Ensiling as pretreatment of grass for lignocellulosic biomass conversion

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Ensiling as pretreatment of grass for lignocellulosic biomass conversion
Abstract

Development of sound technologies of biomass conversion will be increasingly important for many years to come as planetary boundaries drive the development towards a biobased society. Pretreatment of lignocellulosic biomass is, in this regard, an essential technology. Current pretreatment methods, based on severe physio-chemical processes, are effective, however, they are also costly and energy demanding. An alternative biological pretreatment method, based on the well-known biomass preservation of ensiling, has been proposed. Ensiling holds potential as an integrated storage and pretreatment method with low cost and low energy requirements, plus brings about multiple advantages with regards to agricultural management. However, the pretreatment effect of ensiling, and the overall effects for further conversion are limited.

In this study, ensiling was evaluated as a method of pretreatment for subsequent enzymatic saccharification of cellulose and hemicellulose, by using the temperate grass *Festuлаlium* Hykor. The method was additionally combined with hydrothermal treatment, in order to decrease the required severity of an industrial applied pretreatment method. The first part of the project was devoted to method development. This resulted in the development of a simple and flexible standard method for laboratory ensiling with a high reproducibility, which is well suited for high-throughput experiments.

A comprehensive study on important parameters in ensiling was conducted to find optimal conditions providing the best possible pretreatment effect. The parameters were biomass composition, varied by ensiling of four seasonal cuts of grass, different dry matter (DM) content at ensiling, and an addition of different lactic acid bacteria species. First of all, the study confirmed that ensiling can act as a method of pretreatment and improve the enzymatic cellulose convertibility of grass. Furthermore, low DM 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 amount of cellulose and vice versa. This led to the conclusion that an optimal maturity of grass can be found, 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 in fact 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 β-xylosidase) nor hemicellulose degrading esterases (acetyl xylan esterase and ferulic acid esterase) showed any
improvements of xylan or glucan convertibility. Furthermore, hemicellulases were added before ensiling in order to assist and improve the pretreatment effect. This resulted in, however, the undesired effect that additionally released monosaccharides were utilized during storage and had a negative impact on sugar release after enzymatic saccharification. In both of the above mentioned experiments on optimization of sugar release by means of enzymes, it was noted that the hemicellulose structure of Festulolium Hykor appeared unusually resistant to enzymatic degradation. Due to the low conversion results on Festulolium Hykor, the last part of the project was based on a new tenet: Ensiling can not provide sufficient pretreatment effect to be a stand-alone pretreatment method.

Ensiling was therefore combined with hydrothermal treatment (HTT), and the pretreatment combination was applied to both grass (Festulolium Hykor) and wheat straw, in order to compare the effect upon two categorically different biomasses.

For wheat straw, it was found that ensiling in combination with HTT increased the severity of HTT and facilitated a reduction in optimum HTT temperature of 10 to 20 °C. This could, however, not be proven for grass, 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 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. Furthermore, the HTT pretreatment of both grass and grass silage gave considerably lower xylan convertibility than HTT of wheat straw and wheat straw silage. 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.

The study concludes that ensiling may provide a pretreatment effect in itself, 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 and should instead 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. However, the improvements largely depends on the loss of WSC and the type of biomass in question. In this regard, it should be duly stressed that ensiling is not merely a pretreatment method, but an integrated storage and pretreatment method with effects on both agricultural management, biomass feedstock logistics, and biomass conversion. This thesis aimed to study only the last issue of biomass conversion.
Dansk resumé


I dette projekt er ensilering blevet evalueret i forhold til forbehandling af biomasse til efterfølgende enzymatisk omdannelse af biomasse kulhydraterne cellulose og hemicellulose. Ensileringen blev udført på græs (Festulolium Hykor) udviklet af DLF TRIFOLIUM. Ensilering blev yderligere kombineret med hydrotermisk forbehandling, der anvendes i den industrielle bioethanol fremstilling. Dette var med henblik på at sænke den nødvendige temperatur i den hydrotermiske forbehandling. Derudover var den første del af projektet dedikeret til metodeudvikling, som resulterede i udviklingen af enkel og fleksibel standard ensilerings metode til laboratorie skala forsøg, som er velegnet til high-throughput eksperimenter.


Det blev tilgengæld også noteret at ensilering også har sine begrænsninger. Ensilering af græs gav nemlig et betydeligt tab af frit tilgængelige kulhydrater, som viste sig at være en lille smule større end den forbedrede frigivelse af glukose. Samtidig kunne mængden af frigivet glukose ikke understøtte en effektiv produktion af bioethanol og omdannelsen af xylan var usædvanlig lav i både græs og græsensilage.
Enzym hydrolysen blev derfor forsøgt optimeret. Desværre hjælp hverken yderligere xylanaser (Cellic HTec2 * og beta-xylosidase) eller esteraser der nedbryder hemicellulose (acetyl xylan esterase og ferouyl esterase). Hemicellulaser blev tilsat før ensilering for at forbedre forbehandlings effekten, men dette havde en direkte negativ indvirkning da tabet af glucose under ensileringen blev højere. Begge forsøg på optimering af sukker frigivelse ved hjælp af enzymer, pegede på at hemicellulose strukturen i Festulolium Hykor kunne være ekstremt svært nedbrydning.

Ensilering blev istedet kombineret med hydrotermisk forbehandling og denne kombination blev både testet på græs og på hvedehalm, med henblik på at sammenligne effekten på to kategorisk forskellige biomasser.


Acknowledgements

Foremost I would like to thank my two main supervisors during the PhD project Anne S. Meyer and Jens Ejbye Schmidt for valuable guidance, support and inspiring enthusiasm. Likewise I sincerely thank my co-supervisor and trusted office mate Zsófia Kádár for all the scientific discussions and the friendly help.

Additionally I have been fortunate to have Katja S. Johansen in Novozymes A/S and Thomas Didion in DLF TRIFOLIUM A/S as co-supervisors. I thank both for inspiring collaboration, assistance and fruitful project meetings.

An indispensable effort was provided by the great technical team of Ingelis Larsen, Tomas Fernquist, Ulla Lilholdt and Annette E. Jensen, which was highly treasured. I wish to thank Julie Kirk for excellent proofreading. Also thanks to Anne Belinda Thomsen/Bjerre whom inspired me to take up the challenge of this PhD project.

Sincere thanks to my colleagues who indirectly contributed with inspiration and good spirit; those not mentioned yet are Hanne, Stefan, Mads, Christina, Tobias, Pablo, Thalia, Henrik, Nadja, Guotao, Ming, Piotr, Zsuzsa, and a special thanks to my scientific partner in crime Sune Tjalfe Thomsen.

Finally I wish to thank my family and friends for their trust, support, and providing good times. Last a truly special thank you to my wife Marie for never-ending support, encouragement, and late dinners and love.

Morten Ambye-Jensen

January 2014
Preface

The PhD study that lead to this thesis was carried out in the Center of Bioprocess Engineering (BIOENG), Department of Chemical and Biochemical Engineering, at the Technical University of Denmark. The PhD project was initiated November 2010 and ended January 2014, with an interruption of three month during the winter of 2013 where I was on a leave to carry out a related bioenergy project. The work was a part of the EUDP project ‘Silage pretreatment of green crops for 2nd generation bioethanol production’ (Jr. no. 64010-0005) funded by the Danish Energy Agency. The part of the work, that included ensiling and hydrothermal treatment of wheat straw did also receive financially support by the European Commission’s Seventh Framework Programme (PROETHANOL2G, Project no.251,151). During my work in the project I have been supervised by Anne S. Meyer (01.09.2012 – present) and Jens Ejbye Schmidt (01.11. 2010 – 01.09.2012), as well as co-supervised by Zsófia Kádár (01.09.2012 – present), Katja S. Johansen and Thomas Didion (whole length of the study).

The presented thesis is divided in three parts. The first part, Chapter one, is an introduction to the topic, and includes background theory of biomass structure, and silage fermentation as well as a short review of published literature on ensiling as a pretreatment method. The second part, Chapter two, consist of a, close to chronological, assessment of my main experimental work, the results and the most important discussions. This chapter contain both published and submitted results as well as results that have not been written into scientific manuscripts. The last part, Chapter three, compile the three papers that were produced from the study, which is refered to as Paper I, Paper II, and Paper III according to the chronological order they were submitted.
List of abbreviations

<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>LAB</td>
<td>Lactic acid bacteria</td>
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<tr>
<td>WSC</td>
<td>Water soluble carbohydrates</td>
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<tr>
<td>DM</td>
<td>Dry matter</td>
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<tr>
<td>CCM</td>
<td>LACTISIL CCM inocula, (Chr. Hansen, Hørsholm, Denmark) (heterofermentative <em>Lactobacillus buchneri</em>)</td>
</tr>
<tr>
<td>GP</td>
<td>LACTISIL Grass Plus inocula, (Chr. Hansen, Hørsholm, Denmark) (homofermentative <em>Pediococcus pentosaceus</em> and <em>Lactobacillus plantarum</em>)</td>
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<td>HTT</td>
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Chapter one
Introduction

The world we live in today is faced with tremendous changes. Changes that inevitably will affect all aspects of our society, some less, and others will have an essential impact on our lives. Several systemic crises have emerged from the vast expansion of human societies, counting an ecological- a climate-, a food supply-, and a fossil energy crisis. A key development that will influence and possibly remedy all of the above crisis is the transition from our all-pervading fossil based society, towards a bio based society, where biomass, in all its diversity, make up the basis of food, feed, chemicals, materials and fuels.

In a bio based society, we will no longer rely on oil refineries to produce our products and supply us with fuels. Instead products and fuels will have to come from biorefineries, an integrated process facility able to refine biomass into an array of different products.

Today, research in biorefinery technology is a huge topic, and the broad definition of a biorefinery implies that biorefinery research includes a wide range of technologies. The biorefinery technology that has received the most attention by far over the past three decades is production of bioethanol. Bioethanol has been produced from sucrose or starch containing crops since the 1970s. However, in spite of its very efficient production and optimized large scale advantages, this technology has the fundamental drawback that it is merely produced from the edible part of the biomass and therefore, it is in direct conflict with the before mentioned food supply crisis and it is inducing increased pressure on land-use (Don et al., 2012; Allen et al., 2013).

Production of ethanol is based on the well-known process of anaerobic fermentation of sugar by yeast, and thus it can be categorized as a sugar platform biorefinery. The term sugar platform biorefinery refers to any biomass conversion that relies on the release of monosaccharides followed by fermentation or chemical catalysis of these into other products (Kamm et al., 2006).

An alternative to the sucrose and starch based bioethanol merely requires another source of sugars. Conveniently, the building block of biomass structure happens to be exactly that - monosaccharides, forming the far majority of lignocellulose, bound in the two polysaccharides cellulose and hemicellulose.

Bioethanol production from the non-edible lignocellulosic part of biomass has been a joint focus of researchers, companies and governments in an international technology-push towards large scale production of what is here referred to as cellulosic ethanol. The technology development has proven successful, and the first large scale cellulosic ethanol plants are in place, and others are
currently under construction (www.demoplants.bioenergy2020.eu). However, the economics of large scale cellulosic ethanol are still vulnerable due to high capital costs, a low prized bulk product, fluctuating energy prizes and uncertainties of legislation on long term (Zhang et al., 2013; Festel et al., 2013; McCormick and Kautto, 2013).

The overall process of cellulosic ethanol production involves biomass cultivation, harvest, storage, conversion, and distribution. The conversion has evidently been the main focus for research and technology development, but as large scale production has come closer, an effort has certainly also been put into cultivation and logistics (Hess et al., 2007; Kudakasseril Kurian et al., 2013).

Lignocellulosic biomass is ‘engineered’ from nature to resist degradation, and it is therefore highly recalcitrant. Thus, technical conversion of lignocellulosic biomass has proven to be very challenging. Conversion of biomass to ethanol therefore requires therefore a comprehensive pretreatment step, where the tight lignocellulosic structure is ‘unlocked’. Pretreatment is followed by an enzymatic saccharification, where enzymes of cellulases and hemicellulases, by means of pretreatment, are allowed access to their substrates and can release monosaccharides for the subsequent fermentation of ethanol.

Biomass pretreatment has been intensively studied, and exciting scientific results have developed into efficient methods. Methods, which by physical means of high temperatures, and pressure, as well as chemical addition of acids, alkali or solvents alter the biomass structure significantly (Galbe and Zacchi, 2012).

However, the capital and operational cost of such pretreatments can be very significant due to the severe conditions requiring high tech process equipment, and the need for energy and chemicals, depending on the method. Accordingly, pretreatment has been estimated to be one of the most costly processes in cellulosic ethanol (Zhang et al., 2013; Festel et al., 2013; Yang and Wyman, 2008; Bals et al., 2011). Thus, as a main challenge, pretreatment remains to be a key area of optimization.

An alternative method of pretreatment, based on ensiling, has emerged from the well-known agricultural practice of feed preservation. Several researchers have stated the possibility for ensiling to become a low cost, low energy intensive and combined storage and pretreatment method, due to its ambient conditions (Chen et al., 2007; Ren et al., 2007; Pakarinen et al., 2008; Thompson et al., 2005). Additionally, ensiling poses several advantages for agricultural management as opposed to dry storage of biomass, such as less dependence on weather conditions, hence, better harvest-timing and no need for drying.

Ensiling comprises a moist solid state anaerobic fermentation by lactic acid bacteria (LAB), leading to production of organic acids and decrease in pH. The low pH together with the high concentration
of organic acids inhibit growth of microorganisms and prevent biomass degradation (Buxton et al., 2003). The pretreatment effect of ensiling is ascribed to the enzymatic and acid hydrolysis of structural carbohydrates of mainly hemicellulose (Dewar et al., 1963; Morrison, 1979).

Regarding ensiling as pretreatment, several studies have shown promising results. However, a large variety of treatment conditions, different additives and combinations with various other pretreatments make it difficult to evaluate on the actual pretreatment effect of ensiling. Thus, new knowledge is needed.

This PhD project was initiated to evaluate and study the effect of ensiling as a pretreatment method, and especially, with regards to the effect on cellulose conversion in the subsequent enzymatic saccharification.

The biomass that was chosen for this purpose was Festulolium Hykor, a high yielding temperate grass, developed by DLF TRIFOLIUM A/S through crossbreeding of tall fescue (Festuca arundinacea) and Italian rye (Lolium multiflorum). These types of grass have been shown to hold high yield potentials around 18 Mg/ha and high persistency throughout the season (www.DLF.com).

Temperate grass was chosen for two main reasons. Firstly, ensiling of temperate grass is thoroughly documented in forage preservation research, and it is a well-known practice in current agriculture. Furthermore, temperate grass matches extremely well with ensiling, considering the high moisture of grass at harvest, and the considerable amounts of readily available carbohydrates in grass to supply substrate for the LAB.

Secondly, it was hypothesized that temperate grass, with its moist immature cell walls and low content of lignin (<10%), would be better exposed to pretreatment using ensiling. It has been noted that the pretreatment conditions of ensiling are relatively mild compared to the established physio-chemical pretreatments. This means that ensiling of highly rigid and lignified biomasses like true lignocellulosics of cereal straw or corn stover might not be able to facilitate a significant effect. This was seen in the modest improvements of enzymatic saccharification, carried out in the study by Chen et al. (2007), of maximum 9.3% on barley, wheat, and triticale straw (Chen et al., 2007).

However, considering temperate grass for a sugar platform biorefinery has two disadvantages compared to agricultural residues. Since temperate grass is not an agricultural residue, extensive cultivation of grass for bioenergy implies the risk of land use change effects having several negative effects on society instead of the advancement it should represent (Don et al., 2012; Allen et al., 2013). The other drawback is a significantly lower content of cellulose, ranging from 20-40% compared to 30-50% in a typical agricultural residue. Lower cellulose content means lower ethanol
potential from the lignocellulose. Instead, grass has a high content of non-structural carbohydrates, which also constitutes a potential for sugar platform biorefineries.

On the other hand, temperate grass offers several highly valued traits to obviate the need for a sustainable biomass supply for biofuels and other biorefinery products. Temperate grass has a low required energy input, high yield potential and a vast availability in temperate regions. Additionally, temperate grass is a perennial crop with ecological multi-functionality, as it can benefit the ecological system through sequestration of carbon into the soil preventing agricultural degradation of arable land (Tilman et al., 2006), as well as increase biodiversity and preserve ground and surface water quality (Prochnow et al., 2009). Cultivation of temperate grass can therefore be a valuable part of sustainable agriculture. Moreover, carbon sequestration also favors the overall carbon balance of the biofuel.

1 Objectives of the study
The overall aim of the study was to investigate and evaluate ensiling as a pretreatment method.

The measure for pretreatment effect involved primarily the degree of enzymatic saccharification of lignocellulose.

The study was primarily conducted using temperate grass. However, one study was also carried out on wheat straw, which is the typical biomass concerning cellulosic bioethanol production in Denmark.

Hypothesis

- Ensiling can be used as pretreatment method for conversion of lignocellulosic biomass.

  o Thus ensiling can facilitate a higher yield of monosaccharides after subsequent enzymatic saccharification, compared to dry storage.
  o And the consumption of readily available sugars associated with silage fermentation is less than the facilitated higher yield.
2 Biomass structure

Conversion of biomass for the sugar platform biorefinery can be seen as a carbohydrate ‘slaughterhouse’. The biomass as a whole, contain a large pool of carbohydrates that are spread out in different parts with different functions. The objective of the conversion is to get the maximum amount of that carbohydrate pool out, but as intact monosaccharides to be used for fermentation. However, for this to take place intelligent separation and ‘cutting’ are needed, based on the structure and content of the biomass. A thorough review of biomass structure is therefore required. The following section will briefly cover each important cell wall component as well as important non-structural components in relation to temperate grass and wheat straw, which were the two biomasses used in the study.

The structural architecture surrounding dead and living cells make up the far majority of the dry physical mass in all plants. This structure, the plant cell wall, is often divided into primary and secondary walls, latter being thick and lignified, and the former being thin and allows for cell growth. However, nature displays a vast diversity of cell wall structure as according to the specialized function of the cell they are surrounding. Thus, it is hard to categorize cell wall structure since reality most likely is more complex. As it is stated by K. Keegstra (2010): ‘In reality, all differentiated cells contain walls with distinct compositions, resulting in a spectrum of specialized cell walls with primary and secondary walls as two extremes.’ (Keegstra, 2010).

The cell wall components include cellulose, hemicellulose, pectin and lignin. When the wall is in the primary end of the spectra it contains more pectin and hemicellulose, and when cells mature or die, the wall turns to the secondary end of the spectra and contain more cellulose and lignin. This has specifically importance in the maturation of the growing biomass, and makes the stage of maturity at harvest very important to the conversion.

Lignocellulose

As the main structure that provides strength and resistance to the plant, lignocellulose is an important and abundant component, and obviously also the main target in lignocellulosic biomass conversion.

Lignocellulose is describing the heterogeneous, structural matrix, comprised of the polysaccharides, cellulose and hemicellulose, and the polyphenolic structure of lignin (Figure 1).
Cellulose

Cellulose is a linear homopolymer made from glucose monosaccharides forming β-1,4-glucosidic bonds. Cellulose polymers bind together in bundles by intra- and intermolecular hydrogen bonding, which in turn aggregate in cellulose microfibrils. The strong structure of linear covalent bonding and branching hydrogen bonding results in a high tensile strength and insolubility (Rubin, 2008). The principal structure of cellulose does not vary in-between different biomasses, and it is therefore the only class of structural plant polymers which is well defined (Scheller and Ulvskov, 2010).

Hemicellulose

Hemicellulose is a highly heterogeneous structure of several polysaccharides. It is traditionally defined from biomass extraction, however, since extractions obviously vary between different biomasses with different hemicellulosic structure, it is un-useful as definition. It has instead been proposed that hemicellulose should be defined as only polysaccharides with β-(1-4) linked backbones, which are not cellulose (Scheller and Ulvskov, 2010). These include xyloglucan, xylan, mixed linkage glucan, mannan, and glucomannan.
Hemicellulose of the plant family Poaceae including all temperate grasses and cereals also referred to as true grasses, is known to primarily comprise of xylan based structures, and minor amounts of xyloglucans and mixed linkage glucans (Figure 2) (Scheller and Ulvskov, 2010).

Xylans are the main hemicellulosic polysaccharide that apply strength to the lignocellulose of true grasses. Xylans are a highly diverse group of polysaccharides, without a systematically repeating polymeric structure, and thus many variations are still not well known (Scheller and Ulvskov, 2010). The xylans in true grasses are built on a backbone of xylose with a high degree of arabinose residues attached to the backbone as well as glucuronic acids and 4-O-methyl glucuronosyl residues, and are therefore often referred to as arabinoxylan or glucuronoarabinoxylans (Figure 2). Other common branching structures are substitutions of α-galactose, and O-acetyl- and O-feruloyl esters (Figure 2). The latter substitution of ferulic acid is known to make covalent cross-links between xylan and the phenolic lignin structure, which increase recalcitrance of the lignocellulose (Yu et al., 2005).

Xyloglucans are only found in limited amounts in true grasses, where it figures in the structure of the more primary cell walls often in association with pectin structures of rhamnogalacturonans (RG).

**Figure 2:** Schematic drawing of the three main types of hemicelluloses found in cell walls of true grasses. The letters under the xyloglucan (XyG) molecule illustrate the symbols used for the most common side chains. The structure of the hemicelluloses varies greatly in different plant species and tissue types. “Fer” represents esterification with ferulic acid. Adapted from (Scheller and Ulvskov, 2010)
They comprise of a backbone of glucose with differently positioned xylose side chains, and have a repeating polymeric structure (Figure 2) (Scheller and Ulvskov, 2010).

Mixed linkage glucans are β-(1-4) linked glucans with intercepting β-(1-3) linkages which make the polymer non-linear as opposed to cellulose (Figure 2). In true grasses, mixed linkage glucans have traditionally been known to function as a storage carbohydrate and play a role in cell expansion during plant growth, and its abundance are therefore highly related to growth stage. However, new research is pointing out that mixed linkage glucans most likely also have a role and abundance in mature secondary cell walls (Vega-Sánchez et al., 2013)

**Lignin**

Lignin is often referred to as the glue that holds the lignocellulosic matrix together. Lignin is a hydrophobic phenolic polymer that provides mechanical support and water impermeability to secondary cell walls. When cells mature, the wall becomes more lignified, which induce biomass recalcitrance, and it is generally accepted that the amount of lignin is a significant factor for the pretreatment efficiency (Hu and Ragauskas, 2012). Lignin is primarily formed from three hydroxycinnamyl alcohols, namely p-coumaryl alcohol (H), coniferyl alcohol (G) and sinapyl alcohol (S) (Figure 1). The polymeric structure of lignin is not ordered and includes a high diversity in series of linkages. Lignin is therefore traditionally, like hemicellulose, defined according to chemical procedures. Lignin has in this work exclusively been analyzed as Klason lignin, which is the volatile acid insoluble residue after a two-step acid hydrolysis using 72% and 4% H2SO4 as according to (Sluiter et al., July 2011).

**Pectin**

Pectin is a third polysaccharide, which is not considered in the stringent term of lignocellulose. The reason for this is that pectin mainly exists in primary cell walls, where there are no or limited amounts of lignin, and are thus not lignocellulose. Furthermore, pectin is not abundant in true grasses, which is a monocot plant, but is instead a major polysaccharide in dicot plants such as vegetable plants and legumes. Pectin has an even more complex structure than hemicellulose. The main structure is a backbone of α-(1-4)-d-galactoronic acid residues interrupted by (1-2)-l-rhamnose residues, from where arabinosyl and galactosyl residues form complex sidechains. However, the before mentioned definition of hemicellulose, implies that all polysaccharides that do not have a β-(1-4) backbone and are not cellulose, are instead pectin structure. Thus,
polysaccharides like galactans, arabinans, and arabinogalactans are also considered pectin polysaccharides (Ridley et al., 2001; Willats et al., 2006).

**Cutin**

The outer layers of terrestrial plants consist of structural lipid materials, collectively known as wax, which creates a hydrophobic coating around the cell walls (Kolattukudy et al., 1981). The barrier helps the plant to reduce water loss and protects against pathogenic fungi and bacteria. Cutin is a main polymeric constituent of this waxy layer and is constructed of primarily C16 and C18 hydroxy acids interlinked via ester bonds forming a polyester. Cutin is the primary polymer in the cuticle proper whereas, in the cuticular layer cutin is associated with pectins and carbohydrates (Figure 3).

![Figure 3: The hydrophobic layers surrounding the plant cell wall. Adopted from (Bargel et al., 2006)](image)

**Non-structural carbohydrates**

Non-structural carbohydrates are, as the name reveal, not a part of the structural cell wall. However it is certainly a part of the carbohydrate pool, and are therefore of high importance to ensiling and the sugar platform biorefinery. Non-structural carbohydrates are normally quantified by extraction with water, and are thus referred to as water soluble carbohydrates (WSC), also in this thesis.

Temperate grasses contain considerable amounts of the non-structural carbohydrates, which offer an easily accessible source of carbohydrates. The type and function of non-structural carbohydrates
include (I) monosaccharaides, serving as building blocks for plant synthesis, (II) disaccharides, which are transport sugars, and (III) reserve and storage oligo/polysaccharides (Rooke and Hatfield, 2003) (Figure 4). These functions all relate to processes with a high flux, thus the amount and composition of the nonstructural carbohydrates can change considerably over time (Figure 4).

In temperate grass the carbohydrates include glucose and fructose, sucrose, and fructans, and the total content varies from a few percent up to 30% of DM content (Buxton and O’Kiely, 2003). The variation is determined by growth conditions and stage of maturity (King et al., 2012).

![Figure 4: Illustration of the structural and non-structural carbohydrate pool, and their flux.](image)

**Biomass composition and maturity**

Biomass composition changes significantly in a growing plant. The composition of a newly harvested biomass will therefore depend on the stage of maturity. This is in particular important regarding temperate grasses and its usage, since temperate grasses change considerably due to maturity, and it is possible to adapt the harvest strategy of several cuts over a season according to the usage. The general development of the plant composition during maturation is, that the total cell contents including proteins, lipids, non-structural carbohydrates and minerals decreases, while the content of cell walls increases, and in particular lignocellulosic cell walls. This is illustrated in Figure 5.
King et al. studied change in chemical composition of temperate grasses at five different stages of maturity, and found that the WSC reduced as the grasses matured, instead the cellulose and lignin content increased, while the amount of hemicellulose stayed rather constant. The increased proportion of secondary cell walls in mature grass was also reported by (Morrison, 1980). Keating and O’Kiely (2000) studied the effect of maturity on the DM digestibility of perennial rye grass and reported a decreased dry matter digestibility in animal feed experiments with increasing stage of maturity. Thus suggesting higher recalcitrance to microbial degradation at for late harvested grass. It is therefore likely that cellulose convertibility also increases at late maturity of grass, primarily due to the increased lignin content and cross-linkages between lignin and structural carbohydrates (Hu and Ragauskas, 2012).
Table 1: Composition of two temperate grasses at five different stages of growth. Numbers are in (w/w)% of DM. Cellulose, hemicellulose and lignin content is based on feed analysis, of neutral detergent fiber (NDF), acid detergent fiber (ADF) and Acid detergent lignin (ADL). Calculated from (King et al., 2012).

<table>
<thead>
<tr>
<th>Harvest date (dd.mm)</th>
<th>WSC</th>
<th>Cellulose</th>
<th>Hemicellulose</th>
<th>Lignin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Italian ryegrass</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12.05</td>
<td>26.2</td>
<td>23.5</td>
<td>21.1</td>
<td>0.7</td>
</tr>
<tr>
<td>26.05</td>
<td>26.8</td>
<td>25.0</td>
<td>20.9</td>
<td>1.1</td>
</tr>
<tr>
<td>09.06</td>
<td>22.4</td>
<td>28.9</td>
<td>21.3</td>
<td>2.4</td>
</tr>
<tr>
<td>23.06</td>
<td>18.5</td>
<td>31.2</td>
<td>21.7</td>
<td>2.7</td>
</tr>
<tr>
<td>07.07</td>
<td>14.7</td>
<td>32.9</td>
<td>22.2</td>
<td>3.2</td>
</tr>
<tr>
<td><strong>Tall fescue</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12.05</td>
<td>19.6</td>
<td>24.7</td>
<td>23.5</td>
<td>1.4</td>
</tr>
<tr>
<td>26.05</td>
<td>15.7</td>
<td>28.6</td>
<td>24.0</td>
<td>1.6</td>
</tr>
<tr>
<td>09.06</td>
<td>12.6</td>
<td>33.5</td>
<td>24.1</td>
<td>2.5</td>
</tr>
<tr>
<td>23.06</td>
<td>12.9</td>
<td>33.6</td>
<td>25.2</td>
<td>3.3</td>
</tr>
<tr>
<td>07.07</td>
<td>12.7</td>
<td>33.4</td>
<td>24.7</td>
<td>3.2</td>
</tr>
</tbody>
</table>
3 Ensiling

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 (Wilkinson et al., 2003).

3.1 The agricultural practice of feed preservation

The purpose of ensiling is to preserve agricultural crops and prevent them from rotting. Crops with a moisture content higher than 20% (<80% dry matter) 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 (Figure 6). 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 6: pH and bacterial growth as a function of days after ensiling. (Van Soest, 1994)](image)

The ability to store biomass is of great general importance in agriculture, due to the conflict of a pulsing biomass supply from seasonal harvests, and a constant demand of feed and food. Ensiling is uniquely connected to preservation of ruminant animal feed, where silage is an essential part of the diet, in most of the world (Wilkinson et al., 2003). Feed crops such as temperate grasses, legumes, sugar beet tops, and immature whole crop corn and cereals are therefore traditional crops used for ensiling.

Since ensiling takes place at high moisture it is not needed to extensively dry the crops after harvest. Crops for silage can be ensiled directly after harvest at a dry matter (DM) of 20-25% or wilted (drying on the field) to 35-40% DM, which is normally no more than 24 hours depending on the weather (Buxton and O'Kiely, 2003), where after it is concealed in a silo of varying shape and size.
Alternatively, feed crops can be stored dry, as hay, at a DM above 70%. The high DM is normally also reached by field drying, and do therefore require more steady and dry weather conditions as compared to ensiling. The extended field drying pose a considerable risk of high DM loss due to extended plant respiration, leaching, and microbial activity. The DM loss is highly dependent on weather conditions and can range from below 10% to as much as 50% (Muck et al., 2003; Emery and Mosier, 2012). To reduce the period of field drying it is necessary to harvest the feed crop at relatively late maturity, where the DM is higher, but also necessary to time the harvest according to the weather forecast, as opposed to the optimal biomass composition.

![Diagram](image)

*Figure 7*: General correlation between moisture content in the stored biomass and DM loss. Storage loss (grey) is the DM loss taking place during storage after the biomass reached desired DM. Harvest loss is the all DM loss before storage, thus including field drying.

The moist conditions of ensiling, pose therefore multiple advantages concerning agricultural management, allowing for a better harvest timeliness, thus better feed quality, increased number of seasonal cuts, thus higher yields, and reduced harvest losses due to shorter field drying, less machine operations and no dust formation (Buxton and O’Kiely, 2003). On the other hand, ensiling at low DM can likewise result in a high dry matter loss. Here, the risk is not during the harvest and field drying, but during the actual storage, where high amounts of water can result in seepage of an effluent containing high amounts of soluble DM. The general relation between moisture content at storage and DM loss is illustrated in Figure 7.

Concerning preservation quality, ensiling is known to have higher nutrient and protein content and increase DM digestibility. The higher nutrient and protein content is due to the freshness of the crop when it is ensiled. Both nutrients and protein content drops significantly during field drying.
(Rooke and Hatfield, 2003). The DM digestibility is often higher for silage than for hay, since the total lignocellulosic fiber content decreases during long term storage under acidic conditions (Thomas et al., 1969). This is a consequence of microbial and acid hydrolysis of structural components and is in fact the same phenomenon hypothesized to have an effect as pretreatment. Table 2 provides an overview of advantages and disadvantages of ensiling.

Table 2: Overview of advantages and disadvantageous of ensiling regarding agricultural management, preservation quality, and practical concerns

<table>
<thead>
<tr>
<th></th>
<th>Advantageous</th>
<th>Disadvantageous</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Agricultural management</strong></td>
<td>Better harvest timeliness</td>
<td>Higher capital investment compared to hay</td>
</tr>
<tr>
<td></td>
<td>Several cuts</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Faster regrowth</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Higher yields</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Less harvest losses</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Better weed control</td>
<td></td>
</tr>
<tr>
<td><strong>Preservation quality</strong></td>
<td>High protein</td>
<td>Risk of low DM intake (animal feed)</td>
</tr>
<tr>
<td></td>
<td>High nutrition</td>
<td></td>
</tr>
<tr>
<td></td>
<td>High DM digestibility (animal feed)</td>
<td></td>
</tr>
<tr>
<td><strong>Practical</strong></td>
<td>Uniform biomass</td>
<td>High water content (for transport)</td>
</tr>
<tr>
<td></td>
<td>Less bulk</td>
<td>High effluent losses at low DM</td>
</tr>
<tr>
<td></td>
<td>No risk of fire</td>
<td></td>
</tr>
</tbody>
</table>

3.2 The silage fermentation process

The silage fermentation process is in principal a spontaneous biological reaction, which initiates as soon as supply of oxygen becomes limited. The process rely basically on the presence of LAB and its substrate in WSC, which both are naturally present on/in most forage crops.

Silage fermentation is however, highly dynamic and many factors are influencing the biological process. Extensive research efforts have been conducted throughout past and present century to map this complexity, and most of that research is gathered in the thorough works of P. McDonald, et al. (1991) and D. R. Buxton et al. (2003) (Buxton et al., 2003; McDonald et al., 1991). Obviously, not all aspects will be covered here, instead a brief overview of the key factors that relate directly to the current work are presented below.

**Lactic acid bacteria**

As stated above, LAB is a natural part of the epiphytic microflora on the standing crop however, numbers and genera can vary considerably and over a wide range (Pahlow et al., 2003). LAB inocula
is therefore often used as an additive in order to secure a successful silage fermentation. The genera of the dominating LAB has large influence on the silage fermentation, since different fermentation pathways are used by different LAB. LAB is generally divided into two categories, classified according to their hexose metabolism, as either homo- or heterofermentative. Homofermentative LAB ferment hexoses into exclusively lactic acid, using the Embden-Meyerhof-Parnas pathway, whereas heterofermentative LAB ferment hexoses into both lactic- and acetic acid, ethanol and carbon dioxide, using the pentose phosphate pathway. Furthermore, homofermentative LAB are either classified as obligate or facultative, meaning that they are or are not able to ferment pentoses depending if they possess phosphoketolase or not. Most heterofermentative LAB are considered facultative and do therefore ferment both hexoses and pentoses (McDonald et al., 1991).

Table 3 contains a selection of the most common anaerobic fermentation reactions taking place during silage storage, and the respective recovery of DM for each reaction. The table includes reactions carried out by LAB, but also the three possible contaminants of enterobacteria, clostridia, and yeast. From this it can be concluded that homofermentative fermentation of hexoses and homo- and heterofermentative fermentation of pentoses gives the least DM loss, and that contamination of clostridia and yeast gives the most.

Table 3: Summery of anaerobic fermentation reactions encountered during ensiling by different microorganisms, and their respective DM recovery. As according to (Wilkinson et al., 2003), (Rooke and Hatfield, 2003), and (Savoie and Jofriet, 2003).

<table>
<thead>
<tr>
<th>Organism</th>
<th>Substrate</th>
<th>Product</th>
<th>DM Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAB, Homofermentative</td>
<td>Glucose/Fructose</td>
<td>2 Lactate</td>
<td>100</td>
</tr>
<tr>
<td>LAB, Homo-and Heterofermentative</td>
<td>Pentose</td>
<td>Lactate + Acetate</td>
<td>100</td>
</tr>
<tr>
<td>LAB, Heterofermentative</td>
<td>Glucose</td>
<td>Lactate + Acetate + CO₂</td>
<td>83</td>
</tr>
<tr>
<td>LAB, Heterofermentative</td>
<td>Glucose</td>
<td>Lactate + Ethanol + CO₂</td>
<td>83</td>
</tr>
<tr>
<td>LAB, Heterofermentative</td>
<td>3 Fructose</td>
<td>Lactate + 2 Mannitol + Acetate + CO₂</td>
<td>95</td>
</tr>
<tr>
<td>Enterobacteria</td>
<td>Glucose</td>
<td>Acetate + Ethanol + 2 CO₂</td>
<td>95</td>
</tr>
<tr>
<td>Enterobacteria</td>
<td>2 Glucose</td>
<td>2 Lactate + Acetate + Ethanol + 2 CO₂</td>
<td>83</td>
</tr>
<tr>
<td>Clostridia</td>
<td>Glucose</td>
<td>Butyrate + 2 CO₂</td>
<td>66</td>
</tr>
<tr>
<td>Clostridia</td>
<td>2 Lactate</td>
<td>Butyrate + 2 CO₂</td>
<td>49</td>
</tr>
<tr>
<td>Yeast</td>
<td>Glucose</td>
<td>2 Ethanol + 2 CO₂</td>
<td>51</td>
</tr>
</tbody>
</table>
Fast initial acidification is the key of controlling the growth of enterobacteria and clostridia. These main competitors to LAB will continue to grow until inhibited by undissociated acid concentration and/or low pH. Homofermentative ensiling is therefore more efficient and has a more rapid pH drop inducing faster preservation, while heterofermentative ensiling consequently are slower and has a higher mass loss. Heterofermentative fermentation is on the other hand providing better resilience against spoilage of the silage when exposed to air, known as aerobic stability, due to, among others, a higher concentration of acetic acid (McDonald et al., 1991; Nishino and Hattori, 2007).

Addition of LAB inocula is as mentioned standard in modern agriculture, but the strategy of which strains to use varies with the objective and use of the silage product. Many commercial inocula products are on the market. Two of such products are used in this project, namely LACTISIL Grass Plus, and LACTISIL CCM, (Chr. Hansen, Hørsholm, Denmark), containing the homofermentative Pediococcus pentosaceus and Lactobacillus plantarum, and the heterofermentative Lactobacillus buchneri, respectively.

**Dry matter content**

The amount of moisture in the ensiled biomass is naturally affecting all physical, biological, and chemical processes occurring in the silo (Buxton and O'Kiely, 2003). Physically, the water content is a determining factor for biomass compaction and thus the density in the silo. Low DM results in higher density, since the biomass is heavier and more flexible. This leads to a higher pressure in the silo that, together with the higher amount of water, increases production of a liquid effluent. Effluent is a considerable source of silage DM loss during storage (Savoie and Jofriet, 2003).

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. First of all, increased amounts of water increase the mobility of soluble compounds, and thus also the chances of substrate and enzyme/microorganism interaction.

For the fermenting microorganisms, water is a determining factor for both growth rate and minimum pH tolerance. At low DM the growth rate is higher, and the pH tolerance lower. This means that lactic acid production is faster and terminal pH is lower, at lower DM (Muck et al., 2003).

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
content, clostridia is efficiently inhibited, but here the risk of yeast and fungi contamination increases due to their higher tolerance to low water activity. However, fungi contamination is only a threat if the oxygen is not removed efficiently or if the silo barrier somehow fails (Pahlow et al., 2003).

**Biomass**

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 straightforward 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 biological biomass factors have significant effect on the silage fermentation, such as the amount and specific composition of WSC, buffering capacity of the biomass which is highly related to the protein content, and the epiphytic microflora.

There is, furthermore, a significant difference between ensiling of green crops such as temperate grass and ensiling of lignocellulosic residues such as wheat straw. As mentioned temperate grasses contain large amounts of WSC whereas wheat straw does not. This is crucial to the silage fermentation and without sufficient readily available carbohydrates to facilitate a rapid lactic acid fermentation the biomass will not be preserved and high DM losses will occur. Different strategies have been applied to overcome this when ensiling agricultural residue. Instead of LAB fermentation, organic acids can be added directly (Pakarinen et al., 2011), lignocellulytic enzymes can be added to release fermentable carbohydrates from the lignocellulose (Ren et al., 2007; Chen et al., 2007), or sugars can be added as substrate for LAB fermentation (Yang et al., 2006). The method will have to depend on the specific case, biomass and on the desired outcome.

The need for the above mentioned additives highlights the important point that ensiling is not free. Ensiling requires a certain invested amount of input, and not only on agricultural residues, but also in the case of the spontaneous silage fermentation of e.g. temperate grass. Here, it is just the biomass itself that holds the required investment.
4 Ensiling as pretreatment

The microbial activity and acidic conditions during the fermentation and long-term storage of silage are known to degrade the structure of the biomass to a certain degree.

Dewar et al. (1963) showed, in a study on ensiling of perennial ryegrass, that the hemicellulose was hydrolysed during storage, initially by plant enzymes and during longer storage, from 7 to 28 days, by means of acid hydrolysis at pH 4. Hemicellulose degradation in ensiling of perennial ryegrass were later confirmed by Morrison (1979) whom additionally found that the reduction of arabinose content in the cell wall was greater than the xylose reduction. Furthermore, also the content of both feurulic- and acetic acid associated with hemicellulosic structure, decreased significant (>50%) after 60 days of storage. Since then similar findings have been reported on ensiling of different biomasses. E.g. Singh et al. (1996) on wheat straw mixed with lucerne and Yahaya et al. (2002) on orchardgrass.

These changes in the lignocellulosic structure suggest that ensiling can be applied as a biological pretreatment method for further conversion of lignocellulosic biomass. Several pretreatment methods developed for cellulotic bioethanol, such as hydrothermal treatment, steam explosion, and weak acid hydrolysis, have degradation of hemicellulose as their main pretreatment effect. However, these methods involve 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 (Zhang et al., 2013). Compared to mentioned pretreatment methods, ensiling requires 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 (Digman et al., 2010a; Kitamoto et al., 2011). With the new scientific focus of biomass conversion, ensiling has therefore gained increasing attention as a method for combined storage and pretreatment. A review of existing literature on ensiling in the context of pretreatment prior to either enzymatic saccharification, ethanol fermentation and/or anaerobic digestion is presented in Table 4.

Table 4 gives information on which biomasses, silage conditions, and additives that were applied for ensiling, and states the method for which the pretreatment effect was measured.
Table 4: Literature review on ensiling as pretreatment assessed by enzymatic saccharification (ES), ethanol fermentation (EF), and/or anaerobic digestion (AD). The table provides which biomasses, conditions of ensiling, use of additives, and if an additional pretreatment was applied. Additionally, if or if not (yes/no) the study provided a comparison between non-ensiled and ensiled biomasses, and if ensiling, in that case, gave a positive or negative effect on pretreatment.

<table>
<thead>
<tr>
<th>Ref.*</th>
<th>Biomass</th>
<th>Ensiling condition</th>
<th>Additives</th>
<th>Additional treatment</th>
<th>Assessment</th>
<th>Comparison of raw and silage</th>
<th>Positive effect</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>DM (%)</td>
<td>Storage (Days)</td>
<td>Fiber size (cm)</td>
<td>LAB inocula</td>
<td>Enzymes</td>
<td>Acid</td>
</tr>
<tr>
<td>a)</td>
<td>Corn stover</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>b)</td>
<td>Corn stover</td>
<td>46</td>
<td>90</td>
<td>2</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>c)</td>
<td>Corn stover</td>
<td>n.a.</td>
<td>21</td>
<td>1-3</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>d)</td>
<td>Perennial ryegrass</td>
<td>n.a.</td>
<td>1-3</td>
<td>n.a.</td>
<td>Laccase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>e)</td>
<td>Sugarcane bagasse,</td>
<td>32</td>
<td>1/28/56</td>
<td>n.a.</td>
<td>Lactic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Wood</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>f)</td>
<td>Maize, Whole crop rye,</td>
<td>32</td>
<td>17</td>
<td>30</td>
<td>2-3</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Clover grass</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>g)</td>
<td>Maize</td>
<td>n.a.</td>
<td>10/90</td>
<td>0.8</td>
<td>+</td>
<td>Formic acid</td>
<td>Urea</td>
</tr>
<tr>
<td>h)</td>
<td>Hemp</td>
<td>25</td>
<td>244</td>
<td>2</td>
<td>+</td>
<td>Formic acid</td>
<td>Urea</td>
</tr>
<tr>
<td>i)</td>
<td>Hemp</td>
<td>n.a.</td>
<td>244</td>
<td>1-2</td>
<td>+</td>
<td>Formic acid</td>
<td>Urea</td>
</tr>
<tr>
<td>j)</td>
<td>Forage rye, Triticale</td>
<td>n.a.</td>
<td>34</td>
<td>5-6</td>
<td>+</td>
<td>Formic acid</td>
<td>Urea</td>
</tr>
<tr>
<td>k)</td>
<td>Rice straw, Whole crop rice</td>
<td>14/19/29</td>
<td>28/60/120</td>
<td>0.1</td>
<td>+</td>
<td>Formic acid</td>
<td>Urea</td>
</tr>
<tr>
<td>l)</td>
<td>Whole crop maize, Hemp, Faba</td>
<td>40/60</td>
<td>30-180</td>
<td>0.5</td>
<td>+</td>
<td>H2SO4</td>
<td>Lime</td>
</tr>
<tr>
<td></td>
<td>bean</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>m)</td>
<td>Sugar beet pulp</td>
<td>40/60</td>
<td>30-180</td>
<td>0.5</td>
<td>+</td>
<td>H2SO4</td>
<td>Lime</td>
</tr>
<tr>
<td>n)</td>
<td>Switchgrass, Reed canarygrass</td>
<td>14/19/29</td>
<td>28/60/120</td>
<td>0.1</td>
<td>+</td>
<td>H2SO4</td>
<td>Lime</td>
</tr>
<tr>
<td>o)</td>
<td>Maize</td>
<td>14/19/29</td>
<td>28/60/120</td>
<td>0.1</td>
<td>+</td>
<td>H2SO4</td>
<td>Lime</td>
</tr>
<tr>
<td>p)</td>
<td>Clover grass mix, Italian</td>
<td>14/19/29</td>
<td>28/60/120</td>
<td>0.1</td>
<td>+</td>
<td>H2SO4</td>
<td>Lime</td>
</tr>
<tr>
<td></td>
<td>ryegrass</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>q)</td>
<td>Barley straw, Triticale hay</td>
<td>14/19/29</td>
<td>28/60/120</td>
<td>0.1</td>
<td>+</td>
<td>H2SO4</td>
<td>Lime</td>
</tr>
</tbody>
</table>

* a) (Liu et al., 2013a); b) (Liu et al., 2013b); c) (Chen et al., 2012); d) (Xie et al., 2011); e) (Monavari et al., 2011); f) (Oleskowicz-Popiel et al., 2011); g) (Xu et al., 2010); h) (Sipos et al., 2010); i) (Pakarinen et al., 2012); j) (Herrmann et al., 2011); k) (Shinozaki and Kitamoto, 2011); l) (Pakarinen et al., 2011); m) (Zheng et al., 2011); n) (Digman et al., 2010a); o) (Vervaeren et al., 2010); p) (Pakarinen et al., 2008); q) (Chen et al., 2007)
Additionally, the table show if or if not the study provided a comparison between non-ensiled and ensiled biomasses, and if ensiling, in that case, gave a positive or negative effect on pretreatment. Furthermore, the studies in Table 4 is divided by those that applied ensiling as sole pretreatment, and those that applied ensiling together with additional pretreatment.

**Ensiling as sole pretreatment**

The studies on ensiling as a sole pretreatment, generally confirm the hypothesis that ensiling can provide an effect as pretreatment. However, the studies represent a huge diversity in treatments and include a wide range of biomasses. The large variations in methods makes it difficult to derive consistent correlations between silage fermentation and pretreatment effect, and especially the use of enzymes and acidic or alkaline additives distort the effect of the actual silage fermentation. It can therefore be concluded that more research is needed to look exclusively on the pretreatment effect of the silage fermentation.

One of the studies that singled out the pretreatment effect of the silage fermentation is Shinozaki et al. (2011), whom compared enzymatic saccharification and ethanol fermentation of sun dried- and ensiled rice straw. The ethanol conversion yield on the biomasses increased slightly due to ensiling from 60% to 63% of the maximal theoretical yield for each substrate. However, the actual ethanol concentration after each fermentation was exactly the same (Shinozaki and Kitamoto, 2011). This highlights an important issue for ensiling as pretreatment method for biological conversion based on a sugar fermentation platform. Here, the goal of the pretreatment is to facilitate a higher release of fermentable monomers. However in order to ensile the biomass the silage fermentation requires a certain amount of soluble sugars, and these are irreversibly lost to organic acids. It is therefore of significant importance that a total sugar balance is considered.

Such a balance is presented by A. Pakarinen et al. (2011), showing that ensiling of maize resulted in an overall decreased amounts of monosaccharides, while for hemp and faba bean the amount of lost soluble sugars is equaled out with improved enzymatic saccharification (Pakarinen et al., 2011).

Chen et al. (2007) ensiled four agricultural residues, and one forage type biomass with addition of LAB inocula and with/without cellulases and hemicellulases. Here, they found that ensiling improved saccharification of the agricultural residues by 5.2-9.3%, from 19-24% on untreated to
24-34% on ensiled biomass, and the addition of enzymes prior to ensiling did not improve saccharification. On the other hand, ensiling of triticale hay resulted in a direct decrease of sugars after enzymatic saccharification, in accordance with the mentioned loss of soluble sugars. But here addition of enzymes in the ensiling, resulted in 20% more sugars as compared to the silage without enzymes, thus giving same high yield of enzyme saccharification as for untreated, of up to 50%. This points out another important message from the present literature on the pretreatment effect of ensiling. Namely, that 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.

Digman et al. (2010), did not test the effect of silage fermentation, but compared instead acidic wet anaerobic storage, using H₂SO₄, of switchgrass and reed canarygrass. Here, it was found that the same treatments were much more efficient on reed canarygrass than on switchgrass, and it was concluded to be due to the categorical difference between a C₃ grass and a C₄ grass, respectively, where C₄ grass is known to generally be more recalcitrant (Digman et al., 2010a). This was one of the reasons that a temperate grass was chosen in this study, as it was hypothesized that this type of biomass was less recalcitrant compared to mature dry agricultural residues with a high degree of lignification.

Overall the studies show that ensiling is definitely a method which have potential as a pretreatment method, but the levels of improvement can be low depending on conditions and type of biomass. The presented review (Table 4) shows, maybe also therefore, that ensiling has been used in combination with additional pretreatment, in order to increase pretreatment efficiency.

### Ensiling in combination with other pretreatments

However, for the studies that include ensiling in combination with additional pretreatment, it has not been a specific objective to look at the contribution in pretreatment effect by ensiling as such. Only three studies measured the difference between untreated and ensiled biomass on the additional pretreatment.
Oleskowicz-Popiel et al. (2011) combined ensiling of maize, whole crop rye, and clover grass mixture, with HTT (190°C, 10 min) and studied the effect of the additional pretreatment on the subsequent ethanol yield. The result showed a significant increase from 33%, 27%, and 36% of theoretical ethanol yield, to 78%, 73%, and 80%. However, a comparison to HTT of dry biomasses, was not carried out.

Acetic acid was used as catalyst in HTT of wheat straw by Monavari et al. (2011), where it was shown that addition of 0.04 g/(g DM) acetic acid increased glucose yield at both 190°C and 195°C, however not at 200°C, thus the effect of lactic acid was more significant at lower temperatures.

The effect of wet storage of corn stover without any additives as opposed to dry storage, where recently tested on an additional steam explosion pretreatment (Liu et al., 2013b). Here it was found that the wet storage improved the subsequent enzymatic saccharification, due to the concentration of lactic acid. But also due to the wet storage that retained hydration and flexibility of the biomass as opposed to dry corn stover, where shrinkage and collapse of the cell wall pore structure caused decreased permeability for the high pressure steam.

It can be concluded that ensiling holds many interesting possibilities also for various pretreatment combinations. However, this is a very new area of research with a limited amount of studies and a sparse focus on silage conditions. Thus much more research is needed towards development of applicable pretreatment solutions that take advantage of ensiling, its simple approach, low energy usage and possibilities for cost reduction, as well as advantages for the agricultural management.
Chapter two
Consolidated results & discussions

The following chapter consists of a thorough review containing the core studies of the PhD project, main results and discussions leading to an overall conclusion and outlook. The work is presented in a chronological order and reflects to a large extent the progression of the project. Parts of the presented work have already been written into manuscripts, and other parts have not. A total of three manuscripts have been submitted; two of which have been published, and one which is currently in review. In the following, these articles will be referred to as Paper I, Paper II, and Paper III, according to the chronological order in which they were submitted.

The chapter has been divided into five headlines of main research objectives, and thus are studies with the same objective gathered under the same headline. Since the consolidated results and subsequent discussion have been gathered and written after submission of the manuscripts, emergent new correlations and discussions based on the consolidated data are not necessarily reflected in the Papers.

5 Method development

An initial part of the project was to establish and standardize a functional laboratory method for the ensiling of biomass, at the DTU BIOENG laboratory, Risø Campus.

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 studies in Table 4, 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.

Two general methods of simple lab scale ensiling exist; a traditional method based on (Allan et al., 1937) using air tight test tubes or glass jars and manual compaction of biomass, and a ‘newer’ method based on (MAFF-33, 1986) using vacuum packaging of the biomass in plastic. The latter method was optimized by Johnson et al. (2005) 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 (Johnson et al., 2005). It was therefore decided to use the vacuum based ensiling method in this project.

Vacuum was applied by a food packaging vacuum machine (Variovac EK10, (520x560x180 mm)) and each resulting bag of biomass can be considered as an independent silo. The duration of ensiling was ended by disruption of the bags and followed by analysis of silage quality, biomass analysis and enzymatic saccharification etc.

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, adapted from (MAFF-33, 1986).
Ensiling is a complex biological system and is affected and influenced by numerous dynamic factors, many of which are hard to actively control, such as the amount and origin of epiphytic microflora and the exact chemical composition of the biomass. Such factors are mainly determined by the growth environment, but are also greatly affected by the biomass handling (Buxton and O'Kiely, 2003). Therefore, it is necessary to be extremely aware of the pitfalls during the handling of biomass and the process of silage packaging, in order to gain applicable results with high reproducibility.

During the initial set up of the method, we soon found out how important the practical procedure was to the results. An example of this was seen when initial experiments suffered heavily from a high degree of puncture, and therefore, the silage bags became unusable due to perforation by vertically aligned biomass fibers. Uneven distribution of stem and leaf fraction between the bags was also noted to cause high deviations. Although, the first many experiments could not directly be used to draw scientific conclusions about the silage fermentation, these experiments proved to hold valuable practical knowledge.

*Figure 9: Vacuum packaging machine (Variovac EK10) and two silage bags with Festulolium Hykor*

The laboratory silage fermentation should in the best possible way resemble applied agricultural practices in order to reflect reality and produce applicable results. The processes of logistics and storage, in-between harvest and laboratory ensiling, can be a determining factor for the outcome of the silage fermentation (Cherney and Cherney, 2003). In order to gain a realistic ensiling, compared to normal agricultural practice, it was important to avoid any drying, freezing or prolonged refrigerating of the biomass prior to ensiling. The effect of biomass storages prior to
ensiling has been investigated on corn stover, and significant effects of drying and refrigerating were found, while freezing produced statistically similar results to that of freshly ensiled corn stover (Tanjore et al., 2012), however, freezing is not without consequences (Cherney and Cherney, 2003). Experiments of ensiling were therefore conducted as soon as possible and within 24 hours after the time of harvest.

Fractionation of the biomass is another influential process to the silage fermentation, and both length of the fibers, fractionation method, and timing are important. Especially important, in this regard, is the biological activation of epiphytic LAB, triggered by fractionation of grass and known as ‘chopper inoculation’ (Pahlow et al., 2003). This effect occurs as a large part of the epiphytic LAB are present in a dormant stage under aerobic conditions, but can be re-activated when the bacteria comes into contact with plant cell substances (superoxide dismutase and manganese), which are released under mechanical fractionation of biomass (Archibald and Fridovich, 1981; Daeschel et al., 1987). Several fractionation methods were tested in the initial experiments. Simple manual cutting to a size of approximately 5cm in length, using a customized old fashion bread slicer was found to be the most suitable and convenient method for the laboratory vacuum packaging.

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.
6 Ensiling as biological pretreatment of grass
Factors affecting pretreatment effect (Paper I)

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 (Buxton et al., 2003). The objective of the study was therefore to investigate the relations of these three important factors upon enzymatic saccharification of cellulose after ensiling.

Hypothesis:

- Ensiling can function as a biological pretreatment of grass, due to a mild acid hydrolysis of structural components.
- Composition of grass, dry matter, and type of inocula have a significant effect on the silage fermentation which will affect the degree of pretreatment.

Experiment:

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 ranges of 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.

Results and discussion

The compositional analysis of the four grasses showed that 1st and 3rd cut had comparable compositions, while 2nd and 4th cut had significantly lower cellulose and lignin content, but higher amounts of extractives (Figure 10). The difference in biomass composition was mainly due to the relative maturity of the grass at harvest. When grass matures, it builds up more secondary cell wall within their tissue, and the extractable cell content drops (King et al., 2012). Thus 1st and 3rd cut were more mature than 2nd and 4th cut. As mentioned previously, this can have significant effect on the recalcitrance of the different cuts (Keating and O’Kiely, 2000; Buxton and O’Kiely, 2003)
Silage quality analysis of pH and organic acids after ensiling (Figure 11) 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 would be less recalcitrant (Keating and O’Kiely, 2000), suggesting that substrates for silage fermentation could have come from the structural fiber e.g. hemicellulose.

The pH followed in general the produced organic acid; being low when acid concentration was high and vice versa. pH was especially correlating with the concentration of lactic acid, which is due to the lower pKₐ of lactic acid compared to e.g. acetic acid, 3.86 and 4.76 respectively. Hence pH dropped to around 4 in all treatments with high lactic acid concentrations, >6 (w/w)%. The pH did, however, not correspond to the acid production for medium and high DM ensiling of 4th cut. This can be explained by the higher crude protein content of 4th cut, which is known to result in a higher buffering capacity in silage fermentations (Muck et al., 1991). Dewar et al. (1963) found a significant
increase in hemicellulose degradation for long term storage of perennial ryegrass (*Lolium perenne*) by lowering pH from 5 to 4 (Dewar et al., 1963). It is therefore a desired trait of the silage fermentation to reach close to pH 4.

The effect of different LAB inocula on the silage fermentation was not clear, and only ensiling 3rd cut resulted in a consistent effect. Here, the homofermentative (GP) inoculated silage produced significantly higher amounts of lactic acid and resulted in a significantly lower pH, which is in accordance with a homofermentative silage fermentation. The inconsistent and lacking effect of LAB inocula for the remaining three cuts suggests that the natural epiphytic bacteria dominated the silage fermentation to a large extent. It was thus concluded that the inoculated amount, in these cases, was insufficient.

*Figure 11*: 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.
The organic acid concentration was generally higher for the low DM ensiling (Figure 11). 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 were the dominating acid in all treatments and the main contribution to the trend of high organic acid concentration at low DM (Figure 12A). 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 (Figure 12B). 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). However, more data are needed to describe any correlations further.

The lower organic acid production at high DM could be due to plant cell respiration, which also takes place after harvest (Muck et al., 2003), and therefore, also during the extended drying of the grass for medium and high DM treatments. Extended respiration would imply additional loss of WSC, which in turn is not available for LAB and organic acid production. Loss of WSC during drying was however not quantified in this study.
Compositional analyses of the grass silage were performed only on 1st cut, due to the large number of samples. The results showed almost no changes in the content of cellulose and lignin as were expected, but also, hemicellulose content was exactly the same (Figure 13). This is contrary to the significant reduction in hemicellulose that has been found in previous studies (Dewar et al., 1963; Morrison, 1979; Singh et al., 1996; Ren et al., 2007) and conflicts with the hypothesis that hemicellulose, to a certain degree, is hydrolyzed by organic acids during ensiling. However, the monosaccharide analysis of the water extraction, used for silage quality analysis (Figure 11), showed small amounts of xylose (<1(w/w)%) as opposed to none in the WSC analysis of the fresh grass, thus at least some xylan associated to hemicellulose have been hydrolyzed, but the amount was just not significant enough to be clear in the compositional analysis. Additionally, the compositional analysis does not reflect any destructuring of the lignocellulosic fiber unless soluble

Figure 12: Correlation between organic acid production and DM content of the different silage treatments. A. Main organic acids vs. DM; B. Total organic acids vs. DM, for four cuts.
components are released. The ensiling can therefore still have induced a pretreatment effect on the grass silage in the form of destructuring.

![Figure 13: Lignocellulose in 1st cut grass and grass silage at low (21%), medium (31%) and high (41%) DM content, and averaged over inocula.](image)

The objective in the enzymatic saccharification was to test the effect of ensiling on the enzymatic degradation of cellulose and hemicellulose. The enzymatic saccharification of grass and grass silage was therefore carried out on water extracted fibers, where WSC was removed in order to only analyze the actually enzymatically solubilized monosaccharides. It is thus important to consider the removed WSC after retrieving the result of the enzymatic saccharification to get the overall gain from ensiling.

Enzyme saccharification of the untreated four cuts did, also result in significant differences between the four cuts. 2nd cut gave the highest glucose yield followed by 1st, 4th, and last 3rd cut yielding 9.0, 7.8, 7.5, and 5.5 (w/w)% of DM respectively (Table 5). On the other hand calculation of the cellulose convertibility (converted glucan per total glucan) showed that enzyme saccharification acted most efficiently on the cellulose of 4th cut followed by 2nd, 1st, and 3rd (Table 5), suggesting that the less mature grass was less recalcitrant than the mature. This is in line with general knowledge on DM digestibility of grass, which increases at increasing maturity (Keating and O’Kiely, 2000; Buxton and O’Kiely, 2003)

Advanced maturity increases lignin content concurrently with the increase of cellulose content, consequently, causing higher lignocellulosic recalcitrance. The trade of high cellulose content is
thus related to the decrease of cellulose convertibility, thereby resulting in less released glucose. This counter-relation induces an ideal stage of maturity for grass, where cellulose content and convertibility result in optimal sugar release, in this study represented by 2nd cut.

Table 5: Glucose yield and cellulose convertibility of dry grass

<table>
<thead>
<tr>
<th></th>
<th>1st cut</th>
<th>2nd cut</th>
<th>3rd cut</th>
<th>4th cut</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose yield, g/100gDM</td>
<td>7.8b ±0.16</td>
<td>9.0a ±0.03</td>
<td>5.5c ±0.15</td>
<td>7.5b ±0.04</td>
</tr>
<tr>
<td>Cellulose convertibility, %</td>
<td>28.3c ±0.87</td>
<td>40.4b ±0.38</td>
<td>21.4d ±0.67</td>
<td>47.4a ±0.27</td>
</tr>
</tbody>
</table>

The pretreatment effect of ensiling is retrieved through comparison of the enzymatic hydrolysis of glucose yield and cellulose convertibility between dry grass and ensiled grasses (Figure 14A and Figure 14B).

The results clearly show that ensiling in general have had a positive effect on the release of sugar, and low DM treatments gave the highest glucose yields for all cuts. Ensiling of 2nd cut did, however, only improve enzyme hydrolysis at low DM. Ensiling of 1st cut at high DM gave also insignificant improvements, whereas high DM treatments for both 3rd and 4th cut resulted in considerable improvements.

1st, 2nd, and 4th cut resulted in similar maximum glucose yields, around 11 (w/w)% DM regardless of the glucose yield of the appertaining dry grass. Due to the different cellulose content in the four cuts, the picture changes for cellulose convertibility. Here, the 4th cut had the highest of 69% followed by 2nd, 1st and last 3rd cut, reaching 50%, 40%, and 32% converted glucan respectively, all at low DM and averaged over inoculum.

Oleskowicz-Popiel et al. (2011) ensiled clover-grass and achieved a cellulose convertibility of 42%, which was an improvement of 47% to that of the non-ensiled clover grass. This fits well with the improvements obtained for the low DM ensilage in current study, at low DM and averaged over inocula, of 40%, 23%, 51% and 46% for 1st, 2nd, 3rd, and 4th cut, respectively (Oleskowicz-Popiel et al., 2011). The lower improvement for 2nd cut is a consequence of the already quite efficient convertibility of the untreated dry grass.

The improvements were also significantly higher than what was found by Chen et al. (2007) on more lignocellulosic biomasses of cereal straw, of maximum 9.3%.

Merging the results of organic acid production and enzymatic hydrolysis suggests that high organic acid production during ensiling leads to an improved pretreatment effect, and this can be induced
by ensiling at low DM, which secures a high WSC substrate pool. The low DM can, however, also have been contributing to the improved pretreatment effect in itself, since lower DM in the silage bags allowed for a better mobility of soluble organic acids to act on the biomass during long term storage.

Figure 14: Enzymatic hydrolysis of dry grass and silage grass (washed fiber). A: Glucose yield, w/w % of DM raw grass; B: Cellulose convertibility, released unhydrated glucose as percentage of total cellulose.

The improved convertibility corroborated the theory, that ensiling promotes a gentle hydrolysis of lignocellulosic structures, which in turn increases the access for cellulase enzymes to the cellulose. However, significant decrease of hemicellulose after ensiling could not be found in this study. Thus, the explanation for improved cellulose hydrolysis might be a mere alternation of the hemicellulose
structure, lignocellulosic inter-linkages or the surrounding structures of pectin or cutin as well as proteolysis of structural proteins.

As mentioned, it is important that the glucose release from the grass silage is related to the amount of WSC that was utilized during ensiling. The contents of WSC in the four grasses were 6.1, 10.3, 4.1, and 4.6 (w/w)% for 1st, 2nd, 3rd, and 4th, respectively. The maximum increase in glucose release induced by ensiling was 3.6, 2.3, 2.9, and 3.5 (w/w)%, respectively, and the increase in other monomers (xylose, arabinose, and galactose) are each below 1 (w/w)%. Some WSC are, however, still left in the grass silage, ranging between none and 3 (w/w)%. It is therefore questionable, if ensiling can provide excess amounts of sugars, since this was not the case in this study.

Conclusions

The study confirmed that ensiling of grass can improve cellulose convertibility compared to dry storage. This were seen for all four cuts causing maximum improvements of 40%, 23%, 51% and 46% for 1st, 2nd, 3rd, and 4th cut, respectively.

Among the three factors of biomass composition, DM, and LAB inocula, only composition and DM were found to have a significantly important effect on convertibility of cellulose in subsequent enzymatic saccharification.

Different biomass composition of the same grass species caused considerable differences in pH and organic acid concentration, as well as differences in the level of cellulose convertibility of untreated grass between the four cuts. The biomass composition was largely determined by the relative maturity at harvest, and it was shown how less mature grass had a higher level of cellulose convertibility. The results suggest an optimum stage of maturity for grass, between optimal cellulose content (at late maturity) and optimal cellulose convertibility (at early maturity), which will result in an optimum glucose release. Exactly, when that is will require further studies including several maturity samples of the same cut.

The DM had similar effect on each cut, increasing glucose conversion with decreasing DM. The low DM ensiling resulted in the highest production of organic acids, giving the highest pretreatment effect. Thus low DM ensiling (<25%) resulted in the highest glucose yields for all cuts. Additionally, it is proposed that low DM ensiling improves the pretreatment effect due to a better mobility of the organic acids to act on the biomass.
The added LAB inocula did only have a consistent effect on the organic acid production in one out of four cuts, and it had no significance on the cellulose convertibility. It was concluded that the dosage might have been insufficient to compete with epiphytic LAB. Due to the general result that high organic acid concentration improves pretreatment effect, it is still argued that the addition of LAB can only improve the pretreatment effect, or in worst case, be inconsequential.

The improved convertibility of cellulose did, however, not result in an overall increase in fermentable sugars, since the loss of WSC during ensiling exceeded the improvement.

**Outlook**

The obtained cellulose convertibilities after ensiling in the first study (Paper I) is still very low compared to more severe pretreatment methods developed for cellulosic bioethanol. Methods such as hydrothermal treatment, steam explosion, dilute acid treatment or ammonia fiber explosion are all known to reach cellulose convertibilities of above 90% (Galbe and Zacchi, 2012)(Hu and Ragauskas, 2012).

In relation to ethanol production, cost effective ethanol distillation requires a certain concentration of fermentable sugars for the fermentation. Such a concentration threshold can be calculated using the limitations of maximum DM in the enzymatic hydrolysis and a minimum ethanol concentration for the distillation. By assuming a maximum DM of 30% and a minimum ethanol concentration of 4 (w/w)% as according to (Larsen et al., 2008; Larsen et al., 2012), the required fermentable sugar concentration after enzymatic saccharification should be minimum 26 (w/w)% of DM pretreated material.

The maximum concentration of glucose after enzymatic hydrolysis in Paper I, of 11 (w/w)% is therefore not adequate to sustain an economical production of bioethanol, and improvements are needed.
7 Improvements of sugar release

Optimization of enzyme blend & Ensiling with addition of enzymes (not published)

Two approaches were considered in order to improve the sugar release after enzymatic saccharification of grass silage:

- Firstly, by means of a targeted optimization of the enzymatic blend used in the enzymatic saccharification to gain a better match with grass biomass.
- Secondly, by means of improving the actual pretreatment effect of ensiling using biomass degrading enzymes to act during long term storage.

Consequently, two studies were conducted, and the experiments, results and discussions are presented below. The studies are not included directly in any of the published or submitted papers.

7.1 Optimization of enzyme blend

Background

Enzyme development in biomass hydrolysis have largely been driven by the development of cellulosic ethanol, where severe pretreatment is a prerequisite for high yields. Predominant pretreatments such as hydrothermal- and weak acid- treatments, are known to solubilize major parts of the hemicellulose (Hu and Ragauskas, 2012). Thus, enzymatic hydrolysis of pretreated biomasses does generally not deal with high amounts of more or less intact hemicellulose, and the commercial enzyme blends are benchmarked against pretreated biomasses. In this study, enzymatic hydrolysis was applied on untreated grass and grass silage with a more or less intact lignocellulosic structure. This calls for an optimization of the enzyme blend targeted at grass silage.

A reason for the low cellulose convertibility in Paper I could very likely be found in the even lower conversion of hemicellulose. The conversion of xylan in the enzymatic saccharification performed in Paper I was very low and did not exceed 15% converted xylan per total xylan (Figure 15), and the conversion was not improved consistently by ensiling, as was the case for cellulose.
Figure 15: Xylan convertibility in the enzymatic saccharification of dry grass and grass silage (washed fiber). Xylan convertibility, released unhydrated xylose as percentage of total xylan.

It was therefore suspected, that the low hemicellulose conversion was due to insufficient amounts of hemicellulases in the CTe2*:Cellic HTec® (9:1) enzyme blend. CTe2 contains a ß-xylosidase EC 3.2.1.37 and Cellic HTec® contains mainly endo-1,4ß-xylenase activity EC 3.2.1.8. The strategy for higher xylan conversion was therefore to increase the relative amount of Cellic HTec® and add additional ß-xylosidase EC 3.2.1.37.

Hypothesis

- Increased hemicellulose activity in the enzyme blend will improve xylan conversion and facilitate better accessibility for cellulases to make substrate binding and improve cellulose convertibility.

Experiment

Enzymatic hydrolysis of grass and grass silage from both 1st and 2nd cut used in (Paper I), was therefore performed again with varying CTe2*:Cellic HTec® ratios (9:1; 7:3; 5:5) and additional ß-xylosidase EC 3.2.1.37 (1, 2, 4% (w/w) cellulose), otherwise same enzymatic saccharification procedure as in Paper I.
Results & Discussion

The increased ratios of Cellic HTec2® and additional β-xylosidase did not have any significant effect on either cellulose or xylan conversion (Figure 16). Thus, xylanase activity was not the limiting factor of the low xylan conversion in Paper I.

A logic explanation for the lacking effect of additional xylanase activity is also that the xylan degrading enzymes are hindered in their access to bind their substrate. Substrate binding is often inhibited by a high degree of structural branching, so if the hemicellulose in the grass silage still is highly heterogeneous and branched it is easy to imagine that the xylanase cannot access the xylan backbone. Xylanases have furthermore been found to have an abundant diversity in, structure, mode of action and substrate specificities, due to the heterogeneity and complexity of xylan (Berrin and Juge, 2008). Thus the xylanases present in Cellic HTec2® could also not be an optimal match with the xylans in the grass silage.

Hemicellulases do not only include xylan degrading enzymes. Due to the branched structure of hemicellulose, it is also necessary to have enzymes acting upon the side chains and cross linkages (Yu et al., 2005).

Figure 16: Enzymatic hydrolysis of 1st and 2nd cut grass and grass silage (low DM, CCM inocula) 2011. Different Ctec:Htec ratios (9:1, 7:3, 5:5) and additional β-xylosidase (1, 2, 4% (w/w) cellulose)

Juturu and Wu (2013) (Juturu and Wu, 2013) recently reviewed hemicellulose degrading enzymes with a special focus on the non-xylanases. These include several additional glycosyl hydrolases, but also two carbohydrate esterases, namely acetyl xylan esterase (AXE) and ferulic acid esterase (FAE).
The latter cleaves ferulic acid side chain substitutions of the arabinoxylan, and the former cleaves acetic acid substitutions of the xylan backbone.

Table 6: Native functions of hemicellulases and their action sites on hemicellulose. From: (Juturu and Wu, 2013)

<table>
<thead>
<tr>
<th>Enzyme type</th>
<th>Native function</th>
<th>Action sites</th>
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<tr>
<td><strong>I) Glycosyl hydrolases:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endo-xylanase</td>
<td>cleaves β-1,4 bond of xylan backbone releasing xylooligomers</td>
<td>β-1,4 xylan backbone</td>
</tr>
<tr>
<td>β-Xylosidase</td>
<td>cleaves exo β-1,4 bond of xylooligomers releasing xylose</td>
<td>β-1,4 xylooligomers</td>
</tr>
<tr>
<td>Endo-1,4-mannanase</td>
<td>cleaves β-1,4 bond of mannan releasing mannan oligomers</td>
<td>β-1,4 mannan</td>
</tr>
<tr>
<td>β-Mannosidase</td>
<td>cleaves exo β-1,4 bond of mannan oligomers releasing mannose</td>
<td>β-1,4 mannan oligomers</td>
</tr>
<tr>
<td>α-L-Arabinofuranosidase</td>
<td>cleaves arabinan at O-2 and O-3 positions on xylan back bone</td>
<td>α-L-arabinofuranosyl oligomers</td>
</tr>
<tr>
<td>α-L-Arabinanase</td>
<td>cleaves xylooligomers generating arabinose</td>
<td>α-1,5-arabinan</td>
</tr>
<tr>
<td>α-D-Glucuronidase</td>
<td>cleaves α-1,2 bond between glucuronic acid side chain substitutions releasing glucuronic acid</td>
<td>4-O-methyl-α-glucuronic acid</td>
</tr>
<tr>
<td><strong>II) Carbohydrate esterases:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetyl xylan esterase</td>
<td>cleaves acetyl side chain substitutions releasing acetic acid</td>
<td>2- or 3-O-acetyl xylan</td>
</tr>
<tr>
<td>Feruloyl xylan esterase</td>
<td>cleaves ferulic acid side chain substitutions releasing ferulic acid</td>
<td>Ferulic acid substitutions</td>
</tr>
</tbody>
</table>

Temperate grasses are known to have a significant high degree of ferulate - arabinoxylan cross-links, which have been shown to be a limiting factor for plant cell wall digestion in ruminants (Yu et al., 2005; Xu et al., 2007). Wende and Fry did a comprehensive study on tall fescue grass, one of the temperate grass species in Festulolium Hykor, where they determined the presence of both O-feruloylated and O-acetylated oligosaccharide side chains on the backbone of arabinoxylan (Wende and Fry, 1997c; Wende and Fry, 1997a), and after enzymatic hydrolysis with an enzyme blend based on the fungus Irpex lacteus they noted that the ferulylated arabinoxylan in tall fescue were ‘unusually resistant’ compared to that of maize (Wende and Fry, 1997b).

It was therefore decided to add additional FAE and AXE in the enzyme blend of Cellic CTec2®:Cellic HTec2® (9:1).
Hypothesis II

- FAE and AXE in the enzyme blend will cleave cross-links between lignin structures and hemicellulosic structure and facilitate a better substrate binding of especially endo-1,4β-xylenase and increase xylan conversion, which in turn will increase cellulose conversion.

The result from the enzymatic hydrolysis with additional FAE and AXE did, however, not give any improvements of either xylan- or cellulose conversion, as compared to the original Cellic CTec2®:Cellic HTec2® (1:9) blend (Figure 17).

![Figure 17: Enzymatic hydrolysis of 1st and 2nd cut grass and grass silage (low DM, CCM inocula) 2011. Additional ferulic acid esterase (FAE) and acetyl xylan esterase (AXE) (2, 4% (w/w) cellulose)](image)

Conclusion

The low xylan conversion could neither be improved by additional xylanase activity nor additional esterases. It is still possible that additional glycosyl hydrolases, such as α-L-arabinofuranosidase and α-D-glucuronidase, which cleave arabinan and glucoronic acid -side chain substitutions, respectively (Table 6), could improve the xylan conversion. This was, however, not carried out in this setup due to lack of biomass material. The study confirms that tall fescue grass, here represented in a crossbreed, is unusually resistant to enzymatic saccharification as suggested by Wende and Fry (Wende and Fry, 1997b).
The anticipation of these experiments was, that the low xylan conversion could be resolved in a ‘quick fix’ by adding carefully selected enzymes likely to help hemicellulose hydrolysis. However, this proved unsuccessful and it was therefore concluded that the problem calls for a more comprehensive and detailed study of both the hemicellulosic structure and an extended list of specific hemicellulases to find the structures that could be responsible for the low xylan conversion. This was, however, not within the reach nor was it the aim of this PhD project.

7.2 Ensiling with addition of enzymes

Background

Optimization of the actual pretreatment effect during ensiling would, alternatively, add to the necessary improvement of cellulose convertibility. A new silage study was therefore conducted based on the results in Paper I, but here with addition of biomass degrading enzymes, which could act on the biomass during storage and enhance the pretreatment effect.

Addition of cell wall degrading enzymes to silage is not new and has since beginning of the 1990s been widely used as a silage additive, applied for biomasses, which are difficult to ensile due to low WSC content, such as corn stover and wheat straw ((Kung, Stokes and Lin, 2003). Furthermore, cell wall degrading enzymes have been added to temperate grasses, to reduce fiber content, improve digestibility and DM intake of rumen animal feed (Muck and Bolsen, 1991)(Dehghani et al., 2012). Dehgani et al. (2012) found that by adding multi component cellulase and pectinase enzymes significantly decreased fiber content (measured as neutral detergent fiber, NDF) after ensiling of Maize stover, lucerne and grass clover (Dehghani et al., 2012).

Moreover, researchers have studied ensiling with enzymes in order to improve biomass conversion for biofuel production (Chen et al., 2007; Ren et al., 2007; Ren et al., 2007; Chen et al., 2012; Pakarinen et al., 2012). As mentioned previously Chen et al. (2007) ensiled five biomasses, including four agricultural residues (barley-, triticale- and wheat straw, and cotton stalks) and one forage crop (triticale hay), with an addition of two commercial enzyme blends (Celluclast+Novozym 188 and Spezyme CP+Multifect xylanase) and found that the ensiling of triticale hay with the addition of enzymes increased the amount of released sugars by 20% in subsequent enzyme saccharification, while there was no significant improvement for the agricultural residues. Thus, suggesting that the forage type biomass was less recalcitrant and can therefore more successfully be subjected to enzymes during ensiling. On the other hand, the study showed that enzymatic saccharification of untreated triticale hay gave same high amount of released sugars, which was
explained by the high content of WSC in untreated hay. As it was also the case in this project (Paper I).

Ren et al. (2007) ensiled corn stover with addition of several cellulase and hemicellulase mixtures from different fungal origin at different dosage, and found up to 16% cellulose degradation. High cellulose degradation also yielded higher WSC after ensiling; however, it was not clear if the amount of extra WSC corresponded to the loss of cellulose degradation or if degraded cellulose had also been utilized for production of organic acid by LAB (Ren et al., 2007).

High cellulose degradation has a high risk of considerable sugar loss due to metabolisation of the released glucose by LAB during storage. It was therefore decided to avoid addition of cellulases in this study. The grass was instead treated with a cutinase blend, a hemicellulase blend and a mono-component β-xylosidase.

**Hypothesis**

- Addition of enzymes before ensiling will assist de-structuring of the biomass during long term storage and improve pretreatment effect.

A cutinase blend (NS81023, Novozymes, Bagsværd) was added to improve hydrolysis of the outer layer in grass, in order to expose the cell wall polymers to acid hydrolysis.

Cellic HTec2® was added to directly improve hydrolysis of hemicellulose during ensiling, and thus facilitate a better subsequent enzymatic hydrolysis. As previously discussed, Cellic HTec2® consists of mainly endo-xylanases, but does also contain cellulose side activity.

Mono component β-xylosidase (EC 3.2.1.37) was included in the experiment to assist hemicellulose hydrolysis, and to add additional β-xylosidase activity to Cellic HTec2®.

**Experiment**

The experiment was carried out on 1st cut grass from 2013, ensiled in vacuum bags, at low DM (22%) and addition of homofermentative (GP) inocula (8.0 g/kg fresh grass (double the amount as the amount used in Paper I)). The three different enzymes were added separately and in combination, as well as at a low, medium and high dosage (0.01, 0.1, and 1 (w/w)% DM). Thus the experiment amounted to 21 treatments plus enzyme blanks.
Results and discussion

The amount of soluble carbohydrates in the water extraction after ensiling was used to measure the effect of the enzyme treatment in the grass silage (Figure 18A). The result showed a clear effect of Cellic HTec2®, but no significant effect of cutinase and ß-xylosidase or their combination. All combinations, which included Cellic HTec2®, resolved in a similar effect to that of Cellic HTec2® alone. However, more soluble carbohydrates were observed at low and medium enzyme dosage, when Cellic HTec2® were in combination with ß-xylosidase (Figure 18A).

The distribution of soluble monomers was generally the same in all treatments, namely equal amounts of glucose and xylose. However, the soluble oligomers also included significant amounts of galactose and especially arabinose, which suggests hydrolysis of pectic structures like arabinan and arabinogalactan, and/or hemicellulosic side chain residue from arabinoxylan.

The release of more soluble carbohydrates in the treatments with Cellic HTec2® can, as mentioned above, give rise to an increased LAB fermentation and thus, more organic acid. Accordingly, it was observed how Cellic HTec2® treated grass silage tended to contain higher amounts of organic acids (Figure 18B). On average, the high dosage Cellic HTec2® treated silage produced 1.5(w/w)% more organic acid than the enzyme blank. This was, however, not statistically significant due to the relative high standard deviations.

Enzymatic hydrolysis of the silage fiber did not significantly increase either glucose- or xylose release in any of the enzyme treated grass silages. Instead, and contrary to what was anticipated, the glucose release dropped in all cases with grass silages treated with Cellic HTec2® at high enzyme dosage. The decrease was in general from approx. 7 (w/w)% to 3 (w/w)% (Figure 18C). The release of glucose equaled a cellulose convertibility of 27%, 28%, and 24% for the cutinase treated, the ß-xylosidase treated and the blank silage, respectively. While the cellulose convertibility was only 11% for the Cellic HTec2® treated grass silage at high enzyme dosage.

An explanation for the lower glucose yield could be that the actual glucose release during ensiling was considerably higher than what was found in Figure 18A, but the majority of that glucose was in turn utilized as substrate for LAB and secondary fermentations during storage. The suspected release of glucose and its utilization at high dosage of Cellic HTec2® reduce the glucose yield with approx. 4 (w/w)% (Figure 18C). As mentioned, the total organic acid concentration increased in same treatments with approx. 1.5 (w/w)%, thus 2.5 (w/w)% is unaccounted for. Other metabolites than acids were also observed to be slightly higher for the Cellic HTec2® treated grass silage, namely ethanol and mannitol, and some metabolites were not quantified such as butanol, 2,3-butandiol,
or succinic acid, and last generation of CO₂ should be considered. It is therefore thought to be a plausible theory.

![Figure 18](chart.png)

*Figure 18:* Ensiling of grass (1st cut 2013) with different enzyme treatments. **A:** Soluble carbohydrates after ensiling. **B:** Total organic acids (lactic, acetic, propionic acid). **C:** Enzymatic saccharification, glucose and xylose yield, w/w % of DM raw grass.
A significant release of glucose by Cellic HTec2® can be explained by a cellulase side activity, which is expected to be included in Cellic HTec2®. It seems, however, unlikely that the glucose released by Cellic HTec2® during ensiling originates from cellulose, when xylan is left almost untouched by the enzymes. The glucose could instead origin from mixed linkage β-glucans in the hemicellulosic structure, which as mentioned earlier is normally known to be abundant in the primary cell wall, but recently, also suggested to have a considerable structural function in secondary cell walls of the mature plant (Vega-Sánchez et al., 2013).

The presence of significant amounts of mixed linkage β-glucans, would imply an overestimation of the cellulose in the compositional analysis of grass and grass silage, and thus, raise the question of how much of the cellulose convertibility found in Paper I is actually conversion of cellulose? This is, however, still merely speculations, and specific studies on the presence of mixed linkage β-glucans are necessary to confirm the theory.

It is noteworthy that only release and consumption of glucose and not xylose, give rise to the significant decrease in glucose yield, and that the xylose release, again in this enzyme saccharification, is very low (Figure 18C). It was indeed unexpected that addition of considerable amounts of xylanases before ensiling would have no effect on the xylan conversion. On the other hand, the result corroborate the unaffected xylan conversion in the experiment on optimization of enzyme blend by adding more Cellic HTec2® (Figure 16). The result suggests that the xylan in Festulolium Hykor is highly inaccessible for the xylanases present in Cellic HTec2®, and ensiling does not provide the necessary pretreatment severity to facilitate better xylan accessibility.

**Conclusion**

Addition of enzymes in this study had either no effect (cutinase and β-xylosidase) or negative effect (Cellic HTec2®) on the enzymatic saccharification yield of glucose and no effect at all on xylose yield. It is believed that the negative effect of Cellic HTec2® was due to a significant release of glucose, due to its cellulase side activity, and that the majority of this extra released glucose were utilized during ensiling, therefore causing a considerable reduction in available glucan for the enzymatic hydrolysis.

It is speculated that a large part of the released glucose did not originate from cellulose, but instead from mixed linkage β-glucans. Further studies are however needed to verify that theory.

The low release of xylose correlates with previous results in Paper I and in the optimization of the enzyme blend. It becomes therefore more and more evident that the hemicellulosic xylan in
*Festulolium* hykor and is highly recalcitrant to enzymatic attack. The reason for the inaccessible xylan is believed to be found in a high complexity of branching and cross linkages in the heterogeneous grass hemicellulose, for which we have not until now matched with the right hydrolytic activity in order to unlock the structure.

The pretreatment effect of ensiling could not be improved and the method does still not facilitate enough released sugars to sustain an economical production of bioethanol.

**Outlook**

High cellulose conversion require considerable changes in the lignocellulosic structure. The somewhat disappointing result of the optimization of cellulose and hemicellulose conversion, suggested that ensiling is simply not providing enough structural change to be a sufficiently severe pretreatment to pose as a satisfactory pretreatment in itself.

The approach of the project was therefore changed from considering ensiling as a sole pretreatment method, to instead consider ensiling as a beneficial pre-step pretreatment prior to a more severe pretreatment using hydrothermal treatment (HTT).
8 Combining ensiling and hydrothermal treatment
Wheat straw (Paper II) & grass (Paper III)

Background

One prevailing pretreatment concept based on high temperature steam and no addition of chemicals has proven to be advantages on numerous parameters, and are applied in several pilot and demonstration scale facilities around the world (Galbe and Zacchi, 2012). The concept is applied to two methods known as hydrothermal treatment (HTT) and steam explosion, where the latter involves an additional rapid pressure release to induce mechanical disruption of the biomass, and the former only applies the concurrent pressure of the high temperature steam. A simple approach is advantageous to scale up but at the same time efficient biomass breakdown and low inhibitor formation is a requirement.

HTT is used in the demonstration scale cellulosic ethanol plant, Inbicon, located in Kalundborg, Denmark. A down-scaled pilot version of that HTT, is located at The Technical University of Denmark (DTU), Risø campus and known as ‘Mini IBUS’. This pilot equipment was used in two studies in this PhD project in combination with ensiling of both grass and wheat straw.

The pretreatment effect of high temperature steam is the mechanism of autohydrolysis, where water acts as a weak acid and initiates depolymerization of hemicellulose (Garrote et al., 2002). This process hydrolysis the O-acetyl groups on the hemicellulose and releases acetic acid, which enhance the acid hydrolysis further and hemicellulose is solubilize. Additionally the high temperatures give rise to a dislocation of lignin (Hansen et al., 2011). Both events increases accessibility to cellulose and facilitate improved enzymatic saccharification. It is likely that ensiling in combination with HTT could prove successful. The HTT factors of temperature, holding time and pH can be combined to one factor expressing the severity of the pretreatment. Higher severity result in increased biomass breakdown. This gives a better pretreatment until a certain maximum from where degradation of monosaccharides, and thus inhibitor formation, gets too high and reduces the overall yield as well as hamper further conversion.

Reducing pH through ensiling will increase the severity factor of the HTT without changing the temperature and holding time. Thus ensiling can potentially result in a decrease of HTT temperature. It has however been shown that the use of the one dimensional severity factor to predict sugar yields is not reliable, due to the complexity of biomass pretreatment (Pedersen and Meyer, 2010).
The combination of ensiling and HTT have previously been studied by Xu et al. (2010) and Oleskowicz-Popiel et al. (2011), however the actual supplementary effect of ensiling as opposed to dry storage, was not addressed in these studies.

Monavari et al. (2011) investigated the effect of long term impregnation of sugarcane bagasse with lactic acid and found a significant improvement in both glucose and xylose yields after one month of storage as opposed to no storage.

Xu et al. (2009) studied addition of lactic- and/or acetic acid to HTT of dry corn stover and concluded that acetic acid was a slightly better catalyst than lactic acid, and increased the ethanol yield in a subsequent SSF from 78% to 87% of the theoretical yield.

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 at different operating HTT temperatures, as compared to dry storage. The choice of applying two biomasses was to find consistencies and differences of the new approach, when using two considerably different biomasses.

As mentioned previously 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.

In this chapter both studies are presented together in order to compare the results, which was not a specific aim of the separate manuscripts (Paper II and III).

Hypothesis

- Organic acids in the silage produced during ensiling will induce increased pretreatment effect of the HTT, measured as solubilisation of hemicellulose and convertibility of cellulose in subsequent enzyme saccharification.
- 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: As stated above 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, as according to (Yang et al., 2006), in the form of xylose. Addition of xylose instead of glucose were based on two arguments. Firstly because it favors production of acetic acid by both homo- and heterofermentative LAB, as was seen in Table 3, and acetic acid has been shown to assist HTT better than lactic acid (Xu et al., 2009), Secondly using xylose gives the possibility of an internal process loop, which utilizes a share of the xylose from the liquid fraction after HTT to facilitate ensiling. The necessary addition of xylose require that the improvement of the pretreatment due to ensiling gives rise to a significantly higher sugar release than the 7 (w/w)% needed to facilitate ensiling.

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 (Larsen et al., 2012).

Wheat straw was thus ensiled by addition of 7 (w/w)% xylose, addition of a LAB inoculum consisting of pure heterofermentative Lactobacillus buchneri (CCM), at 35% DM, and stored for 4 weeks.
Grass: Ensiling of grass was based on the results in Paper I and were naturally more straight forward compared to ensiling of wheat straw. Fresh grass were ensiled at low DM (26%) and addition of the homofermentative LACTISIL GP inocula, containing *Pediococcus pentosaceus* and *Lactobacillus plantarum*. Homofermentative inocula was used, in contrast to ensiling of wheat straw, since the results of organic acid production for 3rd cut in Paper I (Figure 11), actually had a significant effect, increasing organic acid production. It was therefore hypothesized that addition GP in larger amount (x2), could have a significant increasing effect on organic acid production. The effect of inocula, was however not measured since only one treatment was carried out in order to get enough uniformly ensiled grass, for the pilot scale pretreatment. The grass silage was also stored for 4 weeks.

Hydrothermal pretreatment of ensiled and dried biomasses were performed on the 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 was 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 more than 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 of any of the two biomasses (Table 7), which was also observed for the grass (1st cut) in Paper I.
Table 7: Composition of biomasses before HTT. The numbers are presented in weight percent of biomass DM, followed by standard deviations.

<table>
<thead>
<tr>
<th>Biomass</th>
<th>Glucan</th>
<th>Xylan</th>
<th>Arabinan</th>
<th>Lignin</th>
<th>Ash</th>
<th>EtOH extractives</th>
<th>H₂O extractives</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat straw</td>
<td>40.2 ±0.2</td>
<td>22.3 ±0.1</td>
<td>3.3 ±0.0</td>
<td>18.6 ±1.1</td>
<td>5.2 ±0.2</td>
<td>6.3 ±0.2*</td>
<td></td>
</tr>
<tr>
<td>Wheat straw silage</td>
<td>39.7 ±0.0</td>
<td>24.1 ±0.4</td>
<td>2.6 ±0.0</td>
<td>17.5 ±1.2</td>
<td>3.1 ±1.1</td>
<td>2.4 ±0.8</td>
<td>4.6 ±0.1</td>
</tr>
<tr>
<td>Grass</td>
<td>25.2 ±0.9</td>
<td>14.1 ±0.5</td>
<td>2.1 ±0.1</td>
<td>9.3 ±0.1</td>
<td>8.0 ±0.5</td>
<td>11.9 ±0.1</td>
<td>21.1 ±1.1</td>
</tr>
<tr>
<td>Grass silage</td>
<td>24.2 ±0.3</td>
<td>14.5 ±0.2</td>
<td>2.4 ±0.1</td>
<td>9.3 ±0.1</td>
<td>5.3 ±0.0</td>
<td>10.5 ±0.4</td>
<td>25.0 ±1.5</td>
</tr>
</tbody>
</table>

*only ethanol extraction

Obviously, wheat straw and grass differ largely in their composition. Wheat straw contains much more lignocellulose (cellulose hemicellulose and lignin), in this case adding up to 84% of the total DM more than grass, which merely adds up to 50% of total DM. Wheat straw has 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, approximately 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, but as argued in the previously the compositional analysis does not reveal any structural details. The quantity of cellulose and hemicellulose is of great importance to e.g. ethanol production as it comprises the potential fermentable sugars. In this regard, wheat straw has a clear advantage over grass for ethanol production.

Instead, the grass contains high amounts of extractives, both as water extractives and ethanol extractives (Table 7). Water extractives include WSC (monomers and short chain oligomers), organic acids, and a large fraction of total crude proteins (Paper I), but will also include minerals and silica on the 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 fibres, leaving an almost pure secondary cell wall (Thammasouk, Tandjo and Penner, 1997).

It is important to consider the non-structural carbohydrates of grass which comprise a significant amount of the biomass as a potential source for fermentation due to the relatively lower amounts of cellulose and hemicellulose. Severe pretreatment could very likely result in a 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 the pretreatment of grass would be relatively more vulnerable to the high HTT temperatures than wheat straw would seem to be.
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 (Table 8). The recovery of wheat straw DM is similar to that of wheat straw silage, but the ensiling of grass led to a more apparent decrease in DM recovery in solid fraction.

The expected solubilisation of hemicellulose due to the autohydrolysis can be observed by three means: (I) a decreasing content of xylan and arabinan in the solid fraction (Table 8), (II) an increasing concentration of xylose and arabinose in the liquid fraction (Tabel 9), and (III) a calculated recovery of hemicellulose based on the two former and the mass balance over HTT (Figure 20). The latter also adds significantly to the degree of hemicellulose degradation during HTT.

Hemicellulose solubilisation is not pronounced for HTT of wheat straw, while HTT of wheat straw silage causes considerably more solubilisation of hemicellulose, especially at 190°C, which also results in a significant hemicellulose degradation of 36% (Figure 20).

For grass, the hemicellulose solubilisation is not much different between HTT of the ensiled and the non-ensiled biomass. The contents in the solid fraction are exactly the same (Table 4), but differences in the xylose concentration in the liquid fractions (Tabel 9), show that solubilisation was more apparent at HTT 170 °C and 180 °C for grass silage, but at 190 °C the xylose concentration is the highest for HTT of grass (Table 5), which is explained by a higher degradation of hemicellulose in HTT of grass silage at 190 °C (Figure 20).

Hemicellulose content was, however, the main distinctive difference in the compositional analysis of the pretreated solid fraction between wheat straw and wheat straw silage. Thus, up-concentration of cellulose is similar and the up-concentration of lignin is only slightly higher for the ensiled wheat straw (Table 4).

The compositional difference in the solid fractions of pretreated grass and grass silage is, however, different than that of wheat straw. Up-concentration of cellulose was in this case 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% H2SO4). Klason lignin can therefore be overestimated pre-precipitation of e.g. protein (Tamaki and Mazza, 2010). Since non-ensiled grass contain considerably more intact protein, it is suspected that an overestimation of lignin in pretreated grass, was 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, due to a higher possible glucan concentration in the fermentation at a certain DM content (Galbe and Zacchi, 2012)(Larsen et al., 2012). Thus, on this parameter, the ensiling of grass provided the best improvement due to the generally higher DM solubilisation.

Table 8: Composition of solid fraction after HTT. The numbers are presented in weight percent 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. Only the pretreatment of wheat straw silage were done in triplicate, thus only standard deviation on this treatment.

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

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

The results of the analysis of the liquid fraction after HTT corroborate the general findings mentioned above. Ensiling of wheat straw combined with HTT gave high xylose concentrations which matched the lower recovery in solid fraction (Table 8 and Table 9). On the contrary, both HTT of grass and grass silage gave high xylose concentrations, but HTT of grass also gave a higher glucose concentration, which is believed to originate from the WSC (Table 9).

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 the highest in the liquid fraction of grass silage, even though grass silage contained less acetic acid. This suggests that the grass hemicellulose is more acetylated.
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 (Galbe and Zacchi, 2012). 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 (w/v)% and 0.65 (w/v)%, respectively, at a solid loading of 25%; and assuming that the liquid and the solid are not separated after pretreatment. Thus the acetic acid concentration could pose a potential problem.

Table 9: Composition of liquid fraction after HTT. The numbers are presented in weight percentages of DM raw biomass.

<table>
<thead>
<tr>
<th>Biomass</th>
<th>Temp. °C</th>
<th>Glucose</th>
<th>Xylose</th>
<th>Arabinose</th>
<th>Lactic acid</th>
<th>Acetic acid</th>
<th>HMF</th>
<th>Furural</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat straw</td>
<td>170</td>
<td>0.38</td>
<td>0.76</td>
<td>0.25</td>
<td>0.00</td>
<td>0.05</td>
<td>0.001</td>
<td>0.009</td>
</tr>
<tr>
<td></td>
<td>180</td>
<td>0.62</td>
<td>1.54</td>
<td>0.49</td>
<td>0.00</td>
<td>0.10</td>
<td>0.003</td>
<td>0.025</td>
</tr>
<tr>
<td></td>
<td>190</td>
<td>0.82</td>
<td>5.18</td>
<td>0.68</td>
<td>0.00</td>
<td>0.21</td>
<td>0.009</td>
<td>0.097</td>
</tr>
<tr>
<td>Wheat straw silage</td>
<td>170</td>
<td>0.37</td>
<td>3.55</td>
<td>0.50</td>
<td>0.27</td>
<td>0.40</td>
<td>0.006</td>
<td>0.086</td>
</tr>
<tr>
<td></td>
<td>180</td>
<td>0.60</td>
<td>6.55</td>
<td>0.71</td>
<td>0.35</td>
<td>0.58</td>
<td>0.014</td>
<td>0.228</td>
</tr>
<tr>
<td></td>
<td>190</td>
<td>0.69</td>
<td>7.51</td>
<td>0.80</td>
<td>0.39</td>
<td>0.68</td>
<td>0.023</td>
<td>0.396</td>
</tr>
<tr>
<td>Grass</td>
<td>170</td>
<td>2.40</td>
<td>0.94</td>
<td>0.51</td>
<td>0.02</td>
<td>0.31</td>
<td>0.108</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>180</td>
<td>3.45</td>
<td>2.76</td>
<td>1.01</td>
<td>0.13</td>
<td>0.59</td>
<td>0.233</td>
<td>0.011</td>
</tr>
<tr>
<td></td>
<td>190</td>
<td>4.49</td>
<td>7.67</td>
<td>1.69</td>
<td>0.10</td>
<td>1.19</td>
<td>0.407</td>
<td>0.055</td>
</tr>
<tr>
<td>Grass silage</td>
<td>170</td>
<td>0.59</td>
<td>1.89</td>
<td>0.82</td>
<td>3.74</td>
<td>0.94</td>
<td>0.052</td>
<td>0.016</td>
</tr>
<tr>
<td></td>
<td>180</td>
<td>1.02</td>
<td>5.22</td>
<td>1.23</td>
<td>5.07</td>
<td>1.39</td>
<td>0.083</td>
<td>0.036</td>
</tr>
<tr>
<td></td>
<td>190</td>
<td>0.97</td>
<td>6.15</td>
<td>0.97</td>
<td>4.65</td>
<td>1.52</td>
<td>0.134</td>
<td>0.079</td>
</tr>
</tbody>
</table>

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 the 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 the 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 (Palmqvist et al., 1999; Palmqvist and Hahn-Hägerdal, 2000; Klinke, Thomsen and Ahring, 2004). Inhibitors can, on the other hand, also function as a contaminant control in the ethanol fermentation, thus prevent large investments in sterile fermentation equipment (Larsen et al., 2012).

The large differences in the compositional analysis of solid and liquid fraction reveal that differences in biomass have a huge effect on the process of combining ensiling and HTT.

![Graph](image)

*Figure 20: 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. Standard deviations are not included for the pretreatments of grass since each treatment were done only once, due to scarce amounts of grass biomass from the demoplot harvest.*

Enzymatic saccharification was performed on all HTT solid fractions and is presented as both the direct yield of glucose and xylose per DM of pretreated solid fraction (Figure 21A and Figure 22A) and as glucan and xylan convertibility in percent converted polycarbohydrate per original content in raw material.

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 (Figure 21A). Glucan convertibility was thus improved through ensiling by a factor of 1.9 and 1.8 at 170 °C and 180 °C, respectively (Figure 21B). The effect
at 190 °C was, however, not significant for glucan, and the xylan conversion was heavily hampered by the hemicellulose degradation. The results from pretreatment of wheat straw are in line with the hypothesized reduction in HTT temperature facilitated by ensiling, since glucan and xylan convertibilities from the combination of ensiling and HTT at 170 °C, equal the convertibility from HTT of dry wheat straw at 190 °C.

The 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 removed considerable amounts of carbohydrate oligomers, which was concluded to be the main source of enzyme inhibition as according to (Kont et al., 2013). Washing improved the release of monosaccharides considerably, and the results shown in Figure 22 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 (Figure 21A and Figure 22A), only distinguished by a lower yield at the high temperature of 190 °C, and thus a less significant increase from HTT 180 °C to 190 °C for grass. The ensiling of grass did however, not have the same effective influence on the conversion, as the ensiling of wheat straw had. The glucan convertibility was 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 20).

The conversion of xylan was in general lower for grass compared to wheat straw. Concerning xylan convertibility, it should be noted that Figure 21B and Figure 22B is given in percent converted xylan per original xylan in raw material, thus including the solubilised xylan. The convertibility of the actual xylan left in solid fraction is therefore not reflected in Figure 21 and Figure 22. 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 for grass were found 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 corroborate previous results of poor xylan convertibility of grass and grass silage (Figure 15, Figure 16, Figure 17, and Figure 18C), and underpins 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 the 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 the results found in preliminary experiments by the authors of Paper II (data not shown). For grass, it was somewhat unexpected that ensiling had no effect, 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 (Figure 22A). 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 (Figure 14A). The absent effect of ensiling in this study (Paper III) supports the theory that particular grass cuts have a significant influence on the effect of ensiling, but it does not support the conclusions on relative maturity and low DM in Paper I.

The total release of monosaccharides and short chain oligosaccharides for the differently pretreated wheat straw and grass was 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 (Figure
The excess release of both C6 and C5 sugars was 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 (Figure 23). It can also be concluded that the gain released sugars at 170 °C and 180 °C, is considerably higher than the 7% xylose spent facilitating the ensiling of wheat straw, and moreover, most of the excess sugar release is 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.

Figure 22: 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. Standard deviations for grass are on the analysis and not on the pretreatment.

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 (Figure 24A), 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 very little difference between the total of released C6 sugars in grass and grass silage (Figure 24). The results on C6 clearly show that the 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 is, however, higher for silage grass both for HTT 170 °C and 180 °C, but not for HTT at 190 °C (Figure 24B). 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 is in total, more or less the same between grass and grass silage at the lower temperatures of 170 °C and 180 °C (Table 10). Here, the loss in C6 equals the gain in C5 which interestingly was opposite to the tendency in wheat straw. Nevertheless, the highest overall release of sugars was observed for grass pretreated at 190 °C, yielding almost 13 (w/w)% of biomass DM more than HTT of grass silage at 190 °C. Whereas for wheat straw, the highest release was observed for HTT at 180 °C of wheat straw silage.

![Figure 23: Overall release of monosaccharides and short chain oligosaccharides after differently pretreated wheat straw. A: C6 monosaccharides (glucose, galactose fructose) in enzyme hydrolysate (turquoise) 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.](image)

It is important to note that the sugars represented in HTT liquid fraction in Figure 24 include mono- and oligosaccharides from both the HTT liquid and the wash water generated in order to remove inhibitory oligosaccharides before enzymatic saccharification. This entails that the solid fraction is washed in order to get the presented amount of sugars into a separated liquid fraction (Figure 24). The wash water contributed with considerable amounts of C6 and C5. The contribution of C5 increased with the increase of HTT temperature and adding to the overall C5 in HTT liquid fraction for both grass and grass silage (Figure 25). The contribution of C6 increased with the decrease of HTT temperature and primarily added to the overall liquid fraction of pretreated grass (Figure 25).
Figure 24: Overall release of monosaccharides and short chain oligosaccharides after different pretreatments of grass. A: C6 monosaccharides (glucose, galactose fructose) in enzyme hydrolysate (turquoise) 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. Standard deviations are on the analysis and not on the pretreatment.

Even though these sugars were removed in order to get a better enzymatic saccharification, they are in this case thought of as potential fermentable sugars. It is, however, important to note that utilization of these sugars could entail great difficulties in terms of cellulose inhibition. Further studies of the inhibitory oligomers on the pretreated fibers are needed in order to know the implications of this finding.

Table 10: Excess sugar release due to ensiling, after HTT and enzymatic hydrolysis. Presented as weight percentages of raw biomass DM

<table>
<thead>
<tr>
<th>Biomass</th>
<th>Temp. °C</th>
<th>C6</th>
<th>C5</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat straw</td>
<td>170</td>
<td>13.7</td>
<td>5.9</td>
<td>19.6</td>
</tr>
<tr>
<td></td>
<td>180</td>
<td>13.0</td>
<td>2.2</td>
<td>15.1</td>
</tr>
<tr>
<td></td>
<td>190</td>
<td>2.8</td>
<td>-5.0</td>
<td>-2.2</td>
</tr>
<tr>
<td>Grass</td>
<td>170</td>
<td>-3.7</td>
<td>2.5</td>
<td>-1.1</td>
</tr>
<tr>
<td></td>
<td>180</td>
<td>-1.4</td>
<td>2.0</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>190</td>
<td>-6.9</td>
<td>-5.7</td>
<td>-12.6</td>
</tr>
</tbody>
</table>
The addition of mono- and oligosaccharides from the wash water bring a substantial amount to the calculated overall sugar release. Looking at the mass balance of C5 sugars of the HTT treated grass at 190 °C, it is clear that the released C5 of 24 (w/w)% (Figure 24B) exceeds the content of C5 in raw grass of 16 (w/w)% (unhydrated, from Table 7). Thus a recovery of C5 of 134%. The addition of carbohydrates from the wash water brings additional uncertainties as it involves several additional analysis and extra mass balance calculations, the added uncertainty could likely have resulted in an overestimation. Another source of the high recovery could be that the C5 content in the compositional analysis is slightly underestimated. It is most likely that the ethanol extraction have removed most of the arabinose associated with pectin in the grass, which then does not contribute to the arabinan.

![Graph showing C6 and C5 mono- and oligosaccharides in wash water from solid fraction of pretreated grass and grass silage. Presented as weight percent of raw biomass DM](image)

**Figure 25:** C6 and C5 mono- and oligosaccharides in wash water from solid fraction of pretreated grass and grass silage. Presented as weight percent of raw biomass DM

**Conclusion**

The comparison of the two separate studies, Paper II and Paper III, revealed great differences in the pretreatment of the wheat straw and the grass, and the outcome turned out to point in separate directions.

The results on pretreatment of wheat straw, Paper II, confirmed the hypothesis of higher hemicellulose solubilisation at 170 °C and 180 °C, and resulted in an improved cellulose convertibility of a factor 1.9, and 1.8, respectively. This gave rise to a possible decrease in HTT
temperature of up to 20 °C. Ensiling of wheat straw alone could however not be proven to have an effect on enzymatic saccharification.

Pretreatment of grass, Paper III, did not confirm a significant improvement of the hemicellulose solubilisation facilitated by ensiling, and the cellulose convertibility only improved by a factor of 1.3.

The enzymatic conversion of xylan was much more efficient for pretreated wheat straw silage than for pretreated grass silage. At all temperatures xylan convertibility of the solid fraction was a factor of two higher for wheat straw silage. This corroborate that the hemicellulose in Festulolium Hykor is highly recalcitrant, and significantly more than wheat straw hemicellulose.

There was a categorical difference in the way of ensiling between the two studies, 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 primary 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 in the two ensiled biomasses, 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, whereas for grass the WSC pool is not limited in the same way. This imply that the loss of valuable carbohydrates was significantly higher in ensiling of grass.

The overall release of monosaccharides showed clearly that the combination of ensiling and HTT was more successful for wheat straw than for grass. The pretreatment that gave the highest release of monosaccharides for wheat straw, was ensiling and HTT at 180 °C. For grass it was instead HTT of dry grass at 190 °C.

**Outlook**

As seen in these studies ensiling can benefit HTT in a combined pretreatment setup by increasing the severity of the HTT. This knowledge can be used in many applications with many biomasses. However, as studies clearly pointed out, ensiling also comes with a cost, and the effect of the
combined pretreatment can vary largely depending on the biomass. Experimental evaluation should therefore be conducted before the approach is applied to other biomasses.

Specifically, the positive results of the pretreatment combination on wheat straw encourage new studies of pretreatment optimisation. It would be interesting to study the effect of even lower HTT temperatures and also include holding time as a variable. Preliminary experiments on the effect of storage time of silage, were in fact also conducted (data not shown). These suggested similar improvements after 2 weeks storage, while a prolonged 8 weeks storage time reduced the monosaccharide release from enzymatic saccharification, presumably due to a loss of DM during storage. The exact implications of storage time should therefore also be investigated.

Further implications of the results are assessed in an overall outlook in section 18.

Enzymatic saccharification is good for the evaluation of structural pretreatment effect, however it does not encompass a full picture of the consequences for the fermentation. This require obviously experimental fermentations.

Due to the difficulties that was encountered in the conversion of grass, and the actual loss in overall released monosaccharides due to ensiling, it was decided to also test the effect of ensiling and combined pretreatment on anaerobic digestion.
Ethanol and biogas tests

Ethanol fermentation and anaerobic digestion were conducted as a preliminary study on the further processing of pretreated grass and grass silage into the two energy-carriers ethanol and methane (not published data).

Since ethanol fermentation were the primary target of the pretreatment in order to confirm the results in Paper III could fermentation tests were performed. On the contrary to ethanol fermentation, biogas production does not only utilize released monosaccharides, but can potentially utilize all other components in the biomass except lignin and minerals.

Anaerobic digestion is carried out by a consortium of different bacteria that performs a cascade reaction of hydrolysis, acidogenesis, acetogenesis and last methanogenesis, producing methane (Figure 26). As it can be seen on Figure 26 organic acids are intermediates in the anaerobic digestion. This means that biogas production is well suited for ensiling, since it easily can utilize the produced organic acids, and therefore the WSC loss is not a drawback. Biogas production of grass silage is therefore also a common commercial practice, especially in Germany where it is co-digested with maize silage (Prochnow et al., 2009)(Nizami, Korres and Murphy, 2009).

Even though it was not the scope of the thesis to investigate grass silage for biomethane production, it was thought to be relevant to test the pretreatment effect on anaerobic digestion, especially seen in the light of the slightly disappointing results on the sugar release.

![Diagram of decomposition pathways of anaerobic digestion from biomass to methane.](image)

*Figure 26: Scheme of decomposition pathways of anaerobic digestion from biomass to methane.*
9.1 Ethanol fermentation

Experiment

Ethanol fermentation was performed on dry grass, grass silage and the HTT pretreated solid fraction together with the respective liquid fraction. The setup was a simultaneous saccharification and fermentation (SSF) batch experiment, with a 100ml volume and 10% DM. The experiment included an initial prehydrolysis with 10% (w/w enzyme/cellulose) Cellic CTec2®:Cellic HTec2® (9:1) of 24 hours at 50 °C where after yeast preculture was added. The yeast was an industrial S. cerevisiae strain (Ethanol Red, Fermentis, France) that was exclusively C6 fermenting. The fermentation were monitored by weight loss, and calculated into ethanol production ($m_{\text{ethanol}} = 1.045 \cdot m_{\text{CO}_2}$). The actual concentration of ethanol were measured by HPLC after the end of fermentation (160 hr), as well as monosaccharides and organic acids. The weight loss were found to correlate very well (less than 10% difference) with the actual ethanol production.

Results and Discussion

The results of ethanol yield (g ethanol per 100 g pretreated DM) confirmed the findings on available C6 sugars in Paper III (Figure 24A), showing that ensiling reduced the ethanol yield for HTT at 170 °C and 190 °C, but did not cause significant difference at 180 °C (Figure 27). Furthermore it is clear that ensiling alone reduce ethanol yield due to the loss of WSC in the silage fermentation. Grass yielded 6.8 g ethanol/100 g DM and grass silage 4.0, corresponding to 45% and 24% of theoretical ethanol yield respectively. This was fairly similar to the ethanol fermentation results found by Oleskowicz-Popiel et al. (2011) for clover grass and clover grass silage of 49% and 36% respectively. The pretreatment, which gave the highest ethanol production were HTT of grass at 190 °C yielding around 14.6 g/100 g DM pretreated fiber, corresponding to 60% of theoretical ethanol yield in raw grass based on total C6 carbohydrates. The best yield of the combined pretreatment was also for HTT at 190 °C resulting in 11.7 g/100 g DM pretreated fiber, corresponding to only 46% of theoretical ethanol yield. This is however considerably lower than the theoretical yield on combined ensiling and HTT for clover grass in Oleskowicz-Popiel et al. (2011), that reached 76%. It could therefore be suspected that the fermentation study suffered from inhibition. This would also match the high amounts of inhibitory oligosaccharides on the pretreated fiber, and the high organic acid concentration in the liquid fraction of the pretreated grass silage. However fermentations of both grass and grass silage HTT at 190 °C were also done without the liquid fraction, and gave 12.2 and 11.9 g/100 g DM pretreated fiber, respectively. Which proves that the liquid fraction did not cause
inhibition, but in the case of pretreated grass the yield were reduced due to the lack of C6 carbohydrates present in the liquid fraction of HTT 190 °C of grass (Figure 24A).

![Ethanol fermentation yield](image)

*Figure 27: Ethanol fermentation yield of differently pretreated grass, in g ethanol per 100g pretreated DM. (A): Includes ethanol yields of grass and HTT treated grass at 170, 180, and 190°C (B): includes ethanol yields of grass silage and HTT treated grass silage at 170, 180, and 190 °C.*

**Conclusion**

Even though the fermentation was a preliminary test and did not thoroughly investigate inhibition or applied C5 fermentation. The results evidently show that ensiling mainly have not enhanced ethanol production from the grass, corroborating the results of Paper III. It would be interesting to see if fermentation of both C6 and C5 could level out the differences for HTT at 170 °C and 180 °C as the results in Paper III suggests (Figure 24).

**9.2 Biogas**

**Experiment**

Anaerobic digestion was performed on the same biomasses as for ethanol fermentation, and additionally on the grass and silage from the experiment of ensiling supplemented with cell wall degrading enzymes. The experiment was carried out in a batch set-up at mesophilic conditions (37 °C), a volume of 200 ml and a solid loading of 2.5% volatile solids (VS) equaling 2.7-2.8% DM. Methane production was measured by GC and related to the amount of volatile solids (VS), as it is
standard in determination of the biomethane potential (BMP). The experiment was kept running until production of methane ceased (58 days).

**Results and discussion**

The anaerobic digestion test showed that severe pretreatment using HTT of grass was unnecessary and in fact lowered the methane potential (Figure 28). Grass and grass silage gave exactly the same BMP of 310 ml CH₄ per g VS, thus ensiling of grass did not improve the total methane production. However, the BMP of grass suffered from high standard deviations. The BMP of *Festulolium* Hykor in this study is in the lower range compared to BMP of other temperate grasses and grass mixtures found in the literature. Pakarinen et al. (2008) reported a BMP of 480 and 410 ml CH₄ per g VS on pre-wilted *Lolium multiflorum* and a grass mixture containing *Festuca arundinacea*, respectively. Wall et al. (2013) studied *Lolium perenne* and reported a BMP of 400 ml CH₄ per g VS. BMP of a grass clover mix was reported to 375 ml CH₄ per g VS by Oleskowicz-Popiel et al. (2010). This could indicate that *Festulolium* Hykor might be more resistant to microbial degradation than other grass species. However, results of BMP can vary significantly between studies depending on test equipment and especially depending on the biological nature of the inoculum (Gerber et al., 2013). The indication of high recalcitrance of *Festulolium* Hykor is therefore far from conclusive, based on the anaerobic digestion, but adds to the general conception of the high recalcitrance of grass in this project as a whole.

The results showed a tendency that the HTT treated grass silage gave consistently higher potentials than the HTT treated grass (Figure 28). This is likely due to the high concentration of lactic acid in the liquid fractions from grass silage. Lactic acid is an easily converted substrate in biogas production in contrast to ethanol fermentation.

An interesting observation was that both grass and grass silage at HTT of 190 °C resulted in significant inhibition of the initial methane production causing a lag phase of 10-15 days. This was presumably due to the higher amounts of the microbial inhibitors HMF and furfural (Table 9), formed at high HTT temperature.
In the anaerobic digestion test of the grass and the grass silage from the experiment on ensiling with enzymes, it was found that the dry grass gave a lower methane potential than fresh grass, which was the same as for grass silage. Addition of Cellic HTec2® and mono component β-xylosidase (HtBx), before ensiling improved the methane yield giving 315 gCH₄ per g VS. The difference was however not statistically significant due to high standard deviations, but the tendency was nevertheless clear (Figure 29). The results of dry vs. fresh grass match with the general acceptance that drying induce additional recalcitrance (Luo and Zhu, 2011). An increased methane potential in the grass silage with added enzymes corroborate the results presented in Figure 18, where significant release of mono- and oligosaccharides was observed, and higher amounts of silage fermentation metabolites were produced, which both are substrates for anaerobic digestion, as opposed to ethanol fermentation.
Conclusion

The preliminary anaerobic digestion study showed no significant improvements of BMP due to ensiling. In fact the methane production curve for grass and grass silage were almost perfectly in line. It can therefore be concluded that the ensiling did not provide any pretreatment effect, which the bacteria in the anaerobic digestion could not provide themselves. No effect of ensiling has, however, been also reported by both (Pakarinen et al., 2008) and (Herrmann et al., 2011).

It was found that severe pretreatment with HTT of grass and grass silage only resulted in reduced BMP, and long lag phases due to inhibitory compounds in the HTT liquid fraction.

Last, the rather low BMP compared to several other studies, suggested that *Festulolium* Hykor is a resistant biomass for the bacteria to digest, thus corroborating the general finding that *Festulolium* Hykor has an unusually recalcitrant structure.
10 Conclusion

The comprehensive study of ensiling as a possible pretreatment method for lignocellulosic biomass conversion, which in this thesis was carried out on grass (Festulolium Hykor) and wheat straw, has produced a number of new findings. The study took several turns along the way, as unexpected results appeared more than once. The study is a good example of how knowledge generation also thrives when a hypothesis is confuted and when the results are more complicated than expected.

In the first study (Paper I), it was confirmed that ensiling can act as a method of pretreatment and improve enzymatic cellulose convertibility of grass. Furthermore, ensiling at low DM was found to improve the effects of pretreatment due to a higher production of organic acids in the silage, and possibly, also due to a better mobility of soluble compounds in the silage. Cellulose conversion was noted to be largely determined by the stage of maturity of the grass, where less mature grass had high convertibility but less amount of cellulose and vice versa. This led to the conclusion that an optimal maturity of grass can be found, which gives an optimal glucose release.

However, several shortcomings of the ensiling as a pretreatment methodology for lignocellulosic biomass also became apparent. First of all, ensiling came with a considerable loss of WSC, which was in fact higher than the improved glucose release. Secondly, the amount of released glucose was not adequate to sustain an efficient production of ethanol. And last, the conversion of xylan was extremely low in both grass and grass silage.

The latter observation was taken as a hint which led to the next study; an optimization of the enzymatic saccharification of grass by improving the content of hemicellulase in the enzyme blend. However, neither additional xylanases (Cellic HTec2® and β-xylosidase) nor hemicellulose degrading esterases (ACE and FAE) showed any improvements of xylan or glucan convertibility. It was therefore suggested that the hemicellulosic structure of Festulolium Hykor was unusually resistant.

In an attempt to improve the effect of pretreatment during silage storage, several biomass degrading enzymes were added to the grass before ensiling. An addition of cutinase and β-xylosidase did not have any effect of pretreatment, and adding hemicellulases (Cellic HTec2®) even had a direct negative effect on cellulose convertibility. The results suggested that the negative effect of Cellic HTec2® was due to significant release of glucose that was utilized during ensiling, and therefore, it caused a considerable reduction in available glucan for the subsequent cellulase treatment. In addition to this, xylan conversion remained unchanged in all treatments, which further emphasized the unusually resistant hemicellulose of Festulolium Hykor.
In Paper II, it was found that ensiling of wheat straw in combination with HTT increased the severity of HTT and facilitated a reduction in HTT temperature of up to 20 °C. This could, however, not be proven for grass in Paper III, since the overall release of mono- and oligosaccharides for the combined pretreatment of grass was generally lower than for HTT of dry grass. In comparison, the ensiling of wheat straw improved cellulose convertibility by a maximum factor of 1.9 at HTT of 170 °C, where the ensiling of grass only improved cellulose convertibility by a factor of 1.3. Additionally, the ensiling of grass induced a significantly higher loss of WSC than the ensiling of wheat straw. Furthermore, pretreated grass and grass silage gave rise to considerable inhibition of cellulose activity, and washing of the pretreated fiber fraction proved to be necessary to achieve acceptable enzyme catalyzed cellulose convertibility.

The HTT pretreatment of both grass and grass silage gave considerably lower xylan convertibility than HTT of wheat straw and wheat straw silage. Thus, the hemicellulose in *Festulolium* Hykor was again proven to be highly recalcitrant. The reason for the inaccessible xylan is believed to be found in a high complexity of branching and cross linkages creating a highly heterogeneous and resistant grass hemicellulose. However, further studies are required to confirm this hypothesis.

Preliminary ethanol fermentation experiments confirmed the results on total release of C6 sugars in Paper III, and showed that ensiling of temperate grass did not benefit bioethanol production. Anaerobic digestion on the same pretreated grass showed no pretreatment effect of ensiling upon BMP, and it was evident, that severe HTT pretreatment did not benefit methane production from either grass or grass silage.

Overall, the study encountered great challenges in the conversion of the many different cuts of *Festulolium* Hykor. Due to the difficulties in the conversion of the already relatively small amount of glucan and the significant loss of WSC during ensiling, it is not recommended that ensiling of *Festulolium* Hykor is used for bioethanol fermentation.

In short and useful terms, it can be concluded from the overall study that:

- Ensiling may provide a pretreatment effect in itself, depending on the silage conditions and the recalcitrance of the biomass in question. However, ensiling comes always at a cost of a WSC loss, 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.
• It is unlikely that ensiling alone can act as a sole method of pretreatment for a sugar platform biorefinery. This was found since the low improvements in this study corroborated the general findings of low levels of cellulose convertibility by ensiling alone (Chen et al., 2007; Pakarinen et al., 2011; Oleskowicz-Popiel et al., 2011). Ensiling is therefore, to a greater extent, a good method for biomass storage with possible benefits to biomass conversion.

• Ensiling can, on the other hand, add significant improvements to a combined pretreatment of ensiling and HTT. For wheat straw, this combination proved successful and facilitated a possible decrease in HTT temperature, whereas the same combination on grass did not. Thus, the improvements depends largely on the loss of WSC and the biomass in question.

These three learnings imply that ensiling is highly case specific; in some cases, it will be favorable, while in others, unfavorable. It is therefore of great importance that, in any case, the implications of ensiling is thoroughly analyzed and tested before implementation.

In this regard, it should be stressed that ensiling is not a mere pretreatment method, but an integrated storage and pretreatment method with effects on both agricultural management, biomass feedstock logistics, and biomass conversion. This thesis aimed to study only the last issue of biomass conversion.

For this reason, it is recommended that implementation of ensiling is viewed in the largest possible perspective.
11 Future 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 and/or using carbohydrate microarrays (Moller et al., 2007; Fangel et al., 2012).

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 (Kromu et al., 2006). In this regard, it would be interesting to look closer at the protein content of Festulolium Hykor and separation of those, as well as lactic acid production from the ensiling. Extensive research are conducted on the concept of green biorefinery, and several pilot/demonstration scale facilities exist (Mandl, 2010; Leiß, Venus and Kamm, 2010; Kamm et al., 2010; O'Keeffe et al., 2011)

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 temperature. 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. Selection of the right solution of implementation is case specific and requires simulation of the different effects in a logistic model, as it has been done by (Shastri et al., 2011).

Ensiling could also be combined with other kinds of pretreatments. In this regard the combination of ensiling and the biological white rot fungi treatment is indeed worth mentioning. While the
pretreatment effect of ensiling relies on biological- and acidic hydrolysis of polysaccharides, the white rot fungi treatments rely on extracellular fungal laccases which degrade lignin. The combination of these two methods could therefore be very interesting. Two studies following this strategy have been published (Chen et al., 2012; Liu et al., 2013b), and both conclude possible benefits of the combined pretreatment, however, for (Liu et al., 2013b) the results were hampered by high DM loss in the fungal treatment, and for (Chen et al., 2012), the reaction conditions of laccase treatment were not optimal. Thus, further studies are needed to confirm if such a combined biological pretreatment would be functional and beneficial. Preliminary results on combined silage and white rot fungi treatment of wheat straw made by the authors of Paper II have, however, shown promising results (Data not shown). The potential benefit of such a combined fully biological pretreatment method could be to reduce the energy use in pretreatment even further.
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Chapter three

Papers
Paper I
Ensiling as biological pretreatment of grass (Festulolium Hykor): The effect of composition, dry matter, and inocula on cellulose convertibility

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ABSTRACT

Grass biomass is a prospective type of lignocellulosic biomass for bioenergy and fuel production, but the low dry matter in grass at harvest calls for new pretreatment strategies for cellulose conversion. In this study, ensiling was tested as a biological pretreatment method of the high yielding grass variety Festulolium Hykor. The biomass was harvested in four cuts over a growing season. Three important factors of ensiling: biomass composition, dry matter (DM) at ensiling, and inoculation of lactic acid bacteria, were assessed in relation to subsequent enzymatic cellulose hydrolysis. The organic acid profile after ensiling was dependant on the composition of the grass and the DM, rather than on the inocula. High levels of organic acids, notably lactic acid, produced during ensiling improved enzymatic cellulose convertibility in the grass biomass. Ensiling of less mature grass gave higher convertibility. Low DM at ensiling (<25%) resulted in the highest cellulose convertibilities, which ranged from 32 to 70% of the available cellulose in the four cuts after ensiling. The study confirms that ensiling can enhance cellulose convertibility of green biomass, and provides new insight to ensiling as a biological pretreatment method for green biomass conversion.

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1. Introduction

Grassland biomass may become an important low cost lignocellulosic raw material for fuels and chemicals in the future, as grassland covers about 69% of the world’s agricultural area [1,2]. Additionally, grassland biomass may add significant ecological value, including protection against soil erosion and habitat creation [2]. Cultivation of temperate grass allows for several harvests (2–4 cuts) during a season contributing to the high yield. It is well known that the
chemical composition of grass changes between cuts over the season and with the stage of maturity at harvest [3,4]. This aspect has not been thoroughly examined in relation to processing of grass biomass for biorefining, but is important to take into account when assessing grass biomass as a feedstock for biofuels or biochemicals, since changes in composition may affect the processing and product yields to a high extent.

Another important aspect of a low cost lignocellulosic biomass supply is efficient storage and pre-processing extent. The fact that grass is harvested at low dry matter (DM) typically of 18–20% DM makes dry storage at >90% DM troublesome. Instead, through ensiling, grass can be stored at lower DM (20–50%). Ensiling is the classical method of forage crop preservation optimised throughout the past two centuries to provide nutrient rich animal feed all year round [6]. 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 [7]. 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 [7].

Silage, the resulting biomass product of ensiling, has gained increased focus as a biomass feedstock for biofuel production in recent years [8]. The method poses several potential advantages as opposed to dry storage. The main advantages include (i) less dependence on dry weather conditions prior to harvest, hence, better harvest-timing, (ii) reduced biomass losses during harvest due to less handling steps and no loss from dust formation, (iii) no need for energy intensive drying, and (iv) possibilities of combined storage and pretreatment [9,10]. Combination of storage and pretreatment at ambient temperature and pressure holds considerable potential cost and energy savings compared to common and more severe pretreatments of chemical or physiochemical means [9].

Already 50 years ago Dewar et al. (1963) [11] showed that during ensiling, hemicellulose from perennial rye was hydrolysed initially by enzymes extracted from the grass and during longer storage (7–28 days) by means of acid hydrolysis at pH 4. These changes in biomass composition suggest that ensiling may be utilised as a biological pretreatment method for cellulose biofuel and biochemicals production.

Four studies on ensiling as a biological pretreatment have reported results of cellulose conversion through enzymatic hydrolysis, all with the aim of producing energy carriers of either ethanol or biogas and the studies have consistently been reporting improved enzymatic saccharification for the ensiled biomass [9,12–14].

It is an obvious tenet that the grass biomass composition, DM, and type of inoculum will influence the ensiling process as well as the silage quality, which in turn may affect the subsequent enzymatic cellulose convertibility. Nevertheless, in the currently available studies, the biomass and the conditions of ensiling have varied considerably, making it difficult to derive consistent rules for optimal ensiling for lignocellulose pretreatment. The objective of this study was to investigate the relations of three factors; biomass composition, initial DM, and addition of LAB inocula, upon enzymatic saccharification of cellulose after ensiling, using Festulolium Hykor as the grassland biomass. Festulolium Hykor is a cross-breed of the temperate grasses tall fescue (Festuca arundinacea) and perennial rye (Lolium perenne) developed by DLF TRIFOILUM for high yield potential (18 tonne/ha) and high persistency throughout the season. However, the possible influence of the differences in the grass biomass composition of Festulolium Hykor across different harvests during a season, i.e. different cuts, on ensiling and silage quality has not been investigated.

2. Materials and methods

2.1. Raw material

The four cuts of the grass biomass, Festulolium Hykor (DLF TRIFOILUM, Denmark), were harvested over the season 2011 (1st cut: 01.06.2011, 2nd cut: 06.07.2011, 3rd cut: 20.09.2011, 4th cut: 01.11.2011) from a DLF TRIFOILUM demo plot, sized 1.5 × 8 m and located in southern Zealand, Denmark (55° 20’ N, 12° 23’ E), with a HALDRUP F-55 harvester (Inotec Engineering GmbH). The grass was collected right after harvest, cut to 2–5 cm pieces and split into four portions. Three of the portions were dried to different DM concentrations by means of different drying times at 25–30 °C (drying time ranged from 2 to 48 h). DM content was monitored by use of a halogen DM analyser (Mettler Toledo HR83 Halogen) and exact measurements where done according to the standard procedure developed by the National Renewable Energy Laboratory (NREL) in the US [15]. The last portion of each cut was dried at 60 °C and stored as hay for raw material comparison in compositional analysis and enzyme hydrolysis (see below).

2.2. LAB inocula

The commercially available inocula LACTISIL Grass plus (GP) and LACTISIL CCM (CCM) (Chr. Hansen, Hørsholm, Denmark) were in freeze dried form, prepared individually in a 0.05 g DM/L water suspension, and added to the grass samples for ensiling at a level equaling 4.0 mg DM inocula/kg fresh grass as according to [13].

GP consists of the lactic acid bacteria (LAB) Pediococcus pentosaceus and Lactobacillus plantarum, which are both homofermentative. CCM consists of pure Lactobacillus buchneri which is heterofermentative. Each grass sample was mixed carefully and thoroughly with each inoculum solution in a large plastic tray and samples were taken for final DM measurements prior to each ensiling.

2.3. Ensiling

The ensiling was carried out using a vacuum based plastic bag system according to [16]. A Variovac EK10 vacuum packaging machine (Variovac Nordic A/S, DK-7100 Vejle, Denmark) and 35 × 45 cm vacuum bags were used to pack approx. 100 g DM grass for each treatment.
2.4. Experimental design

A duplicated 3\(^\text{rd}\) experimental design was used to test the effect of different silage LAB inocula and different DM concentrations, and this was carried out on the four cuts of Festuclum Hykor harvested on 01.06.11, 06.07.11, 20.09.11, and 01.11.11. Three portions of grass at different DM were treated with two types of commercial inocula against treatment without inocula (only water added). All treatments were done in duplicates. A total of 72 bags were prepared and stored at room temperature. DM contents were 21, 31 and 41\% for the 1st cut, 23, 35 and 50\% for the 2nd cut, 24, 28 and 43\% for the 3rd cut and 22, 34 and 49\% for the 4th cut. Storage times were 46, 48, 49 and 49 days for all samples from the respective cuts.

2.5. Chemical analysis

2.5.1. Quantitative analyses of monosaccharides and organic acids

After a two-step H\(_2\)SO\(_4\) hydrolysis of the biomass according to Ref. [17] concentrations of carbohydrates (\(\alpha\)-glucose, \(\alpha\)-xylose, \(\alpha\)-arabinose, \(\alpha\)-fructose, \(\alpha\)-mannose, \(\alpha\)-galactose) were quantified by High Pressure Liquid Chromatography (HPLC) (Shimadzu Corp., Kyoto, Japan) using an HPX-87P column (BioRad) (Hercules, CA; USA) and refractive index (RI) detection, at 80 °C using water as eluent, 0.5 ml/min. Organic acids (lactic-, formic-, acetic-, propionic, and butyric acid) were quantified by HPLC using a Biorad HPX-87H column (Hercules, CA; USA), RI detection, 63 °C and 4 mM H\(_2\)SO\(_4\) as eluent, 0.6 ml/min. Cellulose content were calculated as 90\% of \(\alpha\)-glucose content and hemicellulose content as 88\% of \(\alpha\)-xylose plus 88\% of \(\alpha\)-arabinose plus 90\% of \(\alpha\)-galactose.

2.5.2. DM/ash

The DM and ash analyses were done according to NREL standard laboratory analytical procedures based on oven dry matter measurements [15]. Since silage biomass contains large amounts of volatile compounds, it is critical to correct the measured oven-DM (at 105 °C) for loss of volatiles, to obtain the true DM. The measurements were therefore corrected using coefficients according to Ref. [18].

2.5.3. Water extraction

Aliquots of 0.3–0.4 g DM biomass from freshly disrupted silage bags were extracted in 10 mL MilliQ H\(_2\)O containing cananycin (0.1 mg/mL) to prevent microbial activity during extraction. The extraction samples were shaken for 2 h at 25 °C and 150 rpm. Extracts were analysed for sugars and acids by HPLC as described above. The biomass fibres were freeze dried and weighed to determine the amount of extractives. The levels of water and ethanol extractives were used as a measure of relative maturity in-between cuts [19].

2.5.4. Weak acid hydrolysis of water extract

One step acid hydrolysis was performed on the extract to quantify the content of soluble oligomer carbohydrates. Extracts were autoclaved for 10 min at 121 °C with 4 w/w\% H\(_2\)SO\(_4\). Derived monosaccharides were analysed by HPLC as described above.

2.5.5. Ethanol extraction

Lipophilic extraction was done by Soxhlet extraction in a reflux condenser for 6 h with 99\% ethanol. The amount of ethanol extractives, including volatiles, was defined as the mass of material lost through extraction.

2.5.6. Lignin

Lignin content of the extracted bio residue was assessed a two-step H\(_2\)SO\(_4\) hydrolysis according to Ref. [17].

2.5.7. Total N-determination

The biomass samples were prepared for protein determination by wet milling in a Mannesmann wet mill (Remscheid, Germany) of a 1 g DM/1 H\(_2\)O solution to a particle size of 50 μm. Total nitrogen was measured using a kit from Hach Lange GmbH (Germany); Total Nitrogen LCK 138 (detection range: 1–16 mg N L\(^{-1}\)). The protein content was calculated by multiplying the nitrogen content with 5.6 according to Ref. [20].

2.6. Enzymatic hydrolysis

The enzymatic hydrolysis was done at 1.6% DM (w/v) in a total volume of 25 ml using 50 mM citrate buffer at pH 5.0 with 0.4\% w/w sodium azide. Commercially available cellulolytic and hemicellulolytic enzyme preparations, Cellic\(^*\)CTec2 and HTeC2, from Novozymes A/S ( Bagsværd, Denmark) were used in a 9:1 ratio and added at 10% enzyme/substrate (w/w cellulose). Cellic\(^*\)CTec2 is a commercial cellulase preparation based on the cellulase complex produced by Trichoderma reesei containing at least the two main cellulohydrolases EC 3.2.1.91 (Cel6A and Cel7A), five different endo-1,4-β-glucanases EC 3.2.1.4 (Cel7B, Cel5A, Cel12A, Cel61A, and Cel45A), β-glucosidase EC 3.2.1.21, and a β-xylanase (EC 3.2.1.37) in addition to particular proprietary hydrolysis-boosting proteins. Cellic\(^*\)HTec2 mainly contains endo-1,4-β-xylanase activity (EC 3.2.1.8), but also contains cellulase activity. Treatments were done during shaking for 72 h at 50 °C. The enzymatic hydrolysis was done in triplicate and enzyme blanks were also analysed. Hydrolysates were analysed for glucose levels on HPLC and the glucose yield (GY) is presented per DM original grass biomass. Both ensiled and raw grass was extracted in H\(_2\)O prior to the enzymatic hydrolysis to avoid interference from free sugars on the results for cellulose convertibility. Cellulose convertibility (CC) was calculated as the converted cellulose (derived from GY) divided by the original cellulose content (Equation (1)). A relative improvement ratio of the cellulose convertibility as compared to that for dry grass was also calculated to express the ensiling pretreatment effect (Equation (2)).

Cellulose convertibility (CC) = (GY-0.90)/Cellulose content

Relative improvement ratio = 1 + (CCsilage - CCdry grass)/CCdry grass

2.7. Statistical evaluations

One-way analyses of variances (one-way ANOVA); 95% confidence intervals were compared as Tukey–Kramer intervals.
calculated from pooled standard deviations (Minitab Statistical Software, Addison–Wesley, Reading, MA). Statistical significance of linear correlations was tested by the dose–response F-test at 95% confidence level [21].

3. Results and discussion

3.1. Characterisation of grass

3.1.1. Composition and grass maturity

The constituents of the chemical composition were grouped, according to the one-way ANOVA, in order to differentiate between the four cuts of grass (Table 1). The grouping revealed that 1st and 3rd cut had comparable compositions with all constituents except ash falling in the same group, while 2nd and 4th cut differentiated by having lower contents of cellulose and lignin and higher contents of extractives.

As grass matures the proportion of secondary cell walls increases and the fraction of non-structural cell contents decreases [22]. The total amounts of extractives are therefore a measure of relative maturity [19]. Thus, the four cuts represented different stages of maturity at harvest. 1st and 3rd cut had a similar and more advanced maturity than the 2nd cut, and the 4th cut having highest total extractives and lowest content of lignocellulosics, was the least mature at harvest. The higher content of extractives for the less mature 2nd and 4th cut, could be distinguished by a high concentration of water soluble carbohydrates (WSC) in 2nd cut (10 w/w% DM), and a high concentration of crude protein for 4th cut (21 w/w% DM) (Table 1). WSC does generally not directly correlate with maturity, but low production of WSC is often seen in late season growths [4]. Crude protein on the other hand, has been shown to decrease with advancing maturity for spring and summer growths, while late autumn growths usually have high, constant levels [4,23]. The compositional differences found between the four cuts of the grass were coherent with observations done at harvest. It was thus noted that 1st and 3rd cut both had met flowering stage whereas 2nd and 4th cut had not, and that the 4th cut consisted primarily of leaves.

The four cuts of Festulolium Hykor in this study represent an example of the seasonal change that a biomass producer or biorefinery operator can expect when working with temperate grass. Seasonal change, representing the repeating annual variations in solar radiation, temperature, wind and rain, lead to natural differences between the different cuts. The growth season in 2011 (in Denmark) suffered from unusually high amounts of rain in the summer months (Table 2). The unusual rainfall influenced the timing of harvest, resulting in a slightly late 1st cut and a much delayed 3rd cut. In turn this resulted in a relatively high maturity of the 1st and 3rd cut as compared to the standard cutting strategy for the demo-plots at DLF Trifolium, which is optimised for feed quality of the grass biomass.

The maturity is, as reflected in the results (Table 1), a key parameter for the chemical composition. The exact harvest date is therefore important in grass managing systems. The increased proportion of secondary cell walls in mature grass increases recalcitrance and decreases cellulose convertibility. This effect is primarily due to increased lignin content and cross-linkages between lignin and structural carbohydrates [24,25].

Keating and O’Kiely [4] studied the effect of maturity on different cuts of re-grown perennial rye grass and found that increased maturity decreased the DM digestibility in vivo animal feed experiments, which indicated increased recalcitrance to microbial degradation. The compositions were in general comparable to previous published data of temperate grass like Festulolium Hykor [19]. The compositional relations between cuts of different maturity also match the general knowledge of grass growth and maturation [19].

### Table 2 – Average weather conditions for Denmark 2011, when grass was harvested: spring (March, April, May), summer (June, July, August), fall (September, October). In parentheses: the average norm (1961–1990).

<table>
<thead>
<tr>
<th>April</th>
<th>May</th>
<th>June</th>
<th>July</th>
<th>August</th>
<th>September</th>
<th>October</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (°C)</td>
<td>9.9 (5.7)</td>
<td>11.4 (10.8)</td>
<td>15.1 (14.3)</td>
<td>16.4 (15.6)</td>
<td>16.1 (15.7)</td>
<td>14.1 (12.7)</td>
</tr>
<tr>
<td>Rainfall (mm)</td>
<td>16 (41)</td>
<td>54 (49)</td>
<td>75 (55)</td>
<td>113 (66)</td>
<td>132 (67)</td>
<td>92 (73)</td>
</tr>
<tr>
<td>Sun (h)</td>
<td>253 (162)</td>
<td>239 (209)</td>
<td>252 (209)</td>
<td>171 (196)</td>
<td>150 (186)</td>
<td>135 (128)</td>
</tr>
</tbody>
</table>
3.2. Acid production during ensiling

Each cut of grass responded differently to the experimental ensiling factors, giving four different patterns of organic acid production (Fig. 1). The organic acid concentration was highest for low DM treatments of the less mature grass samples (2nd and 4th cut), reaching around 10 (w/w)%, but in both cases the organic acid production decreased considerably with higher DM. Ensiling of the more mature grass (1st and 3rd cut) resulted in less total organic acid production at low DM and the concentration decreased less significantly with DM.

3.2.1. Water soluble carbohydrates

The water soluble carbohydrates (WSC), which in temperate grass consist of fructose, glucose, sucrose and fructans [26], constitute the metabolic substrate pool for the ensiling. Sufficient WSC is therefore a prerequisite for successful silage fermentation. The concentration of WSC in the four cuts did not correlate directly with the production of organic acids. The significantly higher WSC level found in the 2nd cut gave the same levels of total organic acids as the 4th cut, which contained less than half the amount of WSC (Fig. 1). In the main fermentation phase of ensiling the bacteria rapidly consume readily available simple sugars as well as fructose from hydrolysed fructan which in temperate grasses is degraded by the plant enzyme fructan exohydrolase [27]. If the fermentation has not yet reached the stable anaerobic storage phase after the WSC are metabolised then other substances will act as substrate. Thus hydrolysed structural carbohydrates, primarily from hemicellulose, or amino acids from denatured proteins, will be the new source [11,26]. In the current study, the acid production varied differently in response to the amount of WSC available in the fresh grass. Some, namely the silage samples of 3rd and 4th cut, had a higher acid production than what could maximally be produced from the initial WSC content. For the 1st cut a higher acid production only occurred for the three low DM silage samples, and for the 2nd cut the amount of acids produced only exceeded the WSC content in fresh grass of the CCM inoculated, low DM silage sample. These samples have therefore, utilised other substrates like degraded hemicelluloses. The finding that the response to WSC levels was not consistent across the different cuts, underscored that the total composition of the grass rather than the content of WSC determined the outcome of the ensiling.

3.2.2. pH response

In general, the pH dropped according to organic acid production, in particular according to the concentration of lactic acid, which is also in accordance with its lower pKa of 3.1. Low DM silage, especially for 2nd and 4th cut, reached the lowest pH during ensiling of around 4, whereas pH for the high DM silages reached levels around pH 5.5 for 2nd and 4th cut samples (Fig. 1). The latter results were in accordance with the lower production of organic acids. However, both lactic and acetic acid were in fact produced during the ensiling of the 4th cut at high DM, and moreover in comparable amounts to silage samples from the 1st cut which resulted in significantly lower pH values (around 4.6). Consequently grass from the 4th cut had a higher buffering capacity than the other cuts. The

![Fig. 1](image-url) – Organic acids and pH after ensiling; analysed 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.
reason for this is most likely the high content of crude protein in the 4th cut (Table 1) that is known to facilitate buffer capacity towards silage fermentation [23].

3.2.3. Inocula

The type of inoculum caused significant differences in acid production in only 6 out of 12 cases within same cut and same DM (1st cut low DM; 2nd cut low DM; 2nd cut medium DM; and all three DM’s of 3rd cut) (Fig. 1). For the 3rd cut the GP inoculum clearly improved the ensiling as measured by lactic acid production across the different DM concentrations. However, any such effect was not consistent across the four cuts, thus addition of an inoculum did not affect the silage fermentation under the experimental conditions used in this study. Lactic acid bacteria can be divided into two groups according to their carbohydrate metabolism, the homo- and the hetero-fermentative, the latter producing lactic and acetic acid, ethanol and carbon dioxide, the former producing only lactic acid [7]. Homofermentative ensiling is more efficient due to a more rapid pH drop and therefore faster preservation, while heterofermentative ensiling provides better resilience against spoilage of the silage [3]. It was thus somewhat surprising that the inocula did not affect the acid production of the ensiling to a larger extent despite the categorical differences in the types of microorganisms within the inocula. An explanation for the inconsistent effect of inoculum is obviously that the natural epiphytic organisms on the grass dominated the fermentation processes to a large extent. Accordingly, the inoculated amount may have been too low to dominate the silage fermentations.

3.2.4. DM

DM at ensiling has been shown in several studies to be a main control factor affecting the microbial activity in the fermentation, and therefore affecting silage quality [28,29]. The results in Fig. 1 indicate a negative correlation between DM at ensiling and the production of organic acids during storage. Lactic acid concentration was found to decrease linearly with increasing DM ($p < 0.05$) (Fig. 2A). The acetic acid and propionic acid concentrations were also decreasing with increasing DM at ensiling, however much less than what was seen for lactic acid, and the linear correlations were not statistically significant ($p > 0.05$). Relatively large differences were found in the correlation between total organic acids and DM for the four cuts in-between (Fig. 2B) suggesting that chemical composition and thereby maturity and seasonal change had a huge impact on the results. The production of organic acid did, however decrease at higher DM for each cut (Fig. 2B) but linear correlation did only prove significant ($p < 0.05$) for the 4th cut. The slope of the decrease was higher for the more immature 2nd and 4th cut compared to 1st and 3rd, however more data points are needed within each cut to describe the correlations further.

3.3. Enzymatic hydrolysis

3.3.1. Dried grass

Results of the glucose yield (GY), representing the amount of glucose released per biomass DM, and the cellulose convertibility (CC), representing the converted cellulose yield as percentage of the total cellulose, were calculated for dry grass from each of the four cuts (Fig. 3A and B). The grass from 2nd cut gave the significantly (0.05) highest GY of 9.0 w/w% DM followed by 1st and 4th cut, which yielded 7.8 and 7.5 w/w% DM, and last 3rd cut with 5.5 w/w% DM (Fig. 3A). Thus a combination of less mature grass and relatively high cellulose content gave highest GY. Nevertheless, the CC results of the dried grass (Fig. 3B) show how the low cellulose content of the 4th cut grass led to a higher CC for 4th cut than 2nd cut. Comparing the enzymatic hydrolysis of the four dried grasses showed that the mature grass of 1st and 3rd cut was more recaltrant in terms of cellulose hydrolysis than the less mature 2nd and 4th cut.
Both seasonal change and maturity contributed to the different chemical compositions of the four cuts, and even though the experimental setup did not include a detailed study of maturity, the fact that Festulolium Hykor was cut at different stages of maturity contributed considerably to the result of the study as a whole.

As seen in the current study cellulose content increased with advanced grass maturity, but likewise did lignin, consequently increasing lignocellulosic recalcitrance. The trade of high cellulose content is therefore related with decreasing cellulose convertibility in the enzymatic hydrolysis, thus resulting in less released sugars overall. This counter-relation suggests that there is an optimum stage of maturity for grass, where cellulose content and convertibility results in an optimal sugar release.

The findings related to maturity and enzymatic hydrolysis are in line with a study by Ding et al. [30] concerning the nanoscale architecture of plant cell walls and its direct influence on enzymatic degradation. In this study it was concluded that poor degradation of lignified cell walls was due to blocking of the enzymatic binding to the hydrophobic planar face of the cellulose microfibrils. Harvesting grass at an earlier growth stage, before a high degree of lignin deposition occurs during elongation, may therefore increase accessibility and productive binding of the cellulosic enzymes and increase degradation. Thus, maturity should definitely be a key factor in the optimization of the cellulosic biomass.

![Fig. 3](image)

**Fig. 3** — Enzymatic hydrolysis of dry and silage grass. A: Glucose yield (GY) w/w% of DM; B: Cellulose convertibility (CC), %; C: Relative improvement ratio: cellulose convertibility of silage compared to that for dried grass.
3.3.2. Effect of ensiling on enzymatic hydrolysis

The pretreatment effect of ensiling was measured by enzymatic hydrolysis and the GY and CC were compared to that of the dried grass (Fig. 3A and B). The GY results clearly show that ensiling had a positive effect on the sugar release by generally yielding higher amounts of sugar per g biomass DM. For all cuts the low DM silage treatments gave higher GYs than the high DM silage treatments. However, as discussed above the compositional differences of the grasses caused deviations between the four cuts. The major differences between the cut were that ensiling of 4th cut at medium and high DM also resulted in relatively high GYs as opposed to the other cuts, and 3rd cut silage generally resulted in lower GY, also at low DM. On the other hand, both the 1st, 2nd, and 4th cut resulted in similar maximum GYs, around 11 w/w% DM regardless of the GY of the appertaining dry grass. LAB inocula did not have a consistent and significant effect on GY, as it was also the case for the silage fermentation.

When translated into CC, the picture changed due to the different cellulose content in the four cuts (Fig. 3B). 4th cut had the highest CC of 69% followed by 2nd, 1st and last 4th cut of 50%, 40%, and 32%, respectively, all at low DM and averaged over inoculum. In coherence with CCs of the dried grass the less mature cuts had higher CCs.

The CCs of the silage samples were compared to the CCs of the dried grass and a relative improvement ratio was calculated (Fig. 3C). Here it is even clearer that low DM gave better results than high DM across the four cuts. The highest improvements were found for 1st and 3rd cut of 1.40 and 1.42, respectively and averaged over inocula. 2nd and 4th also improved the CC but not by as much, 1.23 and 1.35 respectively (Fig. 3C). The only silage treatments which did not improve the enzymatic hydrolysis were at medium and high DM ensiling of 2nd cut.

The enzymatic hydrolysis results matched the levels obtained in previous studies including CC of ensiled green biomasses such as clover-grass and reed canary grass [12,13]. Ensiling of these biomasses was found to facilitate a CC of 42% and 30% respectively, which in the case of clover-grass were an improvement ratio of 1.47. This level matches the maximum relative improvements obtained for the low DM ensilage of 1st and 3rd cut in this study.

The results from the enzymatic hydrolysis correlated with the organic acid production in the silage treatments, and the data consistently indicated that high concentration of acids in the silage increased the pretreatment effect (Fig. 4A, B and C). The trend could however not reach statistical significance of a linear correlation, most likely because the variation in the level of hydrolysis between the four cuts diminished the statistical significance of the linear correlations.

The results of higher concentrations of hydrolysing organic acids produced at lower DM (Figs. 2 and 4) corroborate that the pretreatment effect of ensiling improves at lower DM. This also confirm the findings of previous research [9,11–14] that the organic acids produced during ensiling promotes a gentle hydrolysis of lignocellulosic structures, which in turn appear to increase the access of the cellulosic enzymes to the cellulose. Furthermore the present study demonstrates that maximising organic acid production in the silage, by ensiling at low DM, leads to a better pretreatment effect.

The level of GY’s and CC’s found for ensiling in this study, did however not match the performance of more severe pretreatment methods. Preliminary studies, by the authors, using hydrothermal pretreatment at 190 °C, 10 min as according to
[31], in itself gave close to total convertibility of cellulose (data not shown). The conditions of ensiling are apparently not severe enough to reach such high conversion. Opportunities for further optimisation could include addition of structure specific enzymes to the ensiling and/or development of better enzymatic blends adapted to silage grass. Further, more detailed studies of the fate of the cell wall materials during ensiling are required. Ensiling could also be combined with other more severe pretreatments and used as a pre-pretreatment. Ensiling is a promising method but can at this point not stand alone.

4. Conclusions

The abundant production, high annual yields, and low environmental impact of grasses like Festulolium Hykör, and the benefits of low DM storage simultaneously with a pretreatment effect, make ensiling of grass a promising technology for a future biobased production of fuels and chemicals from green biomass. The results from this study confirm and expand the knowledge on the subject of using ensiling as a biological pretreatment method.

- Ensiling improved cellulose convertibility compared to dry storage, through acid hydrolysis of the lignocellulosic matrix.
- Dry matter and chemical composition of the biomass affected the ensiling which affected cellulose convertibility.
- Low DM ensiling (<25%) resulted in highest glucose yield and cellulose convertibility for all cuts of grass.
- The composition is largely determined by the maturity; less mature grass resulted in higher cellulose convertibility both with and without ensiling, due to the lower lignin content. However, less mature grass also has lower cellulose content. This suggests an optimum stage of maturity for grass, where cellulose content and convertibility results in an optimal sugar release.

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REFERENCES


Paper II

Ensiling of wheat straw decreases the required temperature in hydrothermal pretreatment

Morten Ambye-Jensen, Sune Tjalfe Thomsen, Zsófia Kádár* and Anne S Meyer

Abstract

Background: Ensiling is a well-known method for preserving green biomasses through anaerobic production of organic acids by lactic acid bacteria. In this study, wheat straw is subjected to ensiling in combination with hydrothermal treatment as a combined pretreatment method, taking advantage of the produced organic acids.

Results: Ensiling for 4 weeks was accomplished in a vacuum bag system after addition of an inoculum of Lactobacillus buchneri and 7% w/w xylose to wheat straw biomass at 35% final dry matter. Both glucan and xylan were preserved, and the DM loss after ensiling was less than 0.5%. When comparing hydrothermally treated wheat straw (170, 180 and 190°C) with hydrothermally treated ensiled wheat straw (same temperatures), several positive effects of ensiling were revealed. Glucan was up-concentrated in the solid fraction and the solubilisation of hemicellulose was significantly increased. Subsequent enzymatic hydrolysis of the solid fractions showed that ensiling significantly improved the effect of pretreatment, especially at the lower temperatures of 170 and 180°C. The overall glucose yields after pretreatments of ensiled wheat straw were higher than for non-ensiled wheat straw hydrothermally treated at 190°C, namely 74-81% of the theoretical maximum glucose in the raw material, which was ~1.8 times better than the corresponding yields for the non-ensiled straw pretreated at 170 or 180°C. The highest overall conversion of combined glucose and xylose was achieved for ensiled wheat straw hydrothermally treated at 180°C, with overall glucose yield of 78% and overall conversion yield of xylose of 87%.

Conclusions: Ensiling of wheat straw is shown to be an effective pre-step to hydrothermal treatment, and can give rise to a welcomed decrease of process temperature in hydrothermal treatments, thereby potentially having a positive effect on large scale pretreatment costs.

Keywords: Silage, Ensiling, Combined pretreatment, Hydrothermal treatment, Wheat straw, Enzymatic hydrolysis

Background

Lignocellulosic residues such as wheat straw (WS) are an attractive renewable resource for the production of fuel, feed and chemicals. Wheat is the most important crop in the EU with an annual average production of over 130 Mt grain [1] and around 200 Mt of straw residues (using a residue to product factor of 1.5 according to [2]). Replacement of conventional sugar or starch based feedstock with lignocellulosic agricultural residues, such as WS, for ethanol production is advantageous due to a more efficient use of the agricultural area. However, lignocellulosic residues require more advanced processing technologies. Lignocellulose consists of the polysaccharides cellulose and hemicellulose and the polyphenolic structure of lignin; together forming a rigid matrix structure in the secondary plant cell wall. This structure is naturally ‘engineered’ to resist degradation, thus creating great challenges in terms of biorefining. Physical and chemical pretreatments have been developed for lignocellulosic biomass in order to create accessibility for hydrolytic enzymes to hydrolyze the polysaccharides into readily fermentable sugars [3]. Bioethanol production from lignocellulosic residues has been the main driver for the technology development, and production is now on the verge of industrialization [4]. However the industry is facing huge difficulties in creating enough economic viability to engage in full scale production [5]. Pretreatment have been shown to...
cover up to 33% of the processing costs [6-9]. The pretreatment step is most often based on hydrothermal principles of high temperatures (170-220°C) in aqueous solution, and is the most energy intensive and expensive process step in the lignocellulose to ethanol process, due to the need of high temperature, pressure, and/or chemicals as well as specialized equipment. Examples of pretreatment methods are hydrothermal treatment (HTT), dilute acid treatment (using H2SO4), and ammonia fiber explosion. HTT has been widely studied for pretreatment of WS and other cellulosic biomasses, where it facilitates high yields of enzymatic cellulose conversion (70-90%) and its simple approach without additives makes it advantageous to upscale [5,8,10,11]. In the current Inbicon demonstration plant in Kalundborg, Denmark [5] the straw is hydrated to a dry matter (DM) mass fraction of 35% before it is continuously fed to a pressurized pretreatment reactor operating at 180-200°C for a retention time of 10-20 min [5]. Considering the low feed-in DM for lignocellulosic bioethanol, dry biomass storage processing is no longer an advantage as compared to traditional combustion. Furthermore drying of biomass increases the biomass recalcitrance towards biological degradation [12]. Alternatively wet storage (<40% DM) can be applied using ensiling.

Ensiling is the well-known preservation method for forages, based on anaerobic fermentation by lactic acid bacteria (LAB) that produce organic acids, reduce pH, and prevent growth of yeasts, fungi and competing bacteria. Lignocellulosic residues including WS, do not have sufficient available sugars to facilitate the necessary lactic acid fermentation required for preservation at low DM. Organic acids can be added directly instead of LAB fermentation [13]. Lignocellulolytic enzymes can be applied to release fermentable carbohydrates from the lignocellulose [6], or sugars can be added as substrate for LAB fermentation [14]. This study applies the latter of the three strategies. The species of LAB are usually separated into homo- and heterofermentative LAB based on their type of hexose fermentation. The homofermentative utilizes the Empden-Meyerhof-Parnas pathway and produces only lactic acid, while the heterofermentative utilizes the phosphoketolase pathway and produce lactic- and acetic acid, ethanol and carbon dioxide [15]. However when pentoses are used as fermentation substrate, then both types of LAB may produce both lactic- and acetic acid, see Eq. 1, but variation do occur [16,17].

\[
\text{Pentose} \rightarrow \text{Lacti c acid} + \text{Acet i c acid} \\
\text{HOC}_5(\text{CH(OH)}_5)\text{CHO} \rightarrow \text{CH}_3\text{C(\text{OH})COOH} + \text{CH}_3\text{COOH} \tag{1}
\]

Ensiling has in the last 6 years gained increased focus as a method for combined storage and pretreatment in biorefinery applications [6,18-24]. Based on studies of grass ensiling for forage purposes [25], the effect of ensiling as pretreatment is known to be correlated to the produced organic acids that act primarily on hemicellulose.

Oleskowicz-Popiel et al. [26] combined ensiling with HTT (190°C, 10 min) on maize, clover grass, and whole crop rye, which all contain easily fermentable free sugars, however they were not able to prove a positive effect of the ensiling. Xu et al. [27] studied the effect of adding lactic- and/or acetic acid to the hydrothermal pretreatment of dry corn stover and found that addition of acetic acid performed better as a catalyst than lactic acid, and increased the ethanol yield in a subsequent simultaneous saccharification and fermentation from 78% to 87% of the theoretical yield [27].

The pretreatment factors of temperature, holding time and pH, are often combined to one factor expressing the severity of the pretreatment [28]. Reducing pH through ensiling will increase the severity factor of the pretreatment at same temperature and holding time, thus higher severity would result in higher sugar release. It has however been shown by Pedersen et al. [29] that the use of the one dimensional severity factor to predict sugar yields is not reliable, because lignocellulosic pretreatment is much too complex.

Based on the hypothesis that the acid produced during ensiling can assist pretreatment, the aim of this study is to investigate the effect of ensiling prior to HTT in order to decrease pretreatment temperature and thereby decrease energy consumption. The ensiling is facilitated by addition of xylose and a heterofermentative LAB inoculum, which will favor acetic acid production in the silage. The motivation for using xylose as silage fermentation substrate is the availability of cheap C5 sugars in internal biorefinery process streams such as C5 molasses condensed from a HTT liquid fraction.

**Results and discussion**

**Ensiling wheat straw**

Ensiling of WS successfully preserved the biomass, resulting in only 0.35% loss in total DM and produced both acetic and lactic acid which caused the pH to drop from 7.0 to 3.7 (Table 1). The addition of 7 (w/w)% xylose resulted in 2.8 (w/w)% acetic acid and 2.4 (w/w)% lactic acid weight base in relation to the initial WS DM before ensiling. Over 1% of the added xylose was recovered, thus preservation can be carried out with less addition of xylose. Following Eq. 1 and assuming xylose were the only substrate, it can be calculated that 6 (w/w)% of utilized xylose would yield 3.6 (w/w)% lactic acid and 2.4 (w/w)% acetic acid. This is presumably due to the inoculum of *Lactobacillus buchneri* which is capable of a secondary fermentation where lactic acid is converted to acetic acid, thus shifting the ratio between acetic- and
lactic acid [30,31]. The motive to favor acetic acid to lactic acid is that it increases the effect of pretreatment [27]. Production of propionic acid and xylitol (Table 1) is due to minor secondary fermentations, which are still occurring during the stable phase of the ensiling. These secondary reactions can be carried out by a variety of acid tolerant microorganisms such as LAB, *Clostridium-* or *Bacillus-* or *Propioni* bacteria. It is well documented that secondary fermentation often utilizes other carbon sources than sugars including fatty acids, alcohols and amino acids derived from plant proteins [16]. This complicates the mass balance when products become substrates, for example parts of the produced lactic acid has most likely been further metabolized into propionic acid.

The ensiled wheat straw (EWS) was also analyzed for butyric acid, since butyric acid usually is due to presence of *Clostridium* bacteria and is a common indicator of insufficient preservation. The amounts detected were however below 0.01 (w/w)%, showing efficient preservation.

It was not possible in this experimental setup to distinguish between leftover xylose and the xylose released from hemicellulose. Preliminary experiments have shown xylose release during WS ensiling (unpublished observation, M. Ambye-Jensen and S. T. Thomsen), but in amounts less than 0.1 (w/w)%. It is therefore assumed that the released xylose only counts for a negligible fraction compared to leftover xylose. No arabinose was found in the water extractions and only insignificant amounts of released glucose were detected (Table 1).

The DM loss during ensiling was very limited and measured to below 0.5%. This was due to a fast and effective preservation facilitated by the efficient laboratory vacuum ensiling, however, losses cannot be expected to be as low in large scale.

Evaporation of fatty acids needs to be considered when determining DM content of silage, which can be done by using of volatilization coefficients to determine the acids lost during DM-determination [32]. In this work volatilization coefficients and the quantity of the total fatty acids in the EWS were used, to subtract the remaining fatty acids from the DM of the EWS as described at Material and Methods. Fatty acids originated from the added xylose were hereby not taken into account.

**HTT pretreatment**

**Composition**

The composition of the raw WS (RWS) and the solid fractions of hydrothermally pretreated WS (HTT WS) are compared with the EWS and the solid fractions of pretreated EWS (HTT EWS) (Table 2). The effects of increased temperature in the HTTs are up-concentration of cellulose and lignin in the solid fraction (Table 2).

Since xylan and arabinan levels in the solid fractions of HTTs are decreasing with increasing HTT temperature, and since levels are lower on EWS, the solubilisation of hemicellulose is concluded to be intensified when the WS is ensiled and the temperature of the HTT pretreatment is increased.

Comparing the glucan content of RWS with that of EWS confirmed that the ensiling effectively preserves the cellulose (Table 2). Likewise, the total amount of fatty acids produced during ensiling (Table 1) is corresponding to the amount of added xylose. Hence, there is no indication of loss of structural carbohydrates during the 4 weeks of ensiling.

**Mass balance**

The glucan content in the pretreated solid fraction plus the small amounts of solubilized glucan were compared to the amount of glucan in the RWS and a total recovery was calculated. The glucan in the EWS was preserved to the same extent as the RWS after HTT and all pretreatments had a recovery above 90% (data not shown).

The pretreatment effect of HTT lies in the mechanism of autohydrolysis, catalyzed by the high temperature steam; here water acts as a weak acid and initiates depolymerization of hemicellulose [28]. During this process acetic acid is released from the O-acetyl groups on the hemicellulose which further enhance the acid hydrolysis [3,29]. The solubilization of hemicellulose, simultaneously with a dislocation of lignin [33] is the reason for increased accessibility to cellulose that facilitates enzymatic attack. Even though the hemicellulose solubilization is attractive, the hemicellulose carbohydrates still holds potential value in a biorefinery context. The recovery of hemicellulose (xylan and arabinan) is therefore an important factor.

A clear trend was found that temperature increased solubilisation of hemicellulose (Figure 1). For all pretreatments, except HTT EWS 190°C, the hemicellulose was mainly recovered in the solid fraction, and the total recovery for these pretreatments was high (92-97%), while only 64% of the total hemicellulose was recovered.

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**Table 1** Dry matter loss and pH after 4 weeks ensiling: the most significant organic compounds in water extraction after ensiling

<table>
<thead>
<tr>
<th>Composition</th>
<th>DM loss (w/w)%</th>
<th>pH</th>
<th>Glucose</th>
<th>Xylose</th>
<th>Xyitol</th>
<th>Lactic acid</th>
<th>Acetic acid</th>
<th>Propionic acid</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>0.35</td>
<td>6.9</td>
<td>0.06 ± 0.00</td>
<td>1.27 ± 0.02</td>
<td>0.17 ± 0.00</td>
<td>2.46 ± 0.09</td>
<td>2.79 ± 0.08</td>
<td>0.36 ± 0.01</td>
<td>7.06</td>
</tr>
</tbody>
</table>

Total includes the mentioned organic compounds.
from HTT EWS 190°C (Figure 1). The solubilisation of hemicellulose was in general quite low compared to similar studies on hydrothermal pretreatments on WS (e.g. Petersen et al., 2009). This is most likely due to differences in biomass composition; e.g. Petersen et al. had significantly lower lignin and cellulose content compared to the WS used in this study.

It is clear from the results that ensiling significantly increased the solubilisation of hemicellulose, and the increase with pretreatment temperature was more pronounced (Figure 1). The relative high degradation of hemicellulose for EWS at 190°C indicates that severity of this pretreatment was too high.

It is well known that HTT at high temperature and acidic conditions cause degradation of xylose and forms furfural while degradation of glucose mainly forms hydroxymethyl furfural (HMF) and both are potential fermentation inhibitors. Accordingly, the increase in hemicellulose degradation with temperature, enforced by the combination with ensiling, was recorded in the measurements of furfural in the hydrolysates (Figure 2). Although the furfural levels were significantly higher in the HTT EWS samples than the HTT WS samples, the maximum concentration did not exceed 0.53 g/L (HTT EWS 190°C), which is far below the critical inhibition levels of 2.0 g/L. HMF concentrations were found not to exceed 0.03 g/L (data not shown) which is likewise much below inhibition levels.

For both WS and EWS the concentration of organic acid in the HTT liquid increased with temperature as expected (Figure 2) due to the higher biomass degradation at higher temperature. The HTT EWS liquids had significantly higher concentrations of total organic acids than HTT WS, which was due to both higher biomass degradation but also the organic acid content in the biomass before HTT. The levels on Figure 2 in (w/w)% of DM before HTT is

### Table 2 Composition of raw wheat straw (RWS) hydrothermal treated wheat straw (HTT WS), ensiled wheat straw (EWS) and hydrothermal treated ensiled wheat straw (HTT EWS) in the solid fraction after HTT (if pretreated)

<table>
<thead>
<tr>
<th></th>
<th>Glucan</th>
<th>Xylan</th>
<th>Arabinan</th>
<th>Lignin</th>
<th>Ash</th>
<th>Extractives</th>
</tr>
</thead>
<tbody>
<tr>
<td>RWS</td>
<td>40.2 ± 0.2</td>
<td>22.3 ± 0.1</td>
<td>3.3 ± 0.0</td>
<td>18.6 ± 1.1</td>
<td>5.2 ± 0.2</td>
<td>6.3 ± 0.2*</td>
</tr>
<tr>
<td>HTT WS 170°C</td>
<td>40.3 ± 2.4</td>
<td>24.8 ± 0.8</td>
<td>2.3 ± 0.1</td>
<td>21.3 ± 0.1</td>
<td>4.8 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>HTT WS 180°C</td>
<td>45.1 ± 1.5</td>
<td>25.2 ± 0.2</td>
<td>2.0 ± 0.0</td>
<td>21.6 ± 0.3</td>
<td>4.0 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>HTT WS 190°C</td>
<td>50.5 ± 0.2</td>
<td>22.4 ± 0.4</td>
<td>1.5 ± 0.2</td>
<td>23.0 ± 0.2</td>
<td>5.0 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>EWS</td>
<td>39.7 ± 0.0</td>
<td>24.1 ± 0.4</td>
<td>2.6 ± 0.0</td>
<td>17.5 ± 1.2</td>
<td>3.1 ± 1.1</td>
<td>6.9 ± 0.8</td>
</tr>
<tr>
<td>HTT EWS 4w 170°C</td>
<td>40.2 ± 1.0</td>
<td>20.1 ± 1.3</td>
<td>1.3 ± 0.2</td>
<td>23.0 ± 0.4</td>
<td>4.2 ± 0.0</td>
<td></td>
</tr>
<tr>
<td>HTT EWS 4w 180°C</td>
<td>43.2 ± 1.0</td>
<td>18.5 ± 1.2</td>
<td>1.6 ± 0.1</td>
<td>24.5 ± 0.4</td>
<td>4.2 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>HTT EWS 4w 190°C</td>
<td>54.3 ± 0.6</td>
<td>11.8 ± 0.6</td>
<td>0.4 ± 0.0</td>
<td>25.9 ± 0.6</td>
<td>4.0 ± 0.1</td>
<td></td>
</tr>
</tbody>
</table>

*only ethanol extraction.
equivalent to between 1.5-1.9 g/L for HTT EWS and 0.1-0.4 g/L for HTT WS. The distribution of organic acids was also different for the WS HTT and EWS HTT. For HTT of WS it was mainly acetic acid and a bit of formic acid, a distribution of 82% and 15% respectively. For the HTT on EWS the distribution was 54% acetic-, 7% formic-, 34% lactic-, and 5% propionic acid (data not shown). The difference in organic acids in the pretreated liquids suggests that the mechanisms during pretreatment of the two different biomasses appear to be different, which is in line with the clear difference in hemicellulose solubilisation (Figure 1). Organic acids can have inhibitory effect in subsequent ethanol fermentation, but for that the concentrations should exceed 10 g/L [35]. On the other hand, it has been shown that inhibitors can serve as very efficient contamination control in large-scale lignocellulosic bioethanol production, preventing growth of especially *Lactobacillus* and thus avoid the need of expensive sterile fermentation equipment [5].

**Enzymatic hydrolysis**

The enzymatic hydrolysis on the pretreated fiber was effectively acting on both cellulose and hemicellulose due to the addition of both cellulase- and hemicellulase blends. The glucose conversion yields in the pretreated solid fraction of the HTT WS increased with temperature especially from 180°C to 190°C where the conversion yield jumped from 45.9 to 71.5% (Table 3). For the HTT EWS the glucose conversion yield ranged from 73.5-78.7% and did not differ significantly due to the standard deviations (Table 2). When addressing the actual release of glucose in (w/w)% of DM in the solid fraction after HTT it were apparent that HTT EWS 190°C gave the highest release of 43.9 (w/w)% (Table 3).

The glucose conversion yields after enzymatic hydrolysis were clearly improved by ensiling especially at the lower HTT temperature of 170°C and 180°C, which leads to a significant increase in the overall glucose conversion yields (Table 3). E.g. at the HTT at 180°C the overall glucose conversion yield increased from 44.4% to 78.5% of glucose in raw material when WS was ensiled. The data also showed that ensiling alone was not sufficient as pretreatment, since only 13% of the available glucose in the raw material could be enzymatically converted (Table 3). The low overall glucose conversion yield on WS at the two lower pretreatment temperatures shows that the pretreatment severities were insufficient.

The overall conversion yield of xylose (Table 4) showed the same trend as for glucose (Table 3). However for HTT EWS 190°C the released xylose was significantly lower compared to pretreatments at lower temperatures. This can be explained by the thermal degradation of hemicellulose at higher pretreatment severity. Furthermore, the xylose release of HTT EWS 170°C (17.2 (w/w)%) was similar to HTT WS 190°C (18.0 (w/w)%), corroborating that ensiling facilitated high xylose release at lower pretreatment temperature.

The positive effect of ensiling WS prior to HTT can be quantified by comparing the yields over the same pretreatment temperature. At 170°C and 180°C ensiling improves the total yield. Comparing the released glucose and xylose (Table 3 and Table 4) from HTT WS with HTT EWS it can be concluded that we gain substantial more released sugar than the 7% xylose spent facilitating the ensiling process. However, at 190°C this positive sugar balances is not observable due to xylose degradation.

The literature points at two main reasons for the improved sugar release of combining ensiling and HTT.

### Table 3 Glucose conversion after enzymatic hydrolysis of raw wheat straw (RWS), hydrothermal treated wheat straw (HTT WS), ensiled wheat straw (EWS) and of hydrothermally treated ensiled wheat straw (HTT EWS)

<table>
<thead>
<tr>
<th></th>
<th>Released glucose</th>
<th>Glucose conversion yield</th>
<th>Overall glucose conversion yield</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>In (w/w)% of DM in solid fraction</td>
<td>In % of glucose in solid fraction</td>
<td>Liquid fraction</td>
</tr>
<tr>
<td><strong>RWS</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HTT WS 170°C</td>
<td>19.1 ± 0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>43.0 ± 1.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.9 ± 0.0&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>HTT WS 180°C</td>
<td>22.8 ± 1.9&lt;sup&gt;d&lt;/sup&gt;</td>
<td>45.9 ± 3.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.4 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>HTT WS 190°C</td>
<td>39.7 ± 2.9&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>71.5 ± 5.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.8 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>EWS</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HTT EWS 170°C</td>
<td>33.5 ± 2.9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>75.7 ± 6.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.8 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>HTT EWS 180°C</td>
<td>37.4 ± 1.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>78.7 ± 3.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.3 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>HTT EWS 190°C</td>
<td>43.9 ± 2.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>73.5 ± 3.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.6 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Released glucose is expressed as (w/w)% of DM in solid fraction after HTT. Glucose conversion yield is expressed as glucose release in % of glucose in the solid fraction after HTT. Overall glucose yield is the glucose release in the liquid fraction after HTT- and in the solid fraction after enzymatic hydrolysis in % of glucose in the raw wheat straw. The results in each row are grouped according to significance (p = 0.05%), where ‘a’ is significantly higher than ‘b’ and so forth.
First, the improved sugar release is connected to the natural long term impregnation of organic acids on the biomas where the lignocellulosic structure is loosened by weak acid hydrolysis accomplished by organic acids [6]. Due to the addition of xylose as substrate for ensiling, it could not be concluded to which extent hemicellulose was solubilized, but the combined results suggests very little solubilisation. Since this study did not look at the duration of the ensiling or included pretreatment of WS merely soaked in organic acids as a control, it cannot be unequivocally concluded that the improvement of HTT on EWS was directly due to the long term ensiling alone. Monavari et al. [36] did a study on impregnation with lactic acid on bagasse prior to steam explosion and found a significant difference between long term impregnation (4 weeks) and merely soaking, favoring the impregnation, proving that this is in fact a factor. Nonetheless, soaking of the dry wheat straw to a DM of 35%, do cause swelling of the cell wall, which is most likely improving the effect of pretreatment. The second main effect of ensiling prior to HTT is the lowering of pH which causes higher severity, i.e. the action of the produced organic acids within the HTT pretreatment. Especially acetic acid, but also lactic acid has been shown to catalyze the autohydrolysis and improve the process as it was found by Xu et al. [27]. Recently it has been shown that addition of 0.04 g (g DM)⁻¹ acetic acid to HTT of wheat straw increased glucose yield at both 190°C and 195°C, however not at 200°C, thus the effect of acetic acid was more significant at lower temperatures [37]. Results from the present study also determine that improvement by acid catalyzed autohydrolysis increases at decreasing pretreatment temperature. Furthermore, due to the large effect of ensiling at lower HTT temperatures i.e. 170-180°C, it would be interesting to test even lower HTT temperatures than 170°C in future studies.

### Conclusion

Ensiling prior to hydrothermal treatment was shown to significantly increase the effect of the pretreatment, especially at 170°C, and 180°C. An effective ensiling of wheat straw was accomplished with the presented method in which both glucan and xylan was effectively preserved, and where the DM loss during ensiling was under 0.5%. Ensiled wheat straw hydrothermally treated at 180°C gave the highest overall conversion yield regarding both glucan and xylan, 73.6% and 83.5% respectively, but even pretreatment of ensiled wheat straw at 170°C provided satisfying results, 70.4% and 77.4% for glucan and xylan respectively. In both cases, more xylose was gained after the enzymatic hydrolysis than was used in the production of the wheat straw silage. The findings potentially enable a considerable decrease in the necessary process temperature in hydrothermal treatments of wheat straw, thereby having a positive effect on large scale pretreatment costs.

### Materials and methods

#### Raw material

Wheat straw (*Triticum aestivum* L.) was supplied by DONG Energy (Skærbæk, Denmark). The straw was chopped to approximately 10 cm pieces and stored at ambient temperature. Dry matter content of the stored WS was 90%.

#### The process

Combined ensiling and HTT pretreatment was tested against conversion of glucose and xylose after subsequent enzymatic hydrolysis. The combined pretreatment

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**Table 4 Xylose conversion after enzymatic hydrolysis of raw wheat straw (RWS), hydrothermal treated wheat straw (HTT WS), ensiled wheat straw (EWS) and of hydrothermal treated ensiled wheat straw (HTT EWS)**

<table>
<thead>
<tr>
<th></th>
<th>Released xylose</th>
<th>Xylose conversion yield</th>
<th>Overall xylose conversion yield</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>In (w/w)% of DM in solid fraction</td>
<td>In % of xylose in solid fraction</td>
<td>Liquid fraction</td>
</tr>
<tr>
<td>RWS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HTT WS 170°C</td>
<td>11.1 ± 0.3c</td>
<td>40.0 ± 1.0d</td>
<td>3.1 ± 0.0f</td>
</tr>
<tr>
<td>HTT WS 180°C</td>
<td>14.6 ± 0.7b</td>
<td>51.6 ± 2.6c</td>
<td>6.2 ± 0.3a</td>
</tr>
<tr>
<td>HTT WS 190°C</td>
<td>18.0 ± 1.6a</td>
<td>71.8 ± 6.2b</td>
<td>21.1 ± 1.8c</td>
</tr>
<tr>
<td>EWS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HTT EWS 170°C</td>
<td>17.2 ± 1.0a</td>
<td>76.3 ± 4.6ab</td>
<td>14.5 ± 0.0d</td>
</tr>
<tr>
<td>HTT EWS 180°C</td>
<td>16.7 ± 0.8b</td>
<td>81.0 ± 5.0c</td>
<td>26.7 ± 2.3b</td>
</tr>
<tr>
<td>HTT EWS 190°C</td>
<td>11.7 ± 0.7c</td>
<td>88.2 ± 5.5a</td>
<td>30.6 ± 0.0c</td>
</tr>
</tbody>
</table>

Released xylose is expressed as (w/w)% of DM in solid fraction after HTT. Xylose conversion yield is expressed as xylose release in % of xylose in the solid fraction after HTT. Overall xylose yield is the xylose release in the liquid fraction after HTT and in the solid fraction after enzymatic hydrolysis in % of xylose in the raw wheat straw. The results in each row are grouped according to significance (p = 0.05%), where ‘a’ is significantly higher than ‘b’ and so forth.
(HTT EWS) were compared to the conversion in raw wheat straw (RWS), ensiled wheat straw (EWS) and sole HTT pretreated wheat straw (HTT WS).

**Ensiling**

Ensiling was carried out on chopped WS (10 cm) adjusted to 35% final DM content. Due to the low free sugar content of WS, 7 g xylose per 100 g DM was added as determined to be optimal by Yang et al. [14]. Each batch of ensiling contained 1.5 kg DM WS. The ensiling was carried out using a vacuum based plastic bag system [38] and a Variovac EK10 vacuum packaging machine (Variovac Nordic A/S, DK-7100 Vejle, Denmark).

The commercially available inoculum LACTISIL CCM (Chr. Hansen, Hørsholm, Denmark) which consists of freeze dried pure heterofermentative *Lactobacillus buchneri* was applied. A suspension of 0.2 g L\(^{-1}\) water was prepared and added in the amount of 40 ml kg\(^{-1}\) WS to reach an initial inoculum size of 8 mg kg\(^{-1}\).

The plastic bags were opened after 4 weeks. Weight loss was measured for calculation of DM loss. After ensiling, 1 kg DM of the ensiled WS was pretreated hydrothermally.

**Hydrothermal pretreatment**

Hydrothermal pretreatments (HTT) were carried out in the “Mini IBUS” equipment (Technical University of Denmark, Risø campus). 1 kg DM (corrected for volatile fatty acid) of the EWS was treated at different temperatures (170, 180 and 190°C) for 10 min. In order to verify the reproducibility of HTT, the EWS pretreated at 180°C were done in triplicate. After HTT the pretreatment reactor was cooled to below 70°C thereby avoiding evaporation of acids, and the material was separated by pressing. Each solid fiber fraction and each liquid fraction were analyzed separately. The solid fraction was kept in the freezer and used to evaluate the process efficiency by enzymatic hydrolysis.

**Enzymatic hydrolysis**

The enzymatic convertibility assay based on commercial CellicCTec2 (blend of cellulases) and CellicHTec2 (blend of hemicellulases) (Novozymes A/S, Denmark) was used to determine the efficiency of the pretreatment process. Enzymatic conversion of pretreated solids was performed at 5% DM content in a total volume of 25 mL using 50 mM citrate buffer (pH 5) and 0.25 mL sodium azide (2%) at 50°C shaken at 150 rpm for 72 h. Applied enzyme loadings were 15 FPU g\(^{-1}\) DM solids of CellicCTec2 supplemented with xylanase CellicHTec2 (90:10 ratio based on protein loading for all assays). The enzymatic hydrolysis was performed in triplicates and enzyme blanks were included. Samples were analyzed for carbohydrates on HPLC. Cellulose convertibility was calculated as the converted cellulose divided by the original cellulose content.

**Chemical analysis**

Raw wheat straw (RWS), ensiled wheat straw (EWS), hydrothermally pretreated wheat straw (HTT WS) and hydrothermally pretreated ensiled wheat straw (HTT EWS) were analyzed for chemical composition by methods based on standard laboratory analytical procedures developed by National Renewable Energy Laboratory (NREL), US [39]. Deviations from these standard procedures are stated in the following sections. The analysis of the solid fiber fraction included ash content determination, water extraction, ethanol extraction and strong acid hydrolysis for structural carbohydrates and lignin. The liquid fraction of the HTT was analyzed by weak acid hydrolysis.

**DM determination**

DM was determined using a standard method [39]. The contribution of fatty acids produced during ensiling was subtracted from the DM, since the acids originated from the added xylose, which likewise were not included in the original DM content of WS. Huida et al. [40] determined volatilization coefficients describing to which extent different fatty acids were evaporating during determination of DM at specific pH. These volatilization coefficients were used to determine how much of the different acids that were left after DM determination of EWS in order to correct for this amount. Fatty acids in RWS and solid fraction of HTTs EWS were negligible, thus no correction of DM were needed in these cases.

**Analytical method**

Concentrations of carbohydrates (D-glucose, D-xylose, L-arabinose), organic acids (lactic-, formic-, acetic-, propionic, and butyric acid) were quantified by HPLC using a Biorad HPX-87H column (Hercules, CA; USA), RI detector, 63°C and 4 mM H\(_2\)SO\(_4\) as eluent, at flow rate of 0.6 ml min\(^{-1}\).

**Water extraction**

0.3-0.4 g DM biomass from freshly disrupted silage bags was extracted in 10 ml MilliQ H\(_2\)O with 10 μl of the antibiotic ampicillin (10 mg/ml solution) to prevent microbial activity during extraction. The extraction samples were shaken for 2 hours at 25°C and 150 rpm. Extracts were analyzed for sugars, acids by HPLC as described above. Acids produced from additional xylose used for initiating ensiling process, were taken into account.

**Weak acid hydrolysis of hydrolysates**

The liquid fraction of HTT was further analyzed by weak acid hydrolysis to quantify the content of soluble oligomer carbohydrates. 10 ml HTT liquid fraction were
autoclaved for 10 minutes at 121°C with 4 w/w % H₂SO₄. Derived sugars were analyzed by HPLC as described above.

**Ethanol extraction**

Lipophilic extraction was carried out by Soxhlet extraction in a reflux condenser for six hours with 99 w/w% ethanol on water extracted samples of EWS. The amount of ethanol extractives, including volatiles, was defined as the mass of material lost through extraction.

**Determination of structural carbohydrates and lignin**

Strong acid hydrolysis was used to measure the carbohydrate and lignin content of the extracted bio residue, based on the NREL standard laboratory analytical procedure [32].

**Statistical evaluation**

One-way analyses of variances (one-way ANOVA): 95% confidence intervals were compared as Tukey–Kramer intervals calculated from pooled standard deviations (Minitab Statistical Software, Addison-Wesley, Reading, MA).

**Competing interests**

The authors declare that they have no competing interests.

**Authors’ contributions**

MA participated in the experimental design, carried out the ensiling treatment, contributed to acquisition of data and drafted the manuscript. STT participated in the planning and executing the laboratory work, contributed to acquisition of data and reviewing the manuscript. ZK participated in the experimental design, contributed to acquisition of data and review the manuscript. AM and STT contributed equally to this work. All authors read and approved the final manuscript.

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**References**

Paper III

Title page

Ensiling of grass and hydrothermal pretreatment: Consequences for enzymatic biomass conversion and total monosaccharide yields

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Abstract

**Background:** Ensiling has been shown to act as a pretreatment of the lignocellulosic structure and increase enzymatic conversion of structural carbohydrates to fermentable sugars. However, most studies report low conversion efficiencies implying that ensiling alone does not provide sufficient severity to be a stand-alone pretreatment method. Here, ensiling is combined with hydrothermal treatment (HTT), with the aim of improving the conversion and possibly lowering the temperature of HTT.

**Results:** Grass silage (*Festulolium Hykor*) were treated at temperatures of 170, 180, and 190°C, 10 min and compared to that of dry grass. Ensiling increased solubilisation of dry matter (DM) during HTT, resulting in increased glucan content in the solid fraction. Enzymatic hydrolysis of all pretreated fibers showed profound inhibition of cellulase activity, and washing proved to be a necessary step in order to get acceptable yields. Ensiling improved glucose yields in the enzymatic hydrolysis of solid fraction at the lower HTT temperatures of 170°C, from 17.0 to 23.8 (w/w)% (45-57% cellulose convertibility), and of 180°C from 22.0 to 28.8 (w/w)% (54-69% cellulose convertibility). However, HTT of grass at 190°C gave same high glucose yield as for grass silage, of 35 (w/w)% (77% cellulose convertibility), and in fact higher xylan yields, of 7 (w/w)% xylose (27% xylan convertibility). The effect of ensiling prior to HTT was consistently smaller than what was found for wheat straw by Ambye-Jensen et al. (2013) [1]. The improvements by ensiling on enzymatic conversion of cellulose for HTT at 170°C and 180°C, did not make up for the loss of water soluble carbohydrates (WSC), which was utilized for silage fermentation. Thus overall sugar yield (C6+C5) was the same for HTT of grass as for HTT of grass silage at both 170°C and 180°C, but at 190°C overall sugar yield was best for dry grass.

**Conclusions:** The results in this study points out that ensiling of green crops such as grass comes with a cost in WSC and do not necessarily return the investment in terms of better pretreatment. On the other hand, ensiling poses considerable advantages as storage method, and can potentially benefit downstream processing.
Keywords: silage, ensiling, combined pretreatment, hydrothermal treatment, grass, enzymatic hydrolysis
Background

Lignocellulosic bioethanol stand before a substantial growth in Europe, generated by binding EU targets of 10% renewable energy in the transport sector by 2020 [Renewable Energy Directive (28/2009/EC, RED)], and the recent amendments proposed by the European Commission promoting non-food derived biofuels [amending Directive 98/70/EC, and amending Directive 2009/28/EC]. To endorse a sustainable biomass supply, it is necessary to broaden the range of biomasses for biofuel and include multifunctionality of the land use [2].

Temperate grass has a great potential for bioenergy due to a low energy input, high yield potential and a vast availability in temperate regions of northern Europe. Additionally, growth of perennial grassland is multifunctional, as grassland areas can benefit the ecological system through sequestration of carbon into the soil preventing agricultural degradation of arable land [3]. Cultivation of temperate grass can therefore be a valuable part of sustainable agriculture and land management. Secondly carbon sequestration favors the overall carbon balance of the biofuel.

A significant difference between grass and agricultural residues, typically studied for bioethanol, such as wheat straw and corn stover, is the dry matter at harvest. Management of temperate grasses like tall fescue (Festuca arundinacea) and italian ryegrass (Lolium multiflorum) is distinguished by multiple cuts over the season (3-4 times), resulting in the harvest of a moist green grass. Wet storage, by means of ensiling, is therefore preferred, thus avoiding field drying that requires long periods with stable dry weather conditions or energy demanding indoor drying. Ensiling is facilitated by low pH, through anaerobic solid state fermentation by lactic acid bacteria (LAB), preventing yeasts, fungi and other bacteria to grow [4].

Temperate grasses contain considerable amounts of water soluble carbohydrates (WSC), consisting of mainly glucose, fructose, sucrose and fructans [5]. The WSC are important for silage fermentation as it
provides the necessary substrate for the LAB. It is therefore not necessary to add carbohydrates or enzymes in order to ensile temperate grass, as it is the case for agricultural residues like wheat straw and corn stover [1][6, 7]. The utilization of WSC in grass will on the other hand represent a loss of potential sugars for ethanol fermentation.

A main challenge for commercial scale lignocellulosic bioethanol are high overall costs [8][9]. Pretreatment have by several researchers been identified as the most costly process step in the conversion from biomass to bioethanol [8, 10, 11][12]. Thus, as a main challenge, pretreatment remain to be a key area of optimization. Ensiling has in several studies been shown to improve enzymatic conversion of cellulose compared to dry storage [13-16][17], and is therefore suggested as a combined storage and pretreatment method, which can potentially reduce cost of pretreatment due to the ambient temperature. However the conversion efficiencies are rarely exceeding 50% (converted cellulose/original cellulose)[13, 15, 17], which is rarely sufficient to provide enough sugars for a cost efficient ethanol production process [11]. Thus ensiling cannot compete with the more severe physico-chemical pretreatments, such as hydrothermal treatment (HTT), steam explosion, dilute acid treatment or ammonia fiber explosion (AFEX).

To improve the conversion efficiency of cellulose, this study combines ensiling of grass with an additional pretreatment using HTT. In turn the combination can facilitate a lower operating temperature of HTT.

HTT uses steam at high temperatures, ranging from 170-220°C, and at corresponding pressure. The pretreatment effect of HTT on biomass involve primarily an autohydrolysis, where water acts as a weak acid and initiates depolymerization and solubilisation of hemicellulose, and a dispositioning of lignin [18, 19]. HTT is advantageous to scale up and are thus applied at the Inbicon demonstration plant in Kalundborg, Denmark, pretreating wheat straw at a rate of 4 tonne/hour[8].
The combination of ensiling and HTT gives rise to an increased severity of the HTT due to the organic acids in silage, which catalyzes biomass hydrolysis [1]. Oleskowicz-Popiel et al. (2011) [15] previously combined ensiling and HTT on maize (whole crop), rye (whole crop), and a clover grass mixture and evaluated by ethanol fermentation. It was found that HTT increased the ethanol yield of the ensiled biomasses significantly, from 36% to 79% theoretical ethanol for clover grass. However ethanol fermentation of hydrothermally treated non-ensiled biomasses, and loss of WSC’s were not investigated in order to conclude on the actual effect of ensiling. In a study on wheat straw, we recently found that ensiling prior to HTT improved the effect of pretreatment at lower temperatures, measured as released glucose and xylose after enzymatic hydrolysis [1]. The results showed an increment of 80% and 81% glucose and xylose yields, respectively, at 170°C, and 68% and 52% at 180°C.

Encouraged by these results the aim of this study was to consider the potential of grass silage as a biomass for bioethanol production using the advantages storage method for grass of ensiling and combining it with HTT. It was thus hypothesized that combining ensiling and HTT increases overall sugar yields after pretreatment and enzymatic hydrolysis, and the increase in yield should in turn exceed the amount of WSC lost to acids during ensiling.

**Results and Discussion**

**Ensiling**

Ensiling of grass did not cause considerable changes in the biomass composition (Table 1) and no degradation of cellulose or hemicellulose was observed. Thus the natural concentration of WSC before ensiling, provided enough substrate for the LAB to reach stable conservation of the biomass. The lack of significant difference in hemicellulose content does, however, also imply that no hydrolysis of hemicellulosic carbohydrates had occurred due to acid hydrolysis, as it has been observed for ensiling in other studies [20][21].
The main WSC in the grass were glucose and fructose, but also xylose, galactose and arabinose were detected (Table 2). The WSC is utilized as substrate in the anaerobic fermentation and metabolized into primarily lactic- and acetic acid. The produced organic acids in the grass silage exceeds the amount of WSC in the fresh grass. Since no hemicellulose were hydrolyzed the extra substrate had to be found elsewhere. It is likely that additional WSC were released from collapsed plant cells during ensiling, which provided an extra supply of substrate [5]. This is corroborated by the increased amount of water extractives after ensiling, which most likely is a result of plant cell lysis, taking place during ensiling [22].

TABLE 1. Composition of grass and grass silage before HTT.

TABLE 2. Water soluble carbohydrates (WSC) and organic acids analyzed in H₂O extract of grass and grass silage.

Hydrothermal treatment

The biomass were separated in liquid and a solid fraction after HTT pretreatment. The solubilisation of DM during pretreatment increases with the HTT temperature and is generally higher for grass silage (Table 3 and 4). The glucan content in the solid fraction increases at higher temperature, and the levels are significantly higher in the pretreated grass silage (Table 3).

TABLE 3. Composition of solid fraction after HTT.

High solubilisation of the biomass is an advantage to the enzymatic liquefaction process, since less water insoluble fiber will reduce the viscosity of the process stream out of the pretreatment, and reduce complications of enzymatic hydrolysis at high DM [23]. The DM in enzyme hydrolysis is an important limiting factor in cellulosic ethanol production since it determines an upper limit of potential sugars and hence, ethanol concentration in the fermentation broth. At the Inbicon demonstration plant (Kalundborg, Denmark), enzymatic hydrolysis is operated at max. 30% DM. Additionally important are the concentration of glucan in the solid fraction. The higher the glucan concentration in the fiber, the higher potential ethanol concentration. It is however important to note that this mainly apply to the
approach of solely C6 sugar fermentation, where the liquid fraction is separated and C5 sugars are used for other purposes such as molasses or biogas production[8].

Solubilisation of hemicellulose (xylan, arabinan and galactan) is, as expected, increasing at higher HTT temperatures, but here there are no difference in the relative amounts of the solid fraction between dried grass and grass silage. Solubilisation of hemicellulose is also exposed in the calculated hemicellulose recovery from liquid and solid fraction after HTT (Figure 1). However, the results of hemicellulose recovery shows that hemicellulose solubilisation actually do differ between grass and grass silage; at all temperatures it is higher for grass silage. The hemicellulose recovery is calculated in relation to the biomass input and do therefore reflect the actual amounts of hemicellulose. Degradation of hemicellulose sugars occurs only at the high temperature treatments of 190°C, and the degradation is significantly higher for grass silage.

The improved solubilisation and increased degradation for grass silage can be due to; firstly, that the grass silage has become less recalcitrant by the effect of ensiling; secondly, that the severity of HTT on grass silage are increased by high concentrations of organic acids associated with grass silage. Since, changes in the chemical structure after ensiling were limited it is most likely that differences were primarily due to higher severity of the HTT.

FIGURE 1. Recovery of hemicellulose.

TABLE 4. Sugars and organic acids in the HTT liquid fraction.

Between 12-36% of the solubilized DM were recovered as mono- and oligo- saccharides, and 6-18% were recovered as organic acids (Table 4). The rest of the solubilized DM are components like e.g. proteins, amino acids, and cuticular wax, which were not quantified.

Despite the slightly higher solubilisation of hemicellulose for HTT of grass silage it is the pretreatment of grass, which amounts to the highest sugar concentration in the liquid fraction. The main difference is that the liquid fractions from pretreated grass contains significantly higher amounts of glucose and
fructose, derived from the WSC, and this considerably exceeds the surplus xylose from hemicellulose in the liquid fraction of pretreated grass silage (Table 4). At 190°C the hemicellulose sugars (xylose, galactose, and arabinose) are highest in the liquid fraction from pretreated grass, reflecting the higher sugar degradation for grass silage at 190°C (Figure 2), due to the higher severity caused by organic acids. Thus ensiling must give rise to a considerable increase in the enzymatic conversion of the solid fraction to compensate for the loss of WSC.

The amount of organic acids in the liquid fraction were significantly higher for grass silage compared to grass, due to a high lactic acid concentration (Table 4), originating from the silage fermentation. Organic acid in the liquid fraction can potentially inhibit ethanol fermentation, due to diffusion of undissociated acids across the yeast cell membrane. Graves et al. (2006) tested inhibition by both lactic- and acetic acid in ethanol fermentation and found inhibitory concentrations at pH 5 and 25% solids, starting from 4.0 (w/v)% and 0.3 (w/v)% of lactic- and acetic acid, respectively [24]. Thus lactic acid, which has the highest concentrations, is a much less inhibitory acid than acetic acid [24]. The amount of lactic- and acetic acid in the liquid fractions of around 11% and 2.9-3.7% of the solubilized DM, respectively corresponds to concentrations of 0.50 and 0.13 (w/v)%, respectively. The organic acid concentration in the liquid do therefore not pose high risk of inhibition.

**Enzymatic hydrolysis**

Ensiling of grass alone did, surprisingly and in contrast to results obtained in a previous study by Ambye-Jensen et al. [17], not improve neither glucose nor xylose yields in the enzymatic hydrolysis. Both grass and grass silage yielded around 7 (w/w)% glucose (Figure 2A), without additional pretreatment. In the previous study, ensiling improved the yield from 7.8 to 11.4 (w/w)% glucose per DM for the same grass species. In the previous study it was concluded that the pretreatment effect was significantly influenced by DM and biomass composition, which was distinguished by the relative maturity. The absent effect of
ensiling in this study support that the particular grass cut have a significant influence on the effect of ensiling, but it does not support the results of relative maturity and low DM in [17].

FIGURE 2. Glucose and xylose yields after enzymatic hydrolysis of the HTT solid fractions.

The enzymatic hydrolysis were done repeatedly with different sample preparations. When fibers were simply dried prior to enzymatic hydrolysis, the amount of released glucose were strikingly low, ranging from 13-25 (w/w)% glucose per DM pretreated fiber (Figure 2A), equaling 29-46% cellulose convertibility (converted glucan per original glucan in raw grass). Nevertheless, the results showed, as expected, increasing glucose yields with increasing HTT temperature, and furthermore consistently higher glucose yields from the grass silage (Figure 2A). Thus ensiling provided a positive effect on enzymatic cellulose saccharification of the HTT solid fraction.

The reason for the low yields (Figure 2A) was hypothesized to be due to inhibition of the enzymes and due to increased recalcitrance induced by the drying. The enzymatic hydrolysis were therefore repeated twice. First with an additional washing and subsequently drying of the fibers, secondly by washing and no subsequent drying. Washing of the HTT fibers gave a significant increase in yields, now ranging from 18-35 (w/w)% glucose per DM pretreated fiber (Figure 2B), and thus cellulose convertibility increased to 45-78% (converted glucan per original glucan). The glucose yields improved in general by a factor of 1.4-1.5, but for HTT grass at 190°C the improvement were significantly higher increasing glucose concentrations by 1.8. Washing and subsequent drying gave very similar result as on washed wet fibers (results not shown). Thus, washing proved to be a necessary step before enzymatic hydrolysis in order to get acceptable yields, but the effect of drying were insignificant.

Strong inhibition of cellulases have recently been shown to occur in hydrothermally pretreated biomass, due to xylo- and gluco-oligosaccharides [25]. Enzymatic hydrolysis of unwashed fibers would imply that oligomers from the liquid fraction, still ‘sticking’ to the fibers, would stay on the fiber, also after drying, and inhibit cellulases in the enzymatic hydrolysis. Such a high inhibition of cellulases was however not
found to be an issue for the enzymatic hydrolysis of pretreated wheat straw in the previous study by Ambye-Jensen et al. (2013) [1].

Cellulase inhibition can also occur due to degradation products of furans and acids (furfural, 5-HMF, levulinic acid, formic acid) derived from degradation of the carbohydrates. These degradation products can form insoluble lignin-like structures that deposits on the pretreated fiber and therefore decrease accessibility for the cellulases [26].

**TABLE 5. Total mono- and oligo-saccharides and organic acids in wash water of HTT solid fraction.**

**FIGURE 3.** Mono- and oligosaccharides in the wash water of HTT solid fractions divided in and C5 (xylose, arabinose) and C6 (glucose, galactose, fructose) sugars.

The analysis of the wash water showed high concentrations of oligosaccharides, which most likely have caused inhibition on the cellulases in the enzymatic hydrolysis of the unwashed fiber. Kont et al. identified inhibitory oligosaccharides to consist of a mixture of xylo- and glucooligosaccharides with a DP of 7 to 16, and found them to cause a 100-fold stronger inhibition on biohydrolase TrCel7A than cellubiose, which is known to be a common inhibitor for cellulases [25].

Washing of the HTT fibers removed considerable amounts of oligosaccharides in all samples, and increasingly amounts at higher HTT temperature (Table 5). The sugar analysis showed significant difference between grass and silage grass (Figure 3). The wash water of HTT grass contained higher amounts of C6, which decreased with increasing HTT temperature. Whereas for grass silage the concentration of C6 was much lower and constant at all HTT temperatures. The concentration of oligomer C5 sugars were on the other hand increasing with increasing temperatures for both biomasses (Figure 3).

The content of furans in the wash water were higher for the HTT of grass than that of grass silage, and the major part being 5-HMF. 5-HMF is the degradation product of glucose, which explains the higher concentration for grass originating from the free glucose in the WSC. Presence of furans in the wash
water suggests that formation and deposits of insoluble pseudo-lignin also could have contributed to
the cellulose inhibition [26]. Free furans are also potentially inhibitory to the ethanol fermentation [27].
The amounts in the wash water does however not give rise to critical inhibition levels. A solid loading in
a subsequent fermentation of 25%, would lead to a maximum furan concentration of 0.12 (w/v)%.
The significantly highest concentration of inhibitory oligosaccharides and furans were found for HTT of
grass at 190°C, corroborating the significant effect of washing, found for this particular sample.
The necessity of washing the fiber prior to enzymatic hydrolysis suggests a separation of liquid and solid
fraction after HTT.
On washed fibers, the ensiling improved glucose yields at the lower HTT temperatures of 170°C, from
17.0 to 23.8 (w/w)% (45-57% cellulose convertibility) and of 180°C from 22.0 to 28.8 (w/w)% (54-69%
cellulose convertibility) (Figure 2B). Pretreatment of grass silage at 170°C gave same sugar yields as
pretreatment of grass at 180°C. However, there was no effect of ensiling at 190°C. Here HTT of grass
gave same high glucose yield as for grass silage, of 35 (w/w)% (77% cellulose convertibility), and in fact
higher xylan yields, of 7 (w/w)% xylose (27% xylan convertibility). It is evident that the lower xylan
convertibility for grass silage at 190°C is a direct consequence of increased hemicellulose degradation
(Figure 1).
The effect of ensiling prior to HTT is nevertheless consistently smaller than what was found for wheat
straw by Ambye-Jensen et al. [1]. Here the glucose convertibility of HTT pretreated wheat straw were
increased by a factor of 1.9 and 1.8 at 170 and 180°C, respectively. The comparable factor of
improvement for grass were in both cases merely 1.3.
Another noticeable difference to the results on wheat straw from Ambye-Jensen et al. [1] is a significant
and generally lower xylose conversion, which for grass is nearly half of that of wheat straw. An
explanation is that differences between grass- and wheat straw- hemicellulose and its cross-linkages to
lignin, could have a strong influence on the enzymatic hydrolysis. Even though both biomasses are grasses (Poaceae) and have hemicellulosic structure that includes glucuronoarabinoxylan, xyloglucan, and mixed linkage glucan, there are also large differences in terms of relative amounts, degree of branching, and cross-linkages [28].

Temperate grasses are e.g. known to have a significant high degree of ferulate-arabinoxylan cross-links, which have shown to be a limiting factor for plant cell wall digestion in ruminants [29, 30].

It was not within the scope of this study to make detailed structure analysis of the grass however such a study could help to identify the reason for the significant difference between combined ensiling and HTT of grass and of wheat straw. A detailed characterization of temperate grass hemicelluloses were studied by Xu et al. [30].

**Total sugars available after pretreatment**

To summarize on the overall effect of ensiling prior to HTT, all released sugars derived from the solid and liquid fraction have been added (Figure 4). The sugars in liquid fraction includes mono- and oligosaccharides from both the HTT liquid and the wash water. This entail that washing is carried out, in order to get the amount of sugars shown for liquid fraction in (Figure 4). The sugars are divided into C6 and C5 sugars in order to evaluate the result in relation to ethanol fermentation, which is often distinguished by solely C6 fermentation or both C6 and C5 fermentation.

The results on C6 clearly shows that ensiling of grass prior to HTT does not give rise to higher amounts of total C6 sugars (Figure 3A). The improved effect of ensiling on enzymatic conversion of cellulose for HTT at 170°C and 180°C, does not make up for the loss of glucose and fructose associated with the ensiling. However at 180°C it is no more than 1.8(w/w)% C6 sugars that separate grass and grass silage, whereas at 190°C the difference is 7.1(w/w)%.
The total release of C5 sugars are however higher for silage grass both for HTT 170°C and 180°C (Figure 3B). This is due to the increased solubilisation of hemicellulose, but also a slightly better enzymatic conversion of xylose from the liquid fraction in the case of HTT 170°C grass silage. The release of C5 sugars are again highest for grass pretreated at 190°C.

Overall the results show that at the lower temperatures of 170°C and 180°C the total amount of released sugars are exactly the same between grass and silage grass (Table 6). What is lost in C6 sugars during ensiling is gained in C5 (Figure 4A and 4B). If silage grass were to be used for bioethanol fermentation this suggests to include fermentation of both C6 and C5. However, as it was shown in this study, efficient enzymatic hydrolysis required washing of the solid fraction to avoid heavy inhibition of cellulases. This imply that there are great difficulties in terms of inhibition connected to simultaneously saccharification and fermentation of both solid and liquid fraction. Furthermore C5 fermenting yeasts are known to have generally lower inhibitor tolerance than the traditional commercial Saccharomyces cerevisiae [27].

Nevertheless, by far the highest overall released sugars were found for grass pretreated at 190°C. Here ensiling did not improve the enzymatic hydrolysis and furthermore also caused significant degradation of hemicellulose during HTT.

Figure 4: Released sugars after pretreatment and enzymatic hydrolysis of grass and grass silage.

TABLE 6. Total released sugars after pretreatment and enzymatic hydrolysis.

Further studies including simultaneous saccharification and fermentation of the pretreated biomasses should be conducted in order to investigate the actual effect of inhibition from liquid fraction and oligomers on the pretreated fibers. Furthermore, the poor enzymatic hemicellulose conversion calls for a detailed study of the hemicellulose structure in pretreated grass and identification of structures which could be responsible for the inefficient enzymatic conversion.
Conclusion

Ensiling of grass prior to HTT resulted in a higher severity of the HTT which caused increased solubilisation and higher concentration of cellulose in the solid fraction compared to HTT of grass alone. This can be beneficial to an enzymatic liquefaction- and ethanol fermentation process in cellulosic ethanol production.

Secondly, ensiling of grass gave rise to an improvement in enzymatic saccharification of both cellulose and hemicellulose at lower HTT temperatures of 170 and 180°C. The improvement were however significantly lower than previously found for ensiling of wheat straw. This is believed to be due to a pure hydrolysis of the grass hemicellulose and emphasize the huge impact of structural differences on biomass processing.

HTT of grass and grass silage gave rise to profound inhibition in the enzymatic hydrolysis, and washing of pretreated fibers are necessary.

Loss of WSC during ensiling is however a large drawback for ensiling as effective pretreatment method, and the improvement in pretreatment effect were at all HTT temperatures merely equal- or less than the loss of WSC. The overall sugar yield was best for grass pretreated at 190°C.

The results in this study points out that ensiling of green crops comes with a cost in WSC and do not necessarily return the investment in terms of better pretreatment. On the other hand ensiling poses considerable advantages as storage method, and can also potentially benefit downstream processing.

Materials and Methods

Raw material

The grass used in the study was *Festulolium* Hykor, which is a crossbreed of the temperate grasses tall fescue (*Festuca arundinacea*) and italian ryegrass (*Lolium multiflorum*) developed by DLF TRIFOLIUM,
Denmark, for high yield potential (18 tonne/ha) and high persistency throughout the season. *Festulolium* Hykor was harvested 31.05.2012 (1st cut) from a DLF TRIFOLIUM demo plot, sized 1.5x8m and located in southern Zealand, Denmark (55° 20’ N, 12° 23’ E), with a HALDRUP F-55 harvester (Inotec Engineering GmbH). The grass was collected right after harvest and stored in a freezer until use. When thawed the grass was cut manually to 10-15 cm pieces. The chopped grass had a DM of 26%. The grass was split into two portions, one was air-dried at room temperature, and the other was ensiled.

**The process**

Combined ensiling and HTT pretreatment was tested against enzymatic conversion of glucose and xylose. Thus, enzymatic hydrolysis was performed on hydrothermally pretreated ensiled grass and compared to dried grass, grass silage, and hydrothermally pretreated grass.

**Ensiling:** The commercially available ensiling inoculum LACTISIL Grass Plus (Chr. Hansen, Hørsholm, Denmark) that consists of freeze dried pure homofermentative *Pediococcus pentosaceus* and *Lactobacillus plantarum* was applied. A suspension of 0.2 g L\(^{-1}\) water was prepared and added in the amount of 40 mL kg\(^{-1}\) WS to reach an initial inoculum size of 8 mg kg\(^{-1}\), the double amount of what was used in [17] where the amount was concluded to be ineffectual. 5.8 kg of chopped grass was packed in two layers of thin black PET plastic bags and one layer of thick transparent PET plastic. Anaerobic conditions were achieved by removing the air from the plastic bag using an industrial vacuum cleaner. The plastic bags were opened after 4 weeks. Weight loss was measured to be 0.6% and used for calculation of DM mass balances.

**Hydrothermal pretreatment:** Hydrothermal pretreatments (HTT) were carried out in the “Mini IBUS” equipment (Technical University of Denmark, Risø campus). 1 kg DM (corrected for volatile fatty acid) of the grass silage was treated at varied temperatures (170, 180 and 190°C) for 10 min. After HTT, the pretreatment reactor was cooled to below 70°C, and the material was separated by pressing. Weight and DM was measured for each solid and liquid fraction and used for mass balance calculations. Solid and liquid fractions were kept in the freezer until further analysis.
Sample preparation for enzymatic hydrolysis: Enzymatic hydrolysis was done with three different sample preparations on HTT pretreated fibers. (I) using dried and milled (2mm) fiber, (II) using washed, dried, and milled (2mm) fiber, and (III) using washed, wet, and cut (10-5mm) fiber. This was done to examine the effect of removing potential inhibitors from the fiber after HTT drying, and to test the influence of drying, which is typically done during sample preparation in laboratory tests.

Enzymatic hydrolysis: The enzymatic hydrolysis was performed at 2.0% DM (w/v) in a total volume of 20ml using 50mM citrate buffer at pH 5.0 with 0.4 % w/w sodium azide. Commercially available cellulolytic and hemicellulolytic enzyme preparations, Cellic®CTec2 and Cellic®HTec2, from Novozymes A/S (Bagsværd, Denmark) were used in a 9/1 ratio and added at 10 % enzyme/substrate (w/w cellulose). Cellic®CTec2 is a commercial cellulose preparation based on the cellulose complex produced by *Trichoderma reesei* containing at least the two main cellbiohydrolases EC 3.2.1.91 (Cel6A and Cel7A), five different endo-1,4β-glucanases EC 3.2.1.4 (Cel7B, Cel5A, Cel12A, Cel61A, and Cel45A), β-glucosidase EC 3.2.1.21, β-xylosidase EC 3.2.1.37, and GH61 [31] in addition to particular proprietary hydrolysis-boosting proteins. Cellic®HTec2 mainly contains endo-1,4β-xylenase activity EC 3.2.1.8, but also contains cellulase activity. The enzymatic hydrolysis was performed during shaking for 72 h at 50 °C, in duplicates and enzyme blanks were included. Hydrolysates were analysed for glucose and xylose levels by HPLC and the yield is presented per DM fiber in the hydrolysis. Cellulose and xylan convertibility were calculated as the converted cellulose or xylan divided by the original cellulose or xylan content in the grass raw material.

Chemical analysis

Grass, grass silage, hydrothermally pretreated grass, and hydrothermally pretreated grass silage were analyzed for chemical composition by methods based on standard laboratory analytical procedures developed by National Renewable Energy Laboratory (NREL), US [32]. Deviations from these standard procedures are stated in the following sections. The analysis of the solid fiber fraction included ash
content determination, water extraction (only on grass and grass silage), ethanol extraction, and strong acid hydrolysis for structural carbohydrates and lignin. The liquid fraction after HTT and the wash-water from the washing of solid fractions prior to enzymatic hydrolysis, was analyzed directly by HPLC and after weak acid hydrolysis to determining oligosaccharide concentration.

**DM determination:** DM and ash analyses were performed according to NREL standard laboratory analytical procedures based on oven dry matter measurements [33]. Since silage biomass contains large amounts of volatile compounds, it is critical to correct the measured oven-DM (at 105°C) for loss of volatiles, to obtain the true DM. The measurements were therefore corrected using concentrations of volatiles and coefficients according to [34].

**Analytical method:** Concentrations of carbohydrates (D-glucose, D-xylose, L-arabinose, L-rhamnose, D-galactose, D-mannose and D-fructose) were quantified by High Pressure Liquid Chromatography (HPLC) (Shimadzu Corp., Kyoto, Japan) using a HPX-87P column (BioRad) (Hercules, CA; USA) and refractive index (RI) detection, at 80°C using water as eluent, 0.5 ml/min. Organic acids (lactic-, formic-, acetic-, propionic, and butyric acid) were quantified by HPLC using a Biorad HPX-87H column (Hercules, CA; USA), RI detection, 63°C and 4 mM H₂SO₄ as eluent, 0.6 ml/min.

**Water extraction:** 0.3-0.4 g DM biomass from freshly disrupted silage bags was extracted in 10 ml MilliQ H₂O with 10 μl of the antibiotic ampicillin (10mg/ml solution) to prevent microbial activity during extraction. The extraction samples were shaken for 2 hours at 25°C and 150 rpm. Extracts were analyzed for sugars and acids by HPLC as described above. The amount of water extractives, was defined as the mass of material lost through extraction.

**Weak acid hydrolysis:** The liquid fraction of HTT and the wash-water from the washing of solid fractions prior to enzymatic hydrolysis, was analyzed by weak acid hydrolysis to quantify the content of soluble oligomer carbohydrates. 10 ml HTT liquid fraction were autoclaved for 10 minutes at 121°C with 4 w/w % H₂SO₄. Derived sugars were analyzed by HPLC as described above.
**Ethanol extraction:** Lipophilic extraction was carried out by Soxhlet extraction in a reflux condenser for six hours with 99 w/w% ethanol on water extracted samples of grass and grass silage. The amount of ethanol extractives, including volatiles, was defined as the mass of material lost through extraction.

**Determination of structural carbohydrates and lignin:** Strong acid hydrolysis was used to measure the carbohydrate and lignin content of the extracted bio residue, based on the NREL standard laboratory analytical procedure [32].

**Abbreviations**

DM: Dry matter; HTT: Hydrothermal treatment; WSC: Water soluble carbohydrates; LAB: Lactic acid bacteria; HPLC: High-performance liquid chromatography;

**Competing interests**

The authors declare that they have no competing interests.

**Authors’ contributions**

All authors participated in the design of the study. All authors read and approved the final manuscript.

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**References**


FIGURE 1. Recovery of hemicellulose.

Percent recovered hemicellulose (xylan, arabinan, galactan) in solid and liquid fraction after HTT out of total hemicellulose in raw biomass.
FIGURE 2. Glucose and xylose yields after enzymatic hydrolysis of the HTT solid fractions.

Yields are in weight percentages of DM in the HTT solid fraction. (A) were done on dried solid fractions and (B) were done on washed, wet solid fractions.
FIGURE 3: Released sugars after pretreatment and enzymatic hydrolysis of grass and grass silage.

(A) Total C6 sugars (glucose, galactose and fructose) in (w/w)% of DM raw grass (before pretreatment). (B) Total C5 sugars (xylose and arabinose) in (w/w)% of DM raw grass (before pretreatment). Liquid fraction include also washing liquid.
FIGURE 4: Released sugars after pretreatment and enzymatic hydrolysis of grass and grass silage.

(A) Total released C6, mono- and oligomers (glucose, galactose and fructose) in weight percentages of DM raw grass

(B) Total released C5, mono- and oligomers (xylose and arabinose) in weight percentages of DM raw grass in (w/w)% of DM raw grass. Liquid fraction include sugars from liquid fractions and sugars in wash water of solid fractions.
Tables

TABLE 1. Composition of grass and grass silage before HTT.

*Numbers are presented as weight percentages of biomass DM, followed by standard deviations (smaller font size).*

<table>
<thead>
<tr>
<th>Biomass</th>
<th>Grass (w/w)% of DM</th>
<th>Silage (w/w)% of DM</th>
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TABLE 2. Water soluble carbohydrates (WSC) and organic acids analyzed in H₂O extract of grass and grass silage.

*Numbers are presented as weight percentages of biomass DM, followed by standard deviations (smaller font size).*

<table>
<thead>
<tr>
<th>Biomass</th>
<th>Grass (w/w)% of DM</th>
<th>Silage (w/w)% of DM</th>
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<td>Glucose</td>
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<td>Lactic acid</td>
<td>0.10</td>
<td>6.53</td>
</tr>
<tr>
<td>Formic acid</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>0.19</td>
<td>1.73</td>
</tr>
<tr>
<td>Propionic acid</td>
<td>0.01</td>
<td>0.04</td>
</tr>
<tr>
<td>Total organic acid</td>
<td>0.30</td>
<td>8.30</td>
</tr>
</tbody>
</table>
### TABLE 3. Composition of solid fraction after HTT.

*Numbers are presented as weight percentages of DM in the HTT solid fraction, followed by standard deviations (smaller font size), except for ‘DM recovery in solid fraction’ in bold, which is the percentage of DM left in solid fraction after HTT pretreatment, based on mass balance over HTT pretreatment.*

<table>
<thead>
<tr>
<th>Biomass</th>
<th>Grass</th>
<th>Grass silage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>HTT temperature</strong></td>
<td><strong>170</strong></td>
<td><strong>180</strong></td>
</tr>
<tr>
<td></td>
<td>(w/w)% of DM</td>
<td>(w/w)% of DM</td>
</tr>
<tr>
<td>Glucan</td>
<td>31.2 0.45</td>
<td>33.0 0.16</td>
</tr>
<tr>
<td>Xylan</td>
<td>18.7 0.40</td>
<td>17.1 0.05</td>
</tr>
<tr>
<td>Arabinan</td>
<td>2.7 0.01</td>
<td>1.8 0.07</td>
</tr>
<tr>
<td>KIason</td>
<td>18.8 0.59</td>
<td>19.0 0.67</td>
</tr>
<tr>
<td>Ash</td>
<td>7.4 0.52</td>
<td>8.2 0.18</td>
</tr>
<tr>
<td>Ethanol Extractives</td>
<td>11.8 0.35</td>
<td>16.4 0.49</td>
</tr>
<tr>
<td>Residual</td>
<td>9.5</td>
<td>4.4</td>
</tr>
<tr>
<td><strong>DM recovery in solid fraction</strong></td>
<td><strong>75.3</strong></td>
<td><strong>69.6</strong></td>
</tr>
</tbody>
</table>

### TABLE 4. Sugars and organic acids in the HTT liquid fraction.

*Numbers are presented as weight percentages of solubilized DM, ‘Solubilized DM’ in bold italic, is the percentage of DM solubilized during HTT pretreatment, based on mass balance over HTT pretreatment.*

<table>
<thead>
<tr>
<th>Biomass</th>
<th>Grass</th>
<th>Silage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>HTT temperature</strong></td>
<td><strong>170</strong></td>
<td><strong>180</strong></td>
</tr>
<tr>
<td></td>
<td>(w/w)% of DM</td>
<td>(w/w)% of DM</td>
</tr>
<tr>
<td>Glucose</td>
<td>9.7</td>
<td>10.5</td>
</tr>
<tr>
<td>Xylose</td>
<td>3.8</td>
<td>8.4</td>
</tr>
<tr>
<td>Galactose</td>
<td>1.4</td>
<td>2.1</td>
</tr>
<tr>
<td>Arabinose</td>
<td>2.1</td>
<td>3.1</td>
</tr>
<tr>
<td>Fructose</td>
<td>3.0</td>
<td>2.9</td>
</tr>
<tr>
<td><strong>Total sugars</strong></td>
<td><strong>20.0</strong></td>
<td><strong>27.1</strong></td>
</tr>
<tr>
<td>Lactic acid</td>
<td>0.10</td>
<td>0.39</td>
</tr>
<tr>
<td>Formic acid</td>
<td>0.83</td>
<td>0.98</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>1.24</td>
<td>1.81</td>
</tr>
<tr>
<td>Malic acid</td>
<td>3.15</td>
<td>3.64</td>
</tr>
<tr>
<td>Propionic acid</td>
<td>0.21</td>
<td>0.27</td>
</tr>
<tr>
<td><strong>Total organic acids</strong></td>
<td><strong>5.5</strong></td>
<td><strong>7.1</strong></td>
</tr>
<tr>
<td><strong>Solubilized DM</strong></td>
<td><strong>24.7</strong></td>
<td><strong>30.4</strong></td>
</tr>
</tbody>
</table>
TABLE 5. Total mono- and oligo-saccharides and organic acids in wash water of HTT solid fraction.

*Numbers are presented as weight percentages of DM in the HTT solid fraction, followed by standard deviations (smaller font size)*

<table>
<thead>
<tr>
<th>Biomass</th>
<th>Grass</th>
<th>Grass silage</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>HTT temperature</td>
<td>170 (w/w)% of DM</td>
<td>180 (w/w)% of DM</td>
<td>190 (w/w)% of DM</td>
<td>170 (w/w)% of DM</td>
<td>180 (w/w)% of DM</td>
<td>190 (w/w)% of DM</td>
</tr>
<tr>
<td>Monosaccharides</td>
<td>6.81 ± 0.07</td>
<td>4.13 ± 0.20</td>
<td>3.19 ± 0.19</td>
<td>1.10 ± 0.07</td>
<td>1.28 ± 0.04</td>
<td>1.53 ± 0.01</td>
</tr>
<tr>
<td>Oligosaccharides</td>
<td>5.01 ± 0.43</td>
<td>11.47 ± 0.28</td>
<td>18.20 ± 0.45</td>
<td>5.73 ± 0.45</td>
<td>10.25 ± 0.41</td>
<td>13.46 ± 0.21</td>
</tr>
<tr>
<td>C5:C6 ratio of oligomers</td>
<td>na.</td>
<td>1.4 ± 0.1</td>
<td>3.6</td>
<td>3.1 ± 0.14</td>
<td>4.3 ± 0.2</td>
<td>6.4 ± 0.1</td>
</tr>
<tr>
<td>Furans</td>
<td>0.11 ± 0.00</td>
<td>0.24 ± 0.01</td>
<td>0.46 ± 0.03</td>
<td>0.07 ± 0.00</td>
<td>0.12 ± 0.00</td>
<td>0.21 ± 0.00</td>
</tr>
</tbody>
</table>

TABLE 6. Total released sugars after pretreatment and enzymatic hydrolysis.

*Numbers are presented as weight percentages of DM in raw grass, followed by standard deviations (smaller font size)*

<table>
<thead>
<tr>
<th>Biomass</th>
<th>Grass</th>
<th>Grass silage</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>HTT temperature</td>
<td>170 (w/w)% of DM</td>
<td>180 (w/w)% of DM</td>
<td>190 (w/w)% of DM</td>
<td>170 (w/w)% of DM</td>
<td>180 (w/w)% of DM</td>
<td>190 (w/w)% of DM</td>
</tr>
<tr>
<td>Total released sugars</td>
<td>29.5 ± 0.78</td>
<td>39.2 ± 0.61</td>
<td>53.3 ± 0.62</td>
<td>29.6 ± 1.75</td>
<td>39.8 ± 1.03</td>
<td>40.5 ± 0.72</td>
</tr>
</tbody>
</table>