ±0.4	19.1 ±0.1	2.7 ±0.0	13.8 ±0.6	7.8 ±0.1	15.4 ±0.2	67.5
	180	40.6 ±0.0	17.5 ±0.4	1.3 ±0.0	13.5 ±0.1	6.8 ±0.1	18.2 ±0.8	61.5
	190	42.8 ±1.1	10.0 ±0.2	0.5 ±0.0	13.9 ±0.3	6.8 ±0.0	24.5 ±0.6	58.8

* The percentage of DM left as solid fraction after HTT pretreatment, based on mass balance over HTT pretreatment.

Ensiling of wheat straw combined with HTT gave high xylose concentrations matching the lower recovery in solid fraction (Table 2 and 3). In contrast both HTT of grass and grass silage gave high xylose concentrations, but HTT of grass gave in addition also a higher glucose concentration, believed to origin from the WSC.

Concerning organic acids in the liquid fractions, it was evident that the high concentration of lactic acid in the grass silage also gave significantly higher amounts of lactic acid in the liquid fraction after HTT. Acetic acid was likewise observed to be highest in the liquid fraction of grass silage. Organic acids can potentially be inhibitory for the ethanol fermentation, due to diffusion of undissociated acids across the yeast cell membrane which can be relevant if the liquid fraction is utilized for ethanol fermentation in a combined C6 and C5 fermentation. The inhibitory level of lactic- and acetic acid in ethanol fermentation has been reported, at a solid loading of 25% and at pH 5, to start from 4.0 (w/v)% and 0.3 (w/v)% of lactic- and acetic acid, respectively (Graves et al., 2006). The maximum amount of lactic- and acetic acid in the liquid fractions of around 5.07 (w/w)% and 1.52 (w/w)% of raw biomass DM, respectively corresponds to concentrations of 2.05 and 0.65 (w/v)%, respectively, at a solid loading of 25% and assuming that the liquid and solid are not separated after pretreatment.

Table 3: Composition of liquid fraction after HTT. The numbers are presented in weight percentages of DM raw biomass, followed by standard deviations.

Biomass	**Temp. °C**	**Glucose**	**Xylose**	**Arabinose**	**Lactic acid**	**Acetic acid**	**HMF**	**Furfural**
Wheat straw	**170**	0,38	0,76	0,25	0,00	0,05	0,001	0,009
	180	0,62	1,54	0,49	0,00	0,10	0,003	0,025
	190	0,82	5,18	0,68	0,00	0,21	0,009	0,097
Wheat straw silage	**170**	0,37	3,55	0,50	0,27	0,40	0,006	0,086
	180	0,60	6,55	0,71	0,35	0,58	0,014	0,228
	190	0,69	7,51	0,80	0,39	0,68	0,023	0,396
Grass	**170**	2,40	0,94	0,51	0,02	0,31	0,108	0,002
	180	3,45	2,76	1,01	0,13	0,59	0,233	0,011
	190	4,49	7,67	1,69	0,10	1,19	0,407	0,055
Grass silage	**170**	0,59	1,89	0,82	3,74	0,94	0,052	0,016
	180	1,02	5,22	1,23	5,07	1,39	0,083	0,036
	190	0,97	6,15	0,97	4,65	1,52	0,134	0,079

There was a distinctive difference in formation of the degradation products from glucose and xylose, HMF and furfural, respectively, between wheat straw and grass. It was observed that ensiling of wheat straw combined with HTT gave increased amounts of furfural, corroborating the higher release of xylose in these treatments. In contrast mainly HMF was produced during pretreatment of grass, and here ensiling had a significant decreasing effect. The highest concentrations of furans were therefore found in HTT of wheat straw silage and HTT of grass both at 190 °C. At a solid loading of 25% and assuming the corresponding liquid fraction, would result in concentrations of furans of 0.14 (w/v)% and 0.18 (w/v)%, respectively. This is however still below inhibitory levels (Paper II, Paper III).

Hemicellulose solubilisation is not pronounced for HTT of wheat straw, while HTT of wheat straw silage causes a considerably more solubilisation of hemicellulose, especially at 190°C, which also results in a significant hemicellulose degradation of 35%.
For grass, the hemicellulose solubilisation is not much different between HTT of the ensiled or the non-ensiled biomass. The content in the solid fraction are exactly the same (Table 4), but differences in the xylose concentration in the liquid fractions, show that solubilisation were more pronounced at HTT 170 °C and 180 °C for grass silage, but at 190 °C the xylose concentration is highest for HTT of grass which is explained by a higher degradation of hemicellulose in HTT of grass at 190 °C (Fig 6). Hemicellulose content was however the main distinctive difference in the compositional analysis of the pretreated solid fraction between wheat straw and wheat straw silage.

The compositional difference in the solid fractions of pretreated grass and grass silage is different than for wheat straw. Up-concentration of cellulose was here significantly higher for grass silage and the lignin content is much lower. The large difference in lignin content is believed to be partly due to an overestimation of Klason lignin in the analysis of the pretreated grass solid fraction. Klason lignin is defined as the volatile acid insoluble material left after strong acid hydrolysis (72% H_2SO_4). Klason lignin can therefore be overestimated pre-precipitation of e.g. protein. Since non-ensiled grass contains considerably more intact protein, it is suspected that overestimation of lignin in pretreated grass, were the main reason for the difference. High concentration of glucan in the pretreated fiber is an advantage for ethanol production when liquid and solid fraction is separated and solely C6 sugar fermentation is conducted Thus, on this parameter, ensiling of grass provided the best improvement due to the generally higher DM solubilisation.

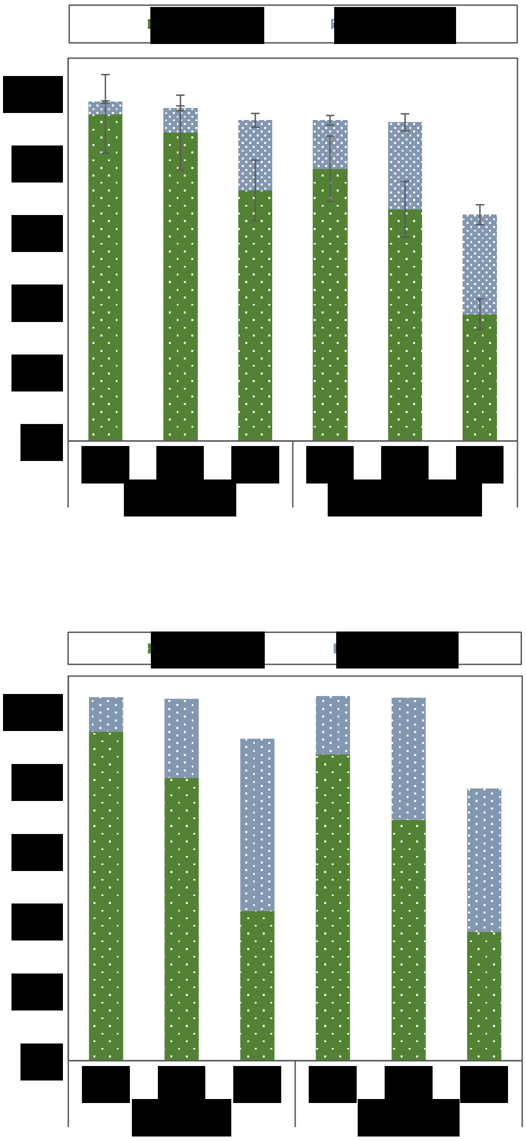

Figure 6 : Recovery of hemicellulose. Recovery of hemicellulose (xylan, arabinan) in solid fraction and liquid fraction on pretreated wheat straw and wheat straw silage at 170 °C, 180 °C and 190 °C.

The enzymatic saccharification of wheat straw, improved with temperature, especially from 180 °C to 190 °C, and the effect of ensiling was found to improve both glucan and xylan conversion significantly at the two lower temperatures. Glucan convertibility were thus improved through ensiling by a factor of 1.9 and 1.8 at 170 °C and 180 °C, respectively. The effect at 190 °C was, however not significant for glucan, and the xylan conversion was heavily hampered by the hemicellulose degradation. The results on wheat straw is in line with the hypothesized reduction in HTT temperature facilitated by ensiling, since glucan and xylan convertibility from the combination of ensiling and HTT at 170 °C, equals the convertibility from HTT of dry wheat straw at 190 °C.

Enzymatic saccharification of pretreated grass was not as straight forward as for the pretreated wheat straw. In Paper III it was found necessary to wash the solid fraction of grass and grass silage before enzymatic saccharification to avoid strong inhibition of the cellulases (Paper III). Washing improved the release of monosaccharides considerably and the results shown are thus from enzymatic saccharification on washed, wet solid fractions.

The release of glucose in the enzymatic saccharification of dry grass showed high similarity to that of dry wheat straw (Fig 7 and 8) only distinguished by a lower yield at the high temperature of 190 °C, and thus a less significant jump from HTT 180 °C to 190 °C for grass. Ensiling of grass did however, not have the same effective influence on the conversion, as ensiling of wheat straw had. The glucan convertibility were improved at 170 °C and 180 °C, but only by a factor of 1.3. At 190°C, ensiling had no effect on glucan convertibility, and xylose release decreased more in HTT of grass silage caused by the higher degradation of hemicellulose.

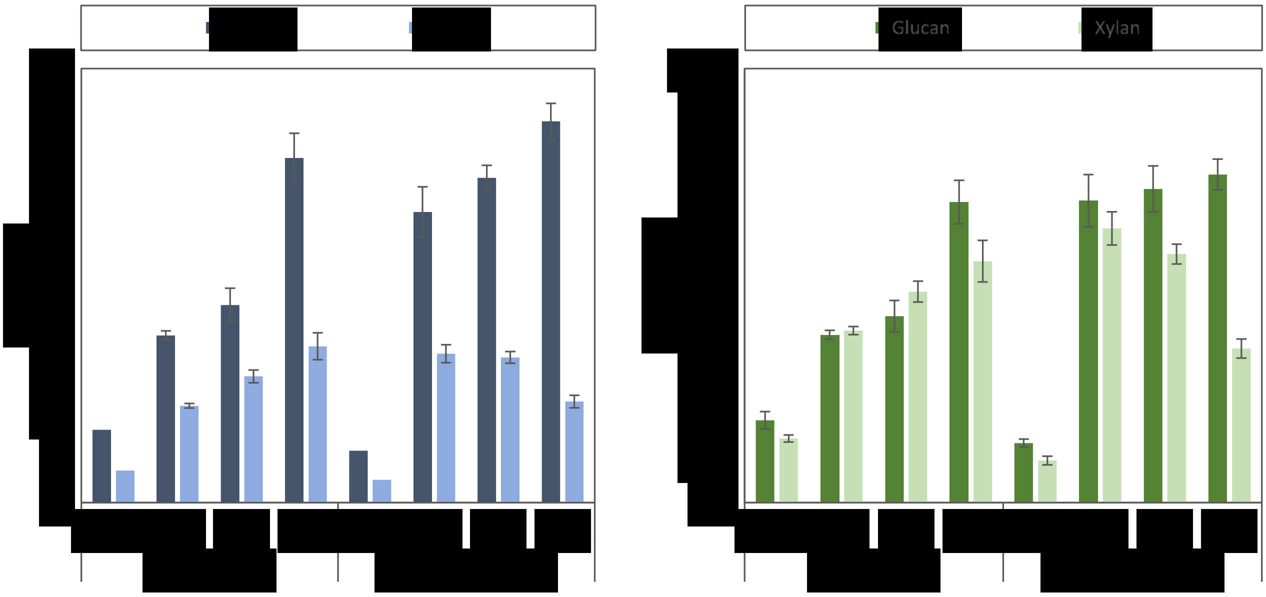

Figure 7: Enzymatic saccharification of HTT solid fractions of wheat straw and wheat straw silage. **A**: glucose (dark blue) and xylose (light blue) yields in weight percentages of DM in solid fraction. **B**: glucan (dark green) and xylan (light green) convertibility in % converted per original content in raw material.

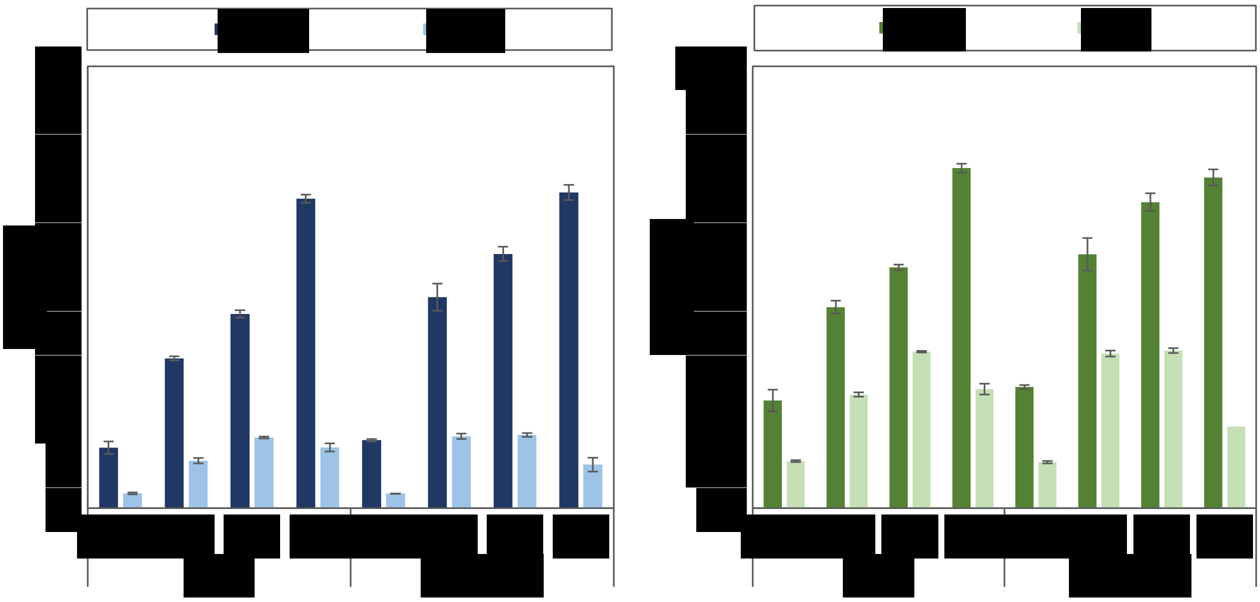

Figure 8: Enzymatic saccharification of HTT solid fractions of grass and grass silage. **A**: glucose (dark blue) and xylose (light blue) yields in weight percentages of DM in solid fraction. **B**: glucan (dark green) and xylan (light green) convertibility in percent converted polycarbohydrate per original content in raw material.

The conversion of xylan was in general lower for grass compared to wheat straw. Concerning xylan convertibility, it should be noted that data in the Figures above are given in percent converted xylan per original xylan in raw material, thus including the solubilised xylan.

The actual convertibility of xylan in solid fraction was found to be 40%, 52%, and 72%, in HTT of wheat straw at 170 °C, 180 °C, and 190 °C, respectively, and improved by ensiling to 76%, 81%, and 88%. The same numbers were found for grass to be much lower: 26%, 42%, and 60% for non-ensiled and 38%, 42%, and 44% for ensiled grass at 170 °C, 180 °C and 190 °C, respectively. It is therefore evident that grass hemicellulose was more recalcitrant than wheat straw hemicellulose. Firstly since less xylan can be converted in general, and secondly since the ensiling and the increased severity of HTT do not affect xylan conversion in the pretreated solid fraction. This result corroborates previous results of poor xylan convertibility of grass and grass silage, and underpin the proposed theory that the grass hemicellulose is highly branched and cross-linked, which induce lignocellulosic recalcitrance of the grass compared to wheat straw. The poor saccharification of xylan is likely to have an additional negative effect on the glucan conversion, since xylan and associated hemicellulosic structure hinder cellulase accessibility to cellulose.

Ensiling alone did not have an effect in any of the two experiments. For wheat straw this was in line with results found in preliminary experiments by the authors of Paper II (data not shown). For grass it was somewhat unexpected as it was in contrast to the results in Paper I. Without additional pretreatment, both grass and grass silage yielded around 7 (w/w)% glucose per DM. In Paper I, ensiling improved the yield from 7.8 to 11.4 (w/w)% glucose per DM at low DM ensiling of the compositionally comparable 1st cut. The absent effect of ensiling in the latter study (Paper III) support that the particular grass cut have a significant influence on the effect of ensiling.

The total release of monosaccharides and short chain oligosaccharides for the differently pretreated wheat straw and grass were quantified in order to evaluate on the overall effect brought by ensiling. Ensiling of wheat straw evidently improved pretreatment and facilitated significantly higher concentrations of both C6 and C5 monosaccharides for the HTT at 170 °C and 180 °C. The excess release of both C6 and C5 sugars were thus found to be 20 (w/w)% and 15 (w/w)% of DM at 170 °C and 180 °C, respectively, while for 190 °C there was a total loss of 2.2 (w/w)% of DM due to the significant degradation of hemicellulose. It can also be concluded that we gain substantially more released sugars at 170 °C and 180 °C, than the 7% xylose spent facilitating the ensiling of wheat straw, and moreover most of the excess sugar release are the easier fermentable C6 sugars. *These results suggest that ensiling very well could induce a considerable decrease in operating HTT temperature of 20°C in pretreatment of wheat straw.*

The results on pretreatment of grass did on the other hand not reflect the clear improvements seen for wheat straw. Concerning total release of C6 sugars, it can be seen that even though there is an improved effect of ensiling on the enzymatic saccharification of glucan for HTT at 170 °C and 180 °C, it does not make up for the loss of glucose and fructose associated with the ensiling, which is still present in the liquid fraction of pretreated grass. However, at 180 °C there is only a very little difference (1.4 (w/w)%) in total released C6 sugars in grass and grass silage. The results on C6 clearly show that ensiling of grass prior to HTT does not give rise to higher amounts of total C6 sugars as it was the case for wheat straw. The total release of C5 sugars are however higher for silage grass both for HTT 170 °C and 180 °C, but not for HTT at 190 °C. This is due to the increased solubilisation of hemicellulose, and a better enzymatic conversion of xylose in the case of HTT at 170 °C of grass silage. The amount of released sugars are in total more or less the same between grass and grass silage at the lower temperatures of 170 °C and 180 °C. Here the loss in C6 equals the gain in C5, which interestingly was opposite to the tendency in wheat straw.

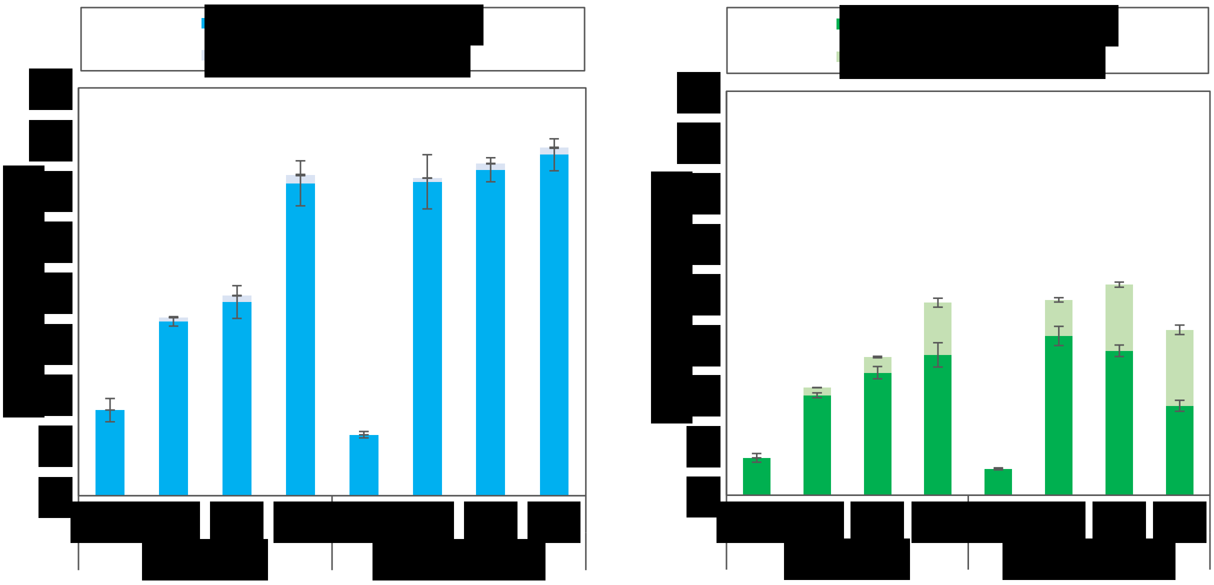

Figure 9 : Overall release of monosaccharides and short chain oligosaccharides after differently pretreated wheat straw. **A**: C6 monosaccharides (glucose, galactose fructose) in enzyme hydrolysate (turquish) and C6 monosaccharides and short chain oligosaccharides in HTT liquid fraction (light blue) presented as weight percentages of DM in raw wheat straw. **B**: C5 monosaccharides (xylose, arabinose) in enzyme hydrolysate (green) and in HTT liquid fraction (light green) presented as weight percentages of DM in raw wheat straw.

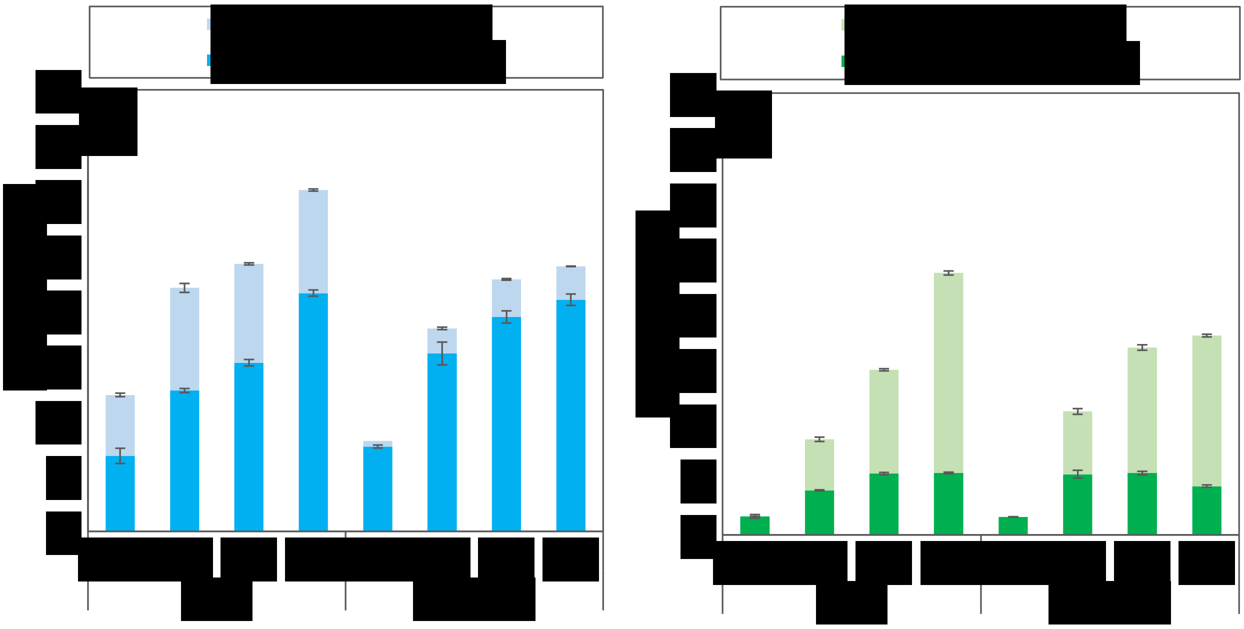

Figure 10: Overall release of monosaccharides and short chain oligosaccharides after different pretreatments of grass. **A**: C6 monosaccharides (glucose, galactose fructose) in enzyme hydrolysate (turquish) and C6 monosaccharides and short chain oligosaccharides in HTT liquid fraction including wash water (light blue) presented as weight percentages of DM in raw wheat straw. **B**: C5 monosaccharides (xylose, arabinose) in enzyme hydrolysate (green) and in HTT liquid fraction (light green) presented as weight percentages of DM in raw wheat straw.

It is important to note that the sugars represented in HTT liquid fraction includes mono- and oligosaccharides from both the HTT liquid and the wash water generated in order to remove inhibitory oligosaccharides before enzymatic saccharification. This entail that the solid fraction is washed, in order to get the presented amount of sugars into a separated liquid fraction (*Figure*). The wash water contributed with considerable amounts of C6 and C5. The contribution of C5 increased with increasing HTT temperature, and added to the overall C5 in HTT liquid fraction of both grass and grass silage. The contribution of C6 increased with decreasing HTT temperature, and added primarily to the overall liquid fraction of pretreated grass.

Table 4: Excess sugar release due to ensiling, after HTT and enzymatic hydrolysis. Presented as weight percentages of raw biomass DM

Biomass	**Temp. °C**	**C6**	**C5**	**Total**
Wheat straw	**170**	13.7	5.9	19.6
	180	13.0	2.2	15.1
	190	2.8	-5.0	-2.2
Grass	**170**	-3,7	2,5	-1,1
	180	-1,4	2,0	0,6
	190	-6,9	-5,7	-12,6

Nevertheless, the highest overall release of sugars were observed for grass pretreated at 190 °C, yielding almost 13 (w/w)% of biomass DM more than HTT of grass silage at 190 °C. Table 4. Whereas for wheat straw the highest release were observed for HTT at 180 °C of wheat straw silage.

There was a categorical difference in the way of ensiling between wheat straw and green grass, which was due to the difference in WSC content in wheat straw and temperate grass. Ensiling of wheat straw only utilized added xylose and had a primarily heterofermentative fermentation, whereas ensiling of fresh grass utilized the natural WSC and had a primary homofermentative fermentation. The amount and distribution of organic acids were therefore different in wheat straw- and grass silage, which might have had a significant effect in the HTT. However the pH was the same and the organic acid concentration was 1.6 times higher in the grass silage, suggesting, a better effect in HTT of grass silage, in contrary to the actual outcome of Paper III. The poorer effect of combining ensiling and HTT for grass, is therefore not believed to be due to the difference in silage fermentation.

The use of valuable WSC during ensiling is easier to control for wheat straw silage than for grass silage, since silage fermentation of wheat straw only utilizes what is added, but for grass the WSC pool is not limited in the same way. The main reason for the differences in the results of the two biomass feedstocks are believed to be due to significant differences in the biomass structure and especially the hemicellulosic structures associated with xylan. The grass hemicellulose is believed to be more complex in its structures, thus highly branched and cross-linked.

Bioenergy production tests

Ethanol fermentation and anaerobic digestion was conducted as a preliminary study on the further processing of pretreated grass and grass silage into energy carriers,
The results of ethanol yield (g ethanol per 100 g pretreated DM) confirmed the findings on available C6 sugars in Paper III, showing that ensiling reduced the ethanol yield for HTT at 170 °C and 190 °C, but did not cause significant difference at 180 °C. Furthermore it is clear that ensiling alone reduce ethanol yield due to the loss of WSC in the silage fermentation. The pretreatment, which gave the highest ethanol production were HTT of grass at 190 °C yielding around 15 g/100gDM pretreated fiber.

The anaerobic digestion study show no significant improvements of biogas production due to ensiling, thus corroborating the general finding that ensiling of *Festulolium* Hykor merely led to limited pretreatment effect.

The project has resulted in 4 scientific publications plus a PhD thesis:

Paper I
Ambye-Jensen, M., Johansen, K.S., Didion, T., Kádár, Z., Schmidt, J.E., Meyer, A.S., 2013. Ensiling as biological pretreatment of grass (*Festulolium* Hykor): The effect of composition, dry matter, and inocula on cellulose convertibility, *Biomass Bioenergy*. 58, 303-312.

Paper II
Ambye-Jensen, M., Thomsen, S.T., Kádár, Z., Meyer, A.S., 2013. Ensiling of wheat straw decreases the required temperature in hydrothermal pretreatment, *Biotechnology for Biofuels*. 6, 116.

Paper III
Ambye-Jensen, M., Johansen, K.S., Didion, T., Kádár, Z., Schmidt, J.E., Meyer, A.S., 2014. Ensiling of grass and hydrothermal pretreatment: Consequences for enzymatic biomass conversion and total monosaccharide yields, *Biotechnol f Biofuels* 7, 95-106

Paper IV
Thomsen, ST, Londono, JEG., Ambye-Jensen, M., Heiske S., Kádár, Z., Meyer, A.S., 2015. Effective combination of ensiling- and fungal pretreatment of wheat straw, paper almost ready for submission.

PhD THESIS: Morten Ambye-Jensen, Jan. 2014: Ensiling as pretreatment of grass for lignocellulosic biomass conversion
ISBN: 978-87-93054-36-3

1.6 Utilization of project results

The production results have been used for decision making re. bioenergy production from temperate grass after ensiling. The project results have also revealed the need for development of better enzymes for green grass biomass if green grass is to be used for bioenergy.

1.7 Project conclusion and perspective

Conclusions: Significant new knowledge was provided in accord with the objectives of the project: Green grass, *Festulolium* Hykor is a tough biomass. The project results *unequivocally establish that ensiling of grass as a biomass pretreatment method comes with a loss of water-soluble carbohydrates (WSC). The loss of WSC by ensiling is not necessarily compensated for by providing a lower temperature requirement for hydrothermal pretreatment for enzymatic monosaccharide release, but ensiling can be an advantageous storage method prior to grass processing.*

Perspective:
The results in the study consistently suggested that *Festulolium* Hykor contains a highly resistant hemicellulosic structure that prevented xylan conversion. It would therefore be extremely interesting to study the hemicellulosic structure in more detail and find the reason for the difficult conversion. This could be done using targeted hemicellulose extraction, specific enzymatic saccharification with a collection of mono component hemicellulases. It was concluded, in the study, that ensiling of *Festulolium* Hykor should not be used for bioethanol production. However, many other biorefinery possibilities exists notably, in the green biorefinery that utilizes separation technologies to extract valuable products from fresh green/silage biomass. The most promising results of the study were the positive effects of combining ensiling with HTT on wheat straw, which gave a possible reduction of HTT temper-

ature. Future optimization should be carried out, and the effects of even lower HTT temperatures, duration of storage, and holding time should be assessed.

A future implementation of a combined pretreatment of ensiling and HTT of wheat straw implies that the ensiling should be incorporated into the logistics and pre-processing of biomass. Ensiling of wheat straw could be carried out decentralized by each farm, or alternatively, be a more controlled continuous process at the biorefinery. The decentralised solution would add value to the product for each farmer and spare the biorefinery of an additional processing and considerable storage space. However, decentralised ensiling would imply transportation of high amounts of water due to the low DM (35%) of the wheat straw silage, which could prove to be a costly practise. On the other hand, silage could possibly also reduce the bulk density of wheat straw bales, which would increase the possible biomass loading on each truck, thus improving the efficiency of biomass supply.

The results of the project hopefully impact the biomass upgrading processing strategies in the future.

Annex

Relevant links (none)