Enzymatic hydrolysis of corn bran arabinoxylan
- theory versus practice

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Enzymatic hydrolysis of corn bran arabinoxylan - theory versus practice

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Preface

The work presented here has been conducted during my time as a PhD student at Department of Chemical and Biochemical Engineering in the group Bioprocess Engineering at the Technical University of Denmark, from March 2007 and until March 2011. The work has been carefully supervised by Professor Anne S. Meyer to whom I am eternally thankful for her ongoing encouragement and enthusiasm about my work. This thesis would never have been a reality without her and her neverending belief in me.

DTU has provided financing for this project and I am again proud to graduate from this world renowned institution that offers grand opportunities to young scientists like my self.

Novozymes A/S has also participated in this project with financing, co-supervision and by supplying relevant enzyme activities for my work. During the project I have been co-supervised by Hanne Risbjerg Sørensen, Anders Viksø-Nielsen and Katja Salomon Johansen and I thank everybody for participating in the work. Finally, FOOD Denmark Research School (LMC) has also provided funding for the project and I appreciate the opportunity that this collaboration has given me to carry out this project.

During my project certain people stand out as extra helpful and these include Ove Nielsen from Novozymes A/S for assisting my in destarching and handling of a large batch of corn bran that provided material enough for my entire PhD project. From DTU Risø, National Laboratory for Sustainable Energy, Tomas Fernqvist who has assisted me in pretreating corn bran in a special loop autoclave and thereby providing material for several important studies during the work. Also Associate Professor Kristian Fog Nielsen from DTU Systems Biology who has performed LC-MS analysis. Last but not least Andrea Lorentzen and Karin Hjernø from Department of Biochemistry and Molecular Biology, University of Southern Denmark who have been of tremendous help by performing MALDI-TOF analysis in a timely and accurate manner. I am truly grateful for everybodies contribution to my final thesis.

And a special thank you to my fellow PhD companions and other colleagues here in the group. It has been a pleasure and a privilege to work with you all.

Finally I would like to thank my family, my dear husband Claus for moral and intellectual support, practical and technical skills and my little Mads for always putting work-related issues in perspective.

Jane Agger
Copenhagen, March 2011

"It is worth remembering that it is human nature to group and classify things to facilitate their comprehension and discourse, whereas Mother Nature simply constructs biological entities,., using material at hand with blatant, pedagogical disregard" (Showalter, 2001)
Summary

This thesis concerns enzymatic hydrolysis of corn bran arabinoxylan. The work has focused on understanding the composition and structure of corn bran with specific interest in arabinoxylan with the main purpose of targeting enzymatic hydrolysis for increased yields.

Corn bran has been used as a model substrate because it represents a readily available agroindustrial side product with upgrading potentials. Corn bran originates from the wet-milling process in corn starch processing, is the outmost layers of the corn kernel and is particularly rich in pentose monosaccharides comprising the major components of arabinoxylan. Corn bran is one of the most recalcitrant cereal byproducts with arabinoxylans of particular heterogeneous nature. It is also rich in feruloyl derived substitutions, which are responsible for extensive cross-linking between arabinoxylan molecules and thereby participate in a complex and rigid cell wall structure.

This thesis contains a thorough examination of the monosaccharide and structural composition of corn bran, which is used to assess and apply the relevant monocomponent enzyme preparations. In this way, the aim is to obtain the most effective minimal enzymatic requirements for hydrolyzing corn bran.

The offset of the work has been a basic set of four hemicellulases consisting of an endo-β-1,4-xylanase (GH10 from H. insolens), a β-xylosidase (GH3 from T. reesei) and two α-L-arabinofuranosidases (GH43 and GH51 from H. insolens and M. giganteus respectively). This set of enzymes have proven efficient in degrading arabinoxylan structures from wheat arabinoxylan and it is also verified in this study that it probably is among the best available hemicellulases for increasing the hydrolysis of corn bran arabinoxylan at present. This set of enzymes creates a solid starting point for hydrolysis of the arabinoxylan structure but is not alone capable of catalyzing complete hydrolysis. Auxiliary enzyme activities that catalyse the hydrolysis of various substitutions are also necessary and several of such enzymes are investigated. This results in the identification of a suitable feruloyl esterase from A. niger (FAE-III) for catalyzing the release of free ferulic acid and diferulic acids to a certain extent. Furthermore, a novel acetyl xylan esterase from Flavolaschia sp. is also found to be important for obtaining higher release of xylose from the arabinoxylan structure. Structural analysis of a soluble fraction of corn bran also confirms the presence of highly acetylated pento-oligosaccharides. All these enzymes together with a commercial cellulase preparation (Cellic™ CTec) are capable of catalyzing the release of up to 36% xylose from a soluble fraction of hydrothermally pretreated corn bran.

Yet enzymatic hydrolysis of corn bran is far from complete and in order to improve the yields, this thesis has thoroughly investigated the need and impact of different pretreatment conditions. Corn bran is a special substrate when it comes to pretreatment conditions because the biomass is mainly composed by heat, acid and alkali labile linkages in arabinoxylan. It therefore becomes a balancing task to find optimum conditions that compromise the advantages and disadvantages. Acidic pretreatments (pH 1.5-2) are found to be particularly effective in promoting the enzymatic hydrolysis, especially with respect to xylose and glucose release, but vast amounts of the valuable monosaccharides are lost during this pretreatment and this is especially evident for arabinose. From a scientific point of view acid catalysed pretreatment...
renders the substrate in a state of disruption where assessment of correct enzyme administration becomes difficult and enzymatic hydrolysis becomes a secondary route to disintegration.

Alkaline pretreatments are less efficient in promoting the enzymatic hydrolysis, but still serve an academic purpose because those conditions chemically remove diferulate cross-linkings between arabinoxylans, which have been believed to be a major obstacle for enzymatic hydrolysis. The chemical removal of these cross-links allows for the interpretation of hindering effects of cross-linking and it is concluded that they do not pose a significant barrier for enzymatic hydrolysis. By this conclusion a major hypothesis of this thesis is rejected.

Because chemically catalysed pretreatments has obvious disadvantages, milder mechanical pretreatments has also be investigated and results show that decreasing the particle size of the insoluble substrate renders it more accessible to enzymatic hydrolysis. The hydrolysis improves with a factor of 3-8 for xylose, arabinose and glucose when comparing the yields in the largest particle size fraction to the yields in the smallest size fraction for native destarched corn bran. This is related to an increased substrate surface area, but it is also observed that different particle size fractions from corn bran are not uniformly composed. The content of monosaccharides varies and results in differences in content and composition of cellulose and arabinoxylan. These differences in biomass composition may very well also be part of the explanation why increased enzymatic hydrolysis is obtained. To further investigate the influence of particle size and other physical parameters on enzymatic hydrolysis, theoretic estimations of how changing particle size influences the enzymatic hydrolysis is made. These estimations point to the observation that other factors than particle size alone governs the enzymatic hydrolysis. It is observed that enzymatic hydrolysis is promoted in certain particle size fractions and inhibited in others. This is likely to be related to the biomass composition.

Corn bran is a recalcitrant substrate and complete hydrolysis is not achieved in this thesis. Instead explanations as to what causes the recalcitrance are sought and it most likely lies within a combination of factors. Firstly, corn bran has an exceptional rigid and tight exterior that leaves it virtually impenetrable to enzymes. Disruption of this outside structure is important if the hydrolysis is at all to commence. In that sense it is important to obtain a higher understanding of the cell wall matrix, the packing of polysaccharides and how they interact with other polymeric structures in the cell wall, eg proteins and lignin. Especially proteins associated with the cell wall may play a significant role in maintaining cell wall strength and preventing enzymatic hydrolysis. Secondly, the heterogeneous nature of arabinoxylan from corn bran makes it difficult even for the correct enzymes to catalyse complete hydrolysis as observed for hydrolysis in a soluble corn bran fraction. Once the arabinoxylan structure is free of the cell wall matrix the hydrolysis seem to be restricted due to steric hindrance or lack of additional enzymes to catalyse the hydrolysis of certain unusual bonds. In particular, it is of outmost importance to target arabinosyl substitutions of arabinoxylan and other possible configurations of arabinose, as this in particular may hold part of the reason for corn bran recalcitrance. Generally, increased arabinose release will most likely also lead to increase in the overall release of xylose. Obstructions by heterogeneous arabinoxylan may be
overcome by completing the knowledge about corn bran arabinoxylan, which can then lead to the identification of missing, central enzyme activities, and thereby also make the work on corn bran generic.

The thesis is based upon the scientific publications produced during the last four years and they represent the development and achievements of this work. To ease the reading the thesis will highlight some of the findings and interpretations from the publications, but also from unpublished work and thereby establish the mindset and progress behind the project.
Dansk sammenfatning

Denne afhandling handler om enzymatisk hydrolyse af majsklid. Projektet har været koncentreret om at forstå kompositionen og strukturen af majsklid, specielt med hensyn til arabinoxylan for med det hovedformål at målrette den enzymatiske hydrolyse mod forhøjede udbytter.

Majsklid har været anvendt som et modelsubstrat, fordi det repræsenterer et direkte tilgængeligt biprodukt fra den agroindustrielle sektor, der endnu ikke er udnyttet til fulde. Majsklid kommer typisk fra våd-formalingsprocessen i majsklidproduktionen, det består af de allerderste lag, som omgiver majskernen og er særlig rigt på pentose monosakkarider, som også udgør hovedparten af arabinoxylan.

Majsklid er et af de mest sværtnedbrydelige cereale biprodukter, hvor arabinoxylanen besidder en særlig heterogen karakter. Det er også rigt på feruloyl-afledte substitueringer, som tildels er ansvarlige for en omfattende krydsbinding mellem arabinoxylanmolekyler, og dermed bidrager til en kompleks og stærk cellévægsstruktur.

Denne afhandling indeholder en grundig gennemgang af monosakkarid- og struktorkompositionen af majsklid med henblik på at vurdere og anvende de relevante mono-komponente enzympræparater. På den måde er det målet at opnå den mest effektive minimale tilsætning af enzymer for at hydrolysere majsklid.


Strukturanalyse af en opløselig fraktion af majsklid fastslår også tilstedeværelsen af højt acetylerede pentose-oligosakkarider. Alle disse enzymer er sammen med et kommercielt cellulasepræparat i stand til at katalysere frigivelsen af op til 36 % xylose fra en opløselig majsklidfraktion efter en hydrotermisk forbehandling.

Men den enzymatiske hydrolyse er endnu langt fra fuldstændig og for at forbedre udbytterne har dette projekt også involveret en grundig undersøgelse for behovet af, og betydningen af forskellige forbehandlingsmetoder. Majsklid er et særligt substrat i forhold til forbehandling, fordi biomassen generelt består af mange varme-, syre- og baselabile bindinger i arabinoxylan. Det er derfor en balancegang for at finde de optimale betingelser for forbehandling, der kan tilgodesse både fordele og ulemper ved en given metode. Sure forbehandlinger (pH 1.5-2) viser sig at være særligt fremmende for
den enzymatiske hydrolyse af specielt xylose og glucose, men det er på bekostning af store mængder monosakkarider, der går tabt, og dette er særlig udtalt for arabinose. Fra et videnskabeligt synspunkt efterlader den syrekatalyserede forbehandling substratet i en forfatning af ødelagte strukturer, hvor det er svært at vurdere den rigtige enzymtilsætning, eftersom substratet ikke længere ligner sig selv, og hvor enzymatisk hydrolyse bliver en sekundær vej til disintegrering.

Basiske forbehandlinger er mindre effektive i at fremme den enzymatiske hydrolyse, men bidrager dog alligevel med et vigtigt videnskabeligt budskab. Den kemiske hydrolyse der her foregår, fjerner diferulatkrydsbindinger mellem arabinoxylanmolekyler, som har været anslået til at være en stor forhindring for enzymatisk hydrolyse. Den kemiske fjernelse af disse krydsbindinger muliggør en fortolkning af betydningen af disse hindringer, og det kan konkluderes, at de ikke udgør en signifikant barriere for den enzymatiske hydrolyse. Med denne konklusion er det muligt at afvise hypothese 1 i denne afhandling.

Fordi kemisk katalyserede forbehandlinger har åbenbare ulemper, er mildere mekaniske metoder anvendt og undersøgt og resultatene viser, at formindskelse af partikelstørrelsen af det uopløselige substrat gør det mere tilgængeligt for enzymhydrolyse. Hydrolysen forbedres med en faktor 3-8 for xylose, arabinose og glucose, når der sammenlignes udbytter fra den største og den mindste partikelstørrelse af det native stivelsesfjernede majsklid. Dette skyldes et forstørret overfladeareal af substratpartiklerne, men det blev imidlertid også observeret, at forskellige partikelstørrelsesfraktioner af majsklid ikke havde samme komposition. Indholdet af monosakkarider varierer og resulterer dermed i forskelligheder i indhold og komposition af både cellulose og arabinoxylan. Disse forskelle i biomassekomposition kan meget vel være en del af forklaringen på, hvorfor udbyttet af den enzymatiske hydrolyse stiger. For nærmere at undersøge hvordan partikelstørrelsen og andre fysiske faktorer influerer enzymhydrolysen, er der foretaget teoretiske estimator af, hvordan ændringer i partikelstørrelse kan påvirke enzymhydrolysen. Disse estimator peger i retning af, at også andre faktorer end partikelstørrelsen alene har betydning for den enzymatiske hydrolyse. Øjensynligt er enzymhydrolysen fremmet i visse partikelstørrelser, men inhiberet i andre. Dette er formentlig relateret til biomassemængden.

Majsklid er et sværtnedbrydeligt substrat og fuldstændig hydrolyse er ikke blevet opnået i dette projekt. I stedet har jeg afsøgt forklaringer på, hvorfor majsklid er så svært nedbrydeligt og det skyldes formentlig en kombination af faktorer. For det første har majsklid et ekstremt hårdt og tæt ydre, som er nærmest uigennemtrængeligt for enzymerne. Ødelæggelse af denne udvendige struktur er nødvendig, hvis hydrolysen overhovedet skal kunne begynde. I den sammenhæng er det derfor nødvendigt at opnå en større forståelse for cellevægsmatricen, arrangementen af polysakkarider og hvordan disse interagerer med andre polymeriske strukturer som f.eks. proteiner og lignin. Specielt proteiner associeret med cellevæggen kan spille en væsentlig rolle i opretholdelsen af cellevæggenes styrke og dermed forebygge enzymatisk hydrolyse. For det andet, den heterogene struktur af arabinoxylan fra majsklid gør det svært selv for de rigtige enzymer at katalysere en fuldstændig hydrolyse, som det er blevet observeret for

Denne afhandling er baseret på de videnskabelige publikationer, som er produceret i løbet af de sidste fire år, og de repræsenterer udviklingen og resultaterne i dette projekt. For at lette læsningen vil afhandlingen fremhæve de vigtigste resultater og fortolkninger i disse publikationer, men også inddrage upUBLISERede data og hermed etablere det tankesæt og den udvikling, som ligger til grund for projektet.
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1 Hypothesis and aims

The overall aim of this project has been to understand and control the enzymatic conversion of corn bran, a model for highly complex cereal plant residues. The enzymatic conversion is aimed at producing readily available monosaccharides for use in relevant industries and thereby upgrade and exploit the potential of using renewable resources.

*The driving philosophy in this work has been that efficient and low-cost conversion of recalcitrant, insoluble cereal plant residues, first and foremost corn bran, can be achieved through targeted enzymatic hydrolysis by designing a mixture of enzymes to selectively catalyse the hydrolysis of specific bonds in the substrate structure. Enzyme catalysed reactions are superior, because they are highly specific, environmentally friendly and have defined product yields.*

In order to achieve efficient enzymatic hydrolysis, understanding of the biomass composition, linkage structure and physical properties are prerequisites for success and in that context the following hypotheses are raised:

1. That diferulate cross-linking in the primary cell wall matrix is an intrinsic barrier for enzymatic hydrolysis of particularly arabinoxylan in corn bran. In turn, opening of these cross-links will render the arabinoxylan more susceptible to enzymatic degradation.
2. That targeting of the linkages in the versatile display of substituents on arabinoxylan from corn bran with specific enzymes will lead to enhanced overall degradation of the polysaccharide structure.
3. Solubility is an issue and increased substrate solubility affects the enzymatic hydrolysis positively. Also other physical properties specifically substrate composition and surface area governed by pretreatment determine the overall enzymatic hydrolysis of corn bran.

The objective of this thesis work has been to test these hypotheses with the overall aim of obtaining enzymatic hydrolysis of corn bran.

Corn bran primarily consists of the pericarp tissues surrounding the corn kernel and is a side product from the wet-milling process in corn starch industries. Corn bran has been used as a model substrate in this work, because the primary cell wall matrix is particularly rich in heavily substituted heteroxylan structures. Furthermore corn bran represents a cereal residue with upgrading potentials. It is a basic motivation for the work, that if desirable hydrolysis of corn bran is achieved, such methods and applications can most probably be directed towards other insoluble cereal residues with similar results, thereby making the approaches generic.
This thesis is structured around the publications resulting from this work and specifically for each publication the subsequent hypotheses are raised:

Paper 1: Enzymatic xylose release from pretreated corn bran arabinoxylan: Differential effects of deacetylation and deferuloylation on insoluble and soluble substrate fractions

a. It is hypothesized that enzymatic deacetylation of arabinoxylan will be important for the overall hydrolysis of arabinoxylan with comparable effects on the xylose release as deferuloylation.

Paper 2: pH catalysed pretreatment of corn bran for enhanced enzymatic degradation of arabinoxylan

b. It is hypothesized that an optimal pretreatment method can be achieved by investigating the influence of pretreatment pH, temperature and time on the enzymatic accessibility of corn bran.

c. It is hypothesized that chemical removal of diferulic acids prior to enzymatic hydrolysis will make it possible to evaluate whether the presence of such cross-linkings are negatively affecting the enzymatic hydrolysis of arabinoxylan.

Paper 3: Biomass composition in response to changing substrate particle size and the consequences for enzymatic hydrolysis of corn bran

d. It is hypothesized that the distribution and composition of non-starch polysaccharides in corn bran is not uniform and that these differences confound the effects of substrate surface area and in turn affect the enzymatic hydrolysis.

During the work other central questions evolves and attempts are made to address these issues. They will be attended to continuously but can generally be divided into the following two categories:

I. Arabinose: Does all arabinose in corn bran originate from arabinoxylan substitutions or can it have other origins like for instance arabinan or arabinogalactan?

II. Cell wall proteins: Is it possible that cell wall proteins obstruct the enzymatic hydrolysis of corn bran, possibly by interacting with or by hydrophobically shielding the polysaccharides? Do glycosylations on structural proteins of corn bran play a significant role in the overall contribution to the carbohydrate profile?
2 Introduction

Agricultural residues and side streams from agro-industrial production obtain increasing attention these years in the search for high quality energy resources due to the ubiquitous climate situation and unstable supply of fossil fuels.

Besides being a complex substrate, corn bran represents a cereal plant residue of particular abundance. Agricultural crop production on a world basis is versatile, but some commodities stand out as exceptionally massive by quantity and these include corn (*Zea mays* L) and others like wheat (*Triticum* spp.) and barley (*Hordeum vulgare*). In 2007 they together contributed approx. 65% of the total world production of cereals (Figure 1). Included in the category of other cereals in figure 1 is rice, which alone contributes approx. 28% of the total world production of cereals and thereby contributes the vast majority of other cereals than corn, barley and wheat.

![Figure 1: Distribution between world wide production of cereals corn, wheat, barley and others (incl. rice, sorghum, rye, oat, millets) in 2007 (FAOSTAT Yearbook 2009 and www.faostat.fao.org)](image)

The United States and China are the world’s two biggest single producers of corn with approx. 343 and 163 mio tons respectively in 2009 out of a total world production of 817 mio tons. Wheat and barley are some of the major crops in Europe and Asia with a total world wide production of 682 and 150 mio tons in 2009.

The vast amounts of cereal grains produced on a yearly basis are the foundation of several grain processing industries that generate high bulk, low value side streams beside the main products. This includes wheat bran from wheat flour and wheat starch productions, barley spent grain (brewers spent grain) from the brewery industry and in particular corn bran from corn starch processing. The major process providing corn bran is the wet-milling process in the starch industries, where the bran is screened from the main process stream after steeping of the corn kernels (Figure 2).
Today corn bran is mixed with semi-condensed steep water and sold as a livestock feed supplement often referred to as 21% gluten feed, wet or dry corn gluten feed (International Starch Institute, Aarhus University, DK). According to a single large American producer of corn starch and derived products (Archer Daniels Midland Co. US) they alone process 66 000 tons of corn every day, which on a yearly basis corresponds to approx. 7% of the total American corn production in 2009. With a cautious estimate that of this 5% by weight ends up as corn bran, approx. 1.2 mio tons of corn bran is generated per year from this one producer alone. Besides the high volume, corn bran possesses the quality of being clean and readily available, minimizing the need for logistic resources.

Corn and other cereals belong to the vascular, angiosperm, monocotyledon plants in the Poaceae family. Vascular plants are characterized by having tissues of differentiated and lignified cells for transporting water and nutrients and for maintaining mechanical strength. All vascular plant cell walls have comparable basic structures with a fibrous matrix of cellulose microfibrils embedded in non-cellulosic polysaccharides of varying composition (Harris, 2005, Carpita and Gibeaut, 1993). Cell walls are commonly divided into two types, the primary and the secondary cell wall. The primary walls are deposited during cell elongation, while the secondary cell walls are deposited inwards on the primary
walls when cell growth ceases, either as lignified or as non-lignified cells. Lignification of the secondary wall brings rigidity and strength to cells, but will also prevent further growth. Non-lignified secondary walls include thickened parenchyma walls surrounding the endosperm in grains (Harris 2005). The primary cell walls can be further subdivided into type I and type II walls (Carpita and Gibeau, 1993), and the occurrence and composition of non-cellulosic polysaccharide of both types depends greatly upon species. Type I primary walls are the more common type within the flowering plants, whereas type II are almost exclusively found in monocot Poaceae (Carpita and Gibeau, 1993).
3  Composition and enzymatic hydrolysis of corn bran

Corn bran consists primarily of the aleurone and pericarp layers of the corn kernel and possibly also parts of the tip cap. Due to starch process imperfections a residual amount of starch granules from the endosperm is also common. The residual starch can contribute up to 20-25% of the dry matter of the raw corn bran after wet-milling (Saha and Bothast, 1999). Corn bran has relatively low lignin content and a high content of arabinoxylan (Saha et al., 1998, Lapierre et al., 2001, Agger et al., 2010 Paper 1) compared to other parts of the corn plant and it is therefore mainly composed of type II primary cell walls, possibly in part containing secondary non-lignified cell walls. Besides residual starch, endosperm associated proteins may also be present in the industrial corn bran fraction (Agger et al., 2010 Paper 1).

![Figure 3: Sketched drawing of possible arabinoxylan structure from corn bran. Red bonds indicate β-linkages, green bonds α-linkages and blue bonds indicate ester linkages.](image)

The major non-cellulosic polysaccharides in type II cell walls are heteroxylans, mainly in the form of arabinoxylan (AX). Arabinoxylan may be further substituted with α-D-glucuronyl (or its methylated form) at the O-2 position on xylose moieties and is therefore often referred to as glucuronoarabinoxylan (GAX) (Kabel et al., 2002). A stylized drawing of arabinoxylan is seen in figure 3. Arabinoxylan from corn bran is known to be exceptionally complex in structure, but the basic structure is a β-1,4-linked D-xylopyranosyl backbone with terminal α-L-arabinofuranosyl substituents either linked to the O-2 or O-3 position of xylosyl units or to both (figure 3). The degree of arabinoferanosyl substitution is generally high in corn arabinoxylan lying in the range of 0.6-0.7 (Schell et al., 2004, Grohmann and Bothast 1997, Saulnier et al., 1995b+2001, Saha et al., 1998) compared to other cereals like wheat or barley, where the A:X ratios lie in the range of 0.5 to 0.6 (Ordaz-Ortiz and Saulnier 2005, Robertson et al., 2010). In 1983 Carpita showed that the hemicellulose fraction of corn cotyleoptiles is dominated by glucuronoarabinoxylan and can be divided into subfractions with different degrees of arabinosyl substitutions ranging from 0.57 in the lowest arabinosyl substituted regions and up to 1.02 in the fractions containing the highest A:X ratio. This
implies that arabinoxylan even though it is composed of relatively few basic components has a spatial distribution and occurrence of these that can be highly differentiated throughout the overall structure of arabinoxylan.

Other complex heterogeneous oligomers consisting of both β-D- and β-L-galactosyls, α- and β-xylosyls linked to feruloylated side chain arabinosyls have also been isolated from corn bran exhibiting various linkage configurations between xylosyls and galactosyls. This truly indicates the complex nature of corn bran xylan (Saulnier et al., 1995a+b; Wende and Fry, 1997, Allerdings et al., 2006).

Besides arabinoxylan, the cell walls of corn bran are also composed of cellulose and mixed-linked β-glucans which constitute 20-23% of the dry matter (Saulnier et al., 1995a, Agger et al., 2010 Paper 1). Cellulose in itself is obviously also a valuable polymer, but more importantly in this context is the fact that arabinoxylan may interact with cellulose, either by non-covalent interactions like hydrogen bonding between lesser substituted regions of xylan and cellulose (Carpita 1983, Kabel et al., 2007), but possibly also by integrating the heteroxylan molecules into the cellulose microfibrils (Whitney et al., 1998). The latter will greatly influence the structure and physical properties of the cell wall.

### 3.1 Other substitutions on arabinoxylan

Arabinoxylan is also known to be substituted with significant amounts of acetyl groups that are esterified directly to the xylan backbone (figure 3) either via the O-2 or O-3 position or possibly by double substitution in both positions (Chesson et al., 1983). It has been reported that up to 50% of all xylosyls is substituted by acetyl groups in wheat and barley arabinoxylan (Chesson et al., 1983). The amounts of acetyl groups reported for corn bran lie in the range of 4-5% of the dry matter, which on a molar basis represents a relatively high degree of substitution of approx 25% (Saulnier et al., 1995a, Agger et al., 2010 Paper 1). The recent paper by Appeldorn et al., 2010 also shows that certain fractions of highly resistant arabinoxylans are extremely substituted by acetyl groups. For certain types of hardwood, the acetylations are much higher, up to 70% (Telemann et al., 2002) proving that they are important to include in the general understanding of the arabinoxylan structure.

Another important group of substitutents is the phenolic compounds in the form of hydroxycinnamates (Carpita and Gibeaut, 1993). In arabinoxylan p-coumaric acid and t-ferulic acid esterify to the O-5 position on terminal arabinofuranosyl moieties as shown in figure 3 (Saulnier et al., 1995b). Corn bran is particularly rich in feruloyl derived substitutions where they constitute approx. 3-5% of the biomass dry weight (Saulnier and Thibault, 1999), whereas the content of p-coumaroyl is approx. 0.3% (Saulnier and Thibault, 1999, Lapierre et al., 2001). Feruloyl units are known to form dehydrodimers with adjacent feruloyls and in that sense cross-links arabinoxylan molecules (figure 3) (Ralph et al., 1994). This dimerization process is catalysed by the action of peroxidases naturally occurring in the plants upon reaction with free radicals and has even lead to the occurrence of trimers and tetramers of feruloyl units in some species (Fry, 1979, Bunzel et al., 2006).
Theoretically, the dimerization process gives rise to several configurations of dehydrodimers, namely the 8,5', 8-0-4', 5,5', 8,8'- and 4-0-5' - coupled as in figure 4 (Bunzel et al., 2001). Analysis of diferulates is often achieved after saponification of plant material, which releases three known forms of the 8,8'- form (the cyclic structure, the open structure and the tetrahydrofuran structure) (figure 4). It is not known if all of these forms are actually present in the original plant material or whether some might be analytical artifacts, but only the cyclic form has been directly isolated yet (Ralph et al., 1994, Bunzel et al., 2008). The 8,5'-benzofuran form generates several forms upon saponification as well, including the non-cyclic 8,5'-form and a decarboxylated form. Neither of the two latter forms are naturally occurring in plants and therefore only the total content of the 8,5'-forms may signify the real content of 8,5'-benzofuran coupled
dimers (Bunzel 2010). Finally the 4-O-5'- configuration has only been isolated in exceptionally small amounts from certain insoluble cereal fiber fractions from wheat, corn and rice (Bunzel et al., 2000). It therefore possibly only represents a minute part of the total phenolic content in corn bran. Various distributions between the dehydrodimers have been reported for corn bran, however the 8,5'-form and the 8-O-4'-form are often dominating (Bunzel et al., 2001, Saulnier and Thibault 1999, Hatfield et al., 1999).

Table 1: Basic biomass composition of destarched corn bran, DCB in g/kg DM. Methods for determination in Agger et al., 2010 Paper 1, which also includes the destarching procedure. All components are given in their hydrated form.

<table>
<thead>
<tr>
<th>Component</th>
<th>Content (g/kg DM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arabinose</td>
<td>267</td>
</tr>
<tr>
<td>Xylose</td>
<td>374</td>
</tr>
<tr>
<td>Galactose</td>
<td>32.3</td>
</tr>
<tr>
<td>Glucose</td>
<td>233</td>
</tr>
<tr>
<td>Glucuronic acid</td>
<td>18.0</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>38.5</td>
</tr>
<tr>
<td>t-ferulic acid</td>
<td>27.3</td>
</tr>
<tr>
<td>p-coumaric acid</td>
<td>5.8</td>
</tr>
<tr>
<td>Dehydrodimers of ferulic acid</td>
<td>18.2</td>
</tr>
<tr>
<td>5,5'</td>
<td>4.9</td>
</tr>
<tr>
<td>- divided</td>
<td>8,5'</td>
</tr>
<tr>
<td>between</td>
<td>8-O-4'</td>
</tr>
<tr>
<td>8,8'</td>
<td>1.4</td>
</tr>
<tr>
<td>Protein</td>
<td>89.1</td>
</tr>
<tr>
<td>Lignin</td>
<td>30.9*</td>
</tr>
<tr>
<td>** Sum</td>
<td>983.4**</td>
</tr>
</tbody>
</table>

* Klason lignin not corrected for protein content is 120 g/kg DM (Agger et al., 2010 Paper 1).
** When carbohydrates are corrected for loss of water in a polymeric form.

Table 1 shows the basic monosaccharide composition of the destarched corn bran (DCB) used in the work presented in this thesis. All work is performed on the same batch, which originates from approx. 15 kg raw corn bran from ADM. The material was destarched and milled prior to use by methods described in Agger et al., 2010 Paper 1. Generally, the composition of this batch of DCB is similar to those reported by others (Saulnier and Thibault, 1999, Saulnier et al., 2001). Also ferulic acid and dehydrodimers of ferulic acid are present in amounts comparable to those reported elsewhere (Saulnier and Thibault, 1999). Traces of dehydro-trimers have also been observed by LC-MS, but more precise qualitative and quantitative determination of the trimers has not been possible.
3.2 Intermolecular interactions

Generally it is believed that dehydrodimers cross-link two polysaccharide chains as a result of dimerization of the esterified monomers, but molecular modeling have shown that intramolecular formation of dimers probably also occur, especially for the 5,5'- and 8,5'-form (Hatfield and Ralph, 1999, Obel et al., 2003) however other forms have also been observed (Lindsay and Fry, 2008). This is particularly important to recognize in the aspect of considering cell wall structure when trimers and tetramers are present. It is believed that trimers of ferulic acid most likely cross-link two polysaccharides chains (figure 5), rather than three even though the latter is theoretically possible. The picture becomes even more complex if formation of cross-linking with tetramers is involved but no evidence of such structural formation exists as yet (Bunzel 2010).

Besides the formation of dehydrodimer cross-linkings feruloyl substitutions have been reported to also participate in oxidative cross-linking between proteins and polysaccharides via dehydroferulic acid-tyrosine linkages (Piber and Koehler 2005) and by linking to lignin structures (Lapierre et al., 2001). Interestingly, speculative structures of such cross-linkings between ferulic acid and tyrosine residues in proteins resemble the structural formations of ferulate dehydrodimer cross-linking due to the relatively large similarities between tyrosine and ferulic acid (figure 6). Finally, the formation of ether linkages to monolignols has been studied and it is generally accepted that the point of nucleation of cell wall lignification is ferulic acid-monolignol linkages in cereal grains (Ralph et al., 1995).

Figure 5: Possible dehydro-trimer cross-linking of two arabinoxylan molecules illustrated via one intramolecular linkage. Stylized drawing with α-linked D-arabinofuranoside moieties (while L-arabinofuranosides are possible those present) to the β-linked backbone of D-xylosepyranoside moities.
Figure 6: t-Ferulic acid, p-coumaric acid, tyrosine and monolignols, corniferyl, p-coumaryl and sinapyl alcohol. Large resemblance between molecules makes them likely to interact through similar configurations to dehydrodimers of ferlic acid via peroxidase mediated coupling.

3.3 Physical properties

Arabinoxylans are often categorized according to their water solubility, and solubility is crucial to enzymatic hydrolysis. There seem to be several factors influencing the arabinoxylan solubility, namely A:X ratio as discussed later, the extent of hydrogen bonding, covalent interactions, molecular size and possibly a combination of these factors. In some of the pioneering work on describing corn bran heteroxylans (Saulnier et al., 1995b) it is discussed if covalent interactions between polysaccharide chains caused by diferulate linkages can be the explanation for corn bran insolubility. But it is concluded that even though they may participate, the intermolecular interactions with cell wall proteins possibly contribute more to the insolubility. This is concluded because significant amounts of protein, particularly hydroxyproline-rich proteins were co-fractionated with certain parts of the arabinoxylan (Saulnier et al., 1995b). When considering such intermolecular interactions it is also evident that the molecular size will increase and possibly lead to aggregate formation and precipitation. Other suggestions of intermolecular bondings have already been mentioned in the form of lignin-polysaccharide interactions. Such interactions will most probably also cause the complexes to become increasingly hydrophobic and this alone can lead to precipitation. Determining the molecular size of insoluble arabinoxylan is difficult by nature, but the molecular weight of alkali extracted arabinoxylan from corn bran has been reported to be in the range of 220-230 kDa (Chanliaud et al., 1995).

Within the field of artificial film preparation from arabinoxylan the effects of excessive hydrogen bondings between unsubstituted xylan molecules are exploited to prepare semicrystalline biofilms (Højie et al., 2008). For the latter arabinoxylan from rye is enzymatically treated with arabinofuranosidases to remove arabinosyl substitutions without affecting the degree of polymerization of the xylan backbone and this process leads to the formation of film structures due to hydrogen bonding between linear xylan chains.
the context of hydrogen bonding it is also known that regions of lesser substituted xylans can interact with cellulose microfilbrils and in that sense become insoluble (Kabel et al., 2007). Summarizing the characteristics of insoluble arabinoxylan is that the molecules are possibly large, lesser substituted than soluble and most probably more interacting with other molecules like adjacent arabinoxylans, proteins, lignin or cellulose.

Soluble arabinoxylan on the other hand is much easier to study and the available data show that the degree of substitution can be very high and even up to 1.06 in certain fractions of soluble wheat and corn arabinoxylan (Dervilly et al., 2000, Agger et al., 2010 Paper 1). This suggests a relatively high occurrence of doubly substituted xylopyranosyls. However, the molecular mass has also been found to be relatively high (up to 590 kDa) informing that even very large molecules may stay in solution if the structure otherwise favors this, for example by being highly substituted. Another study aiming to describe some enzymatically recalcitrant solubilised oligosaccharides from corn bran proved a remarkably high diversity in the monosaccharide composition with considerable amounts of glucose, galactose, mannose and galacturonic acid (Appeldorn et al., 2010). However, it is not proven that it all originates from arabinoxylan. Finally, it is also known, that increasing degree of esterifications by acetyl and feruloyl groups promote the solubility (Poutanen et al., 1990). Summarizing the features of soluble arabinoxylans is that heavy substitutions are important for maintaining solubility and thereby even very large molecules may stay in solution. However, soluble arabinoxylans may actually represent biased structures to study exactly because they are soluble and not insoluble. Solubilization is mostly achieved by chemical modifications of the cell wall leading to breakage of the very molecules of interest, but at present the lack of adequate analytical tools limits the exploration of insoluble structures.

### 3.4 Enzymatic hydrolysis of corn bran

Enzymatic hydrolysis of corn bran has proven exceptionally difficult (Faulds et al., 1995, Saulnier et al., 2001, Appeldorn et al., 2010). Faulds et al., 1995 studied the release of free ferulic acid from destarched corn bran by A. niger FAE-III and obtained only 0.3% of the total amount. Saulnier et al., 2001 applies extreme enzyme loadings of a commercial H. insolens preparation on destarched corn bran and is able to release up to 27% xylose after repetitive enzyme additions. Our own results with a minimal enzyme loading of mono-component hemicellulases and esterases produce approx. 3% xylose release from unpretreated corn bran (Agger et al., 2011, Paper 2). Just recently a thermostable feruloyl esterase with typical type A activity has been isolated from Thermobacillus xylanilyticus and when incubated with xylanase from the same organism it is capable of catalyzing the release of both ferulic acid and diferulic acids (5,5’- and 8-0-4’) from raw corn bran (Rakotoarivonina et al., 2011). This is to our knowledge the first example of release of dehydrodimers from raw corn bran, although the yields are still very limited, approx. 2.5% ferulic acid and less than 1% diferulic acid of the total alkali extractables.

Only a few reports of the enzymatic yields directly from raw corn bran have been made. Instead, nearly all reports involve pretreatment of corn bran prior to enzymatic hydrolysis. Saha et al., 1998 also conclude
that pretreatment is crucial if acceptable yields are to be obtained in order to exploit the potential of corn bran as a feedstock for bioethanol production. These pretreatment methods range from hot-water extractions, dilute acid and alkali pretreatments, AFEX, perioxide extractions, mechanical particle size reduction etc (Saha et al., 1998, Yadav et al., 2007, Dien et al., 2006). All pretreatment methods have advantages and disadvantages, whereas the latter mainly concerns energy, chemical, time and money expenditure. It is therefore equally important to evaluate the yield efficiencies obtained by pretreatment in relation to the process costs. From an academic point of view, the major disadvantage of applying pretreatment is the lack of understanding of the changes introduced by various pretreatment methods (Agger et al., 2011, Paper 2).

**Influence of dehydrodimers of ferulic acid**

For decades it has been a common hypothesis that the presence of dehydrodimer feruloyl cross-linkings between arabinoxylan macromolecules serve to strengthen the cell wall matrix and thereby impede enzymatic hydrolysis (Saulnier and Thibault, 1999, Grabber et al. 1998a+b). It has been a driving hypothesis in the present work that removal of these cross-linkings will leave the arabinoxylan from corn bran more susceptible to enzymatic degradation. However, as reported in the literature it has proven difficult to achieve sufficient release via enzymatic means, even though several feruloyl esterases are known and have been studied intensively (Faulds et al., 1995, Saulnier et al., 2001). Over the time it has been speculated whether diferulate cross-linkings are at all significant in hindering the enzymatic degradation of arabinoxylan (Funk et al., 2007, Agger et al., 2011). These speculations arise because it is difficult to obtain major enzymatically catalyzed release of diferulate in complex substrates, but also because it so far has not been possible to obtain exact and clear data on the effects of diferulic acid removal.

This work was initially set out to investigate feruloyl esterases of various origins for their catalytic capabilities on feruloylated corn bran. Feruloyl esterases (EC 3.1.1.73) belong generally speaking to the carbohydrate esterase family 1 (CE1) and are commonly divided into type A, B, C and some times D. This division depends on their protein sequence homology, affinity towards certain synthetic methylated hydroxycinnamic acid derivatives and their ability to release diferulates (Faulds et al., 2003, Crepin et al., 2004, Benoit et al., 2008). Type A generally has a preference for substrates with a methoxy substitution on the phenolic ring and for O-5-ester-linkages to arabinose (Ralet et al., 2004). A very well-studied type A esterase, FAE-III from *A. niger* has proved especially efficient on this particular configuration of feruloyl substitution, but virtually inactive against the O-2-linkages found in dicots (Ralet et al., 1994). This particular *A. niger* esterase (FAE-III) has also been studied for its ability to release dehydrodimers of ferulic acid and it has been proven, that it can catalyse the release of the 5,5'- and the 8-0-4'- dimers as free diferulous acids from synthetic substrates, whereas it can only open one ester bond to the 8,5'-benz form (Garcia-Conesa et al., 1999). The catalytic efficiency towards the di-ester is generally higher than towards the mono-ester, indicating that even though free dehydrodimers are not produced during an enzymatic reaction, the cross-linking between two polysaccharides may open. Purification and characterization of this FAE-III shows that the apparent molecular weight is around 36 kDa and pH and
The temperature optima of 5 and 55-60 °C respectively (Faulds and Williamson 1994). Both type C and type D esterases have activity towards most of the methyl ester hydroxycinnamates, but type C cannot catalyse release of diferulic acids from synthetic substrates (Wong 2006). Instead a type D esterase from P. fluorescens has been shown to release the 5,5'-form of dehydrodimers even though it is generally recognized to exhibit acetyl xylan esterase activity (Bartolomé et al., 1997, Crepin et al., 2004).

Type B esterases are known to have specific activity against feruloyls O-2 linked to arabinose or O-6 linked to galactose. They are expressed primarily upon fungal growth on sugar-beet pulp and pectins but specifically the type B from A. niger (FAE-I) has also been reported to release feruloyls from the O-5 position on arabinose (Ralet et al., 1994, Crepin et al., 2004). Type B esterases have a catalytic preference for substrates with one or two hydroxyl groups on the phenolic moiety. Type B esterases do not release dehydrodimers of ferulic acid (Crepin et al., 2004, Faulds et al., 2003). However, Faulds et al., 2002 report that a commercial β-glucanase enzyme preparation from H. insolens (Ultraflo™ L) showing mainly type B esterase activity towards methyl ester hydroxycinnamic acid substrates releases 5,5'-, 8-O-4' and 8,5' -forms of dehydrodimers from brewers spent grain. However, the release of dehydrodimers from such commercial blends may rely on the presence of minor side activities and as yet, no release of dehydrodimers with purified type B esterases have been shown. Finally, a tannase (EC.3.1.1.20) from A. oryzae have been shown to release diferulates from diethyl-esters of 5,5'-, 8-O-4'- and the 8,5'-forms (Garcia-Cornesa et al., 2001).

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The hydrolytic activity of feruloyl esterases is known to be greatly enhanced by the presence of glycosyl hydrolases, especially xylanase activity, enhancing both the release of ferulic acid but also the release of dehydrodimers of ferulic acid (Faulds and Williamson 1994, Faulds and Williamson 1995, Bartolomé and Gómez-Cordovés 1999, Faulds et al., 2002).

Acetyl xylan esterases (EC 3.1.1.72) are much more widespread within the CE families, however many falls in the same family as the feruloyl esterases (CE1). In the context of hemicellulose breakdown they are often a neglected group of enzymes, even though acetylations are a widespread phenomenon in xylans, mannans and even in pectinaceous substrates (Poutanen et al., 1990). It has been demonstrated that deacetylation is highly important in the overall breakdown of xylans (Grohmann et al., 1989) and synergistic effects are observed between acetyl xylan esterases and endo/exo-xylanases (Poutanen et al., 1990, Biely et al., 1986). With respect to substrate specificity it is important to distinguish between esterases with activity towards acetylated xylans and those with activity towards other acetylated structures, known as acetyl esterases (EC. 3.1.1.6). Some of the early work with acetyl xylan esterases was based on common cellulolytic fungal enzyme systems like T. reesei, A. niger and S. commune and typically the acetyl xylan esterase activity present in crude culture broths from such fungi have been compared to other esterases, not necessarily of the acetyl xylan esterase kind (Biely et al., 1985, Poutanen and Sundberg 1988). Purified forms of acetyl xylan esterases have been studied from S. commune and T. reesei (Halgasova et al., 1994, Poutanen et al., 1990) and their substrate specificity is described. In the latter study, esterases purified from T. reesei is reported to display large differences in specificity, where some
has preferences towards large molecules while others only catalyse the hydrolysis of C-3 linked acetyl groups of xylobioses.

Other glycosyl hydrolases mainly cellulases are also relevant to include due to the structure of the cell wall matrix. However, degradation of cellulose has not been the major focus in this thesis, and therefore a commercial blend of cellulases has been applied (Cellic CTec™, Harris et al., 2010) mainly consisting of cellobiohydrolase I and II, endo-glucanases and β-glucosidase. This preparation is not a mono component preparation but is based on the well-known T. reesei complex (Rosgaard et al., 2007) and hence also contains residual endo-xylanase and β-xilosidase activity and certain hydrolysis boosting proteins belonging to the GH61 family of proteins, besides the already mentioned cellulases. Other side activities may also be present.

The work presented in this thesis takes its offset in a well described minimal mixture of hemicellulases that has proven efficient in degrading soluble and insoluble arabinoxylan from wheat (Sørensen et al., 2003, Sørensen et al., 2007a). Hemicellulases of this kind must inevitably constitute the basis for evaluating release of monosaccharides and since this set in particular has proven efficient towards arabinoxylan, it has formed the core enzyme activities in all hydrolysis experiments. Here the action of each enzyme is generally known and thereby successful catalysed reactions give information about substrate structure and availability. The minimal mixture contains one β-1,4-endoxylanase (EC.3.2.1.8) GH10 originally from H. insolens, one β-xylidosidase (EC.3.2.1.37, GH3) from T. reesei and two α-L-arabinofuranosidases (EC.3.2.1.55), one GH43 and one GH51 from H. insolens and M. giganteus respectively (table 2). With this set of basic glycosyl hydrolases the main side chain and backbone degrading enzyme activities are present and creates a solid starting point for extensive hydrolysis. The particular GH10 endo-xylanase has been shown to have preferences for solubilized substrates, releasing relatively small xyloooligosaccharides (Sørensen et al., 2007b). Furthermore, GH10 endo-xylanases are commonly known to have broader substrate specificity and to be able to cleave glycosidic linkages closer to substitutions compared to their GH11 counterparts (Biely et al., 1997, Kormelink et al., 1993, Bonnin et al., 2006).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Microorganism</th>
<th>Family/Type</th>
<th>EC number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endoxylanase</td>
<td><em>Humicola insolens</em></td>
<td>GH10</td>
<td>EC.3.2.1.8</td>
</tr>
<tr>
<td>β-xylidosidase</td>
<td><em>Trichoderma reesei</em></td>
<td>GH3</td>
<td>EC.3.2.1.37</td>
</tr>
<tr>
<td>α-L-arabinofuranosidase</td>
<td><em>Meripilus giganteus</em></td>
<td>GH51</td>
<td>EC.3.2.1.55</td>
</tr>
<tr>
<td>α-L-arabinofuranosidase</td>
<td><em>Humicola insolens</em></td>
<td>GH43</td>
<td>EC.3.2.1.55</td>
</tr>
</tbody>
</table>

Notably the GH43 α-L-arabinofuranosidase from *H. insolens* is able to release the (1→3)-α-L-arabinofuranosyls from doubly substituted xylopyranosyls in wheat arabinoxylan and synergistic effects were observed when combined with α-L-arabinofuranosidase GH51 from *M. giganteus*. The latter is capable of removing both (1→2) and (1→3)-α-L-arabinofuranosyls from single substituted xylopyranosyls (Sørensen et al., 2006).
Hence, the bearing philosophy of this PhD thesis work is that with the wide array of enzyme activities already known to this line of work, it should be possible to obtain complete enzymatic hydrolysis of corn bran if the enzymes are combined and administered correctly. Cereal residues and corn bran in particular are recalcitrant in nature and as a result of that enzymatic catalysis in theory is different from that in practice. With the following three scientific publications supplemented by additional results the work has first and foremost strived to answer the three main hypotheses stated at the very beginning, but as the thesis progresses certain other aspects are also investigated in order to obtain an expanded understanding of corn bran in relation to its enzymatic disintegration.


Main issues

Corn bran is used in this study because it is a rich source of ferulic acid and dehydrodimers of ferulic acid. In order to evaluate results of enzymatic hydrolysis it is important to master an analytical method for identification and quantification of as many different components as possible. Therefore reverse phase HPLC equipped with diode array detection is used for identification and quantification of feruloyl species. For the exact elution profile, column and equipment description please refer to Paper 1. It is possible to obtain a method for separation of at least 5 different configurations of dehydrodimers of ferulic acid in the same elution system as free ferulic acid and p-coumaric acid (figure 7). The first identification was based upon authentic standards of each component leading to assignment of retention time and unique spectra. The dehydrodimer standards were acquired from work previously carried out in this group (Andreasen et al., 2000, Larsen et al., 2001). However, gravimetric calibration has not been possible based on these standards and therefore quantification of the dehydrodimers is done indirectly according to response factors reported by Waldron et al., 1996 and calibrated towards an internal t-cinnamic acid standard at 280 nm. p-coumaric and t-ferulic acid is quantified at 316 nm against real standard curves.

Figure 7: RP-HPLC chromatogram (316 nm) for total saponified corn bran sample. (1) p-coumaric acid, (2) t-ferulic acid. (3) c-ferulic acid, (4) 8,5'-diferulic acid, (5) 8,8'-diferulic acid, (6) 5,5'-diferulic acid, (7) 8-0'-diferulic acid. (8) 8,5'-benzofuran diferulic acid, (9) t-cinnamic acid (internal standard, not originating from corn bran). a-j: unidentified peaks with spectra related to hydroxycinnamates.
Figure 8: Spectra for compounds 1 to 9 from 210 nm to 400 nm from HPLC data in figure 7.

Figure 8 illustrates the unique spectra for each of the compounds 1 to 9 in figure 7. Besides compound 1 to 9, compounds a - j is also observed in the chromatogram with spectra similar to those in figure 8, yet no further identification has been possible. Tentative LC-MS data of saponified corn bran hydrolysate also identified the presence of at least two trimers of ferulic acid (m/z of 578) but exact identification could not be obtained due to lack of external standards. However, comparison of the spectral data (figure 9) to literature findings suggest that they are the 5,5’/8-O-4’-dehydrotrifeulic acid and the 8,8’(tetrahydrofuran)/5,5’-dehydrotriferulic acid (Bunzel et al, 2005+2006). However, the molecular weight for the trimers observed in the present work is 578 and not 596 as that reported by Bunzel. The increase of 18 observed by Bunzel is found to be consistent with the addition of a water molecule, most probably induced by the analytical methods.
The presence of ferulic acid and dehydrodimers of ferulic acid in corn bran necessitates the use of feruloyl esterases in the search for extended hydrolysis. Ten candidates of confirmed and putative feruloyl esterases (Novozymes A/S) have been tested in combination with the minimal blend of hemicellulases (Sørensen et al., 2007a) in order to evaluate the release of ferulic acid and xylose. Out of the ten feruloyl esterase candidates the one from *A. niger* known as FAE-III (named FAE in the paper) was found to be the most efficient in releasing ferulic acid with more than 100% higher release than the other esterases tested and was therefore chosen for the experiments in the following paper. At the same time, one of these candidates was found to boost the xylose release without releasing any ferulic acid. Further investigations showed that this esterase, an enzyme derived from *Flavovolaschia* sp., a basidiomycete was in fact an acetyl xylan esterase and based on this finding, the major hypothesis for this particular paper was established, namely that not only feruloyl substitution but also acetyl groups are important to target by the enzymatic hydrolysis in order to improve the hydrolysis.

The hypothesis may also be approached by assessing the extent of feruloyl substitution and dehydrodimer cross-linking in corn bran assuming that feruloylations occur only in the O-5 position on arabinosyls. Based on the data from table 1 (section 3.1) it can be estimated that approx. 8% of the arabinosyls are substituted by feruloyls. Further assuming a polymer size of approx. 230 kDa of arabinoxylan (Chanliaud et al., 1995) and an A:X ratio of 0.71 (table 1), the average DP of the arabinoxylan of DCB is approx. 1000 and it carries approx. 56 substitutions by feruloyl and approx. 18 diferulate cross-linkings per molecule. This estimate is in the range of what other similar estimations reported for corn bran (Saulnier et al., 1995a). Similar estimations for acetylation are that approx. 25% of the xylose moieties (equal to ~250 moieties) of the xylan backbone are mono substituted with acetyl. This number may be lower if some xylosyls are double substituted. In that context and in the light of other findings on the significance of deacetylation (Grohmann et al., 1989, Poutanen et al., 1990, Biely et al., 1986) it is hypothesized that deactylation will be important for the overall hydrolysis of corn bran arabinoxylan with comparable effects to deferuloylation.

The purpose of the work presented in paper 1 is therefore to assess and compare the overall enzymatically catalyzed release of xylose from corn bran when feruloyl esterase and the new acetyl xylan esterase from *Flavovolaschia* sp. are incubated in combination with mono component hemicellulases.
Main results

Corn bran has been heat pretreated in aqueous slurry yielding a soluble and an insoluble substrate fraction. These two substrate fractions are treated as two individual substrates for enzyme hydrolysis. Firstly, the results of monosaccharide compositional analysis show that the soluble residue after pretreatment is more substituted with arabinose than the insoluble, leading to a higher A:X ratio in this fraction (table 2 in Paper 1). Moreover, the amounts of ferulic acid and acetic acid in the soluble fraction is high, thereby adding to the complexity of these oligosaccharides and leading to an extremely high degree of substitution on the xylan backbone of hypothetically 1.08. This must be compared to the insoluble fraction of 0.63. Dehydrodimers of ferulic acid is almost exclusively found in the insoluble fraction.

The results show that deacetylation and deferuloylation do not have the same stimulatory effects on xylose release in the two substrate fractions (table 3). Deacetylation is most efficient in boosting the xylose release in the insoluble fraction where every released mole of acetic acid causes the concomitant release of one mole of xylose. On the contrary, deferuloylation promotes the xylose release in the soluble substrate fraction with the same molar release of 1:1. It was not anticipated that the action of the same enzymes would be distinctively different on the two types of substrates, but it is an example of how specific enzyme reaction may also function as tools for structural clarification rather than just as hydrolysis catalysts.

| Table 3: Xylose yield per released amount of substituent (acetic acid or ferulic acid) |
|----------------------------------|-----------------------------------|
|                                  | AXE (mol xylose/mol acetic acid) | FAE (mol xylose/mol ferulic acid) |
| Insoluble                        | 1.06                             | 0.39                              |
| Soluble                          | 0.51                             | 1.01                              |

A general observation however, is that the extent of enzymatic hydrolysis is markedly higher in the soluble substrate fraction compared to the insoluble (table 4 in Paper 1), despite the fact that monosaccharide composition proves more complex in the soluble substrate. The highest yields of the enzymatic hydrolysis is obtained with the full enzyme loading including the Cellic CTec™ cellulase preparation and amount to approx. 15% xylose, 20% arabinose, 23% acetic acid and 48% ferulic acid release, when compared to the amounts present in the original corn bran. The cellulase preparation mostly promotes the xylose release from the insoluble fraction, which increases from approx. 11% to approx. 15% compared to no cellulase preparation (table 4 in Paper 1). This effect may originate from endo-xylanase side activity in the preparation, but may also be related to the fact that cellulose is most abundant in the insoluble fraction. Otherwise the effects of the cellulase preparation are negligible.

On this pretreated corn bran substrate no synergistic effects between FAE-III and the GH10 endo-xylanase is observed, either on the soluble or the insoluble substrate fraction, even though this is a well-known phenomenon (Faulds et al., 2002). The release of ferulic acid by FAE-III from both substrate fractions is
the same independent of the presence of endo-xylanase (data not shown). This may indicate that the pretreatment has induced alterations to the native substrate in a way so that substrate conditions no longer promote synergism between the two activities.

It has also been tested if this particular the new acetyl xylan esterase is the best available, when evaluated based on boosting effects on xylose release. This has been assessed by testing seven other putative acetyl xylan esterases provided by Novozymes A/S, but none turned out to be significantly better than the one used in the present study (data not shown).

**Conclusion**

The data in this publication corroborate hypothesis 2 by exemplifying the effects of enzymatically removing acetylations and feruloylations. Yet, despite high release of ferulic acid by the feruloyl esterase, no release of dehydrodimers has been observed, thereby making it impossible to either support or dismiss hypothesis 1. The pretreatment induces a significant decrease to the diferulic acid content and it can be speculated that this decrease causes a promotion of the overall enzymatic hydrolysis. Still, the results suggest that this is more likely related to the overall solubilization, compositional changes and disruption of the physical appearance induced by the pretreatment, then directly linked to the diferulic acid content.

The work also generated new questions, namely why complete hydrolysis was not obtained even though efficient pretreatment and relevant enzyme activities had been employed. In that context future work is directed towards understanding and describing the different effects of various pretreatments, keeping in mind that corn bran as a substrate does not contain significant amounts of lignin and therefore probably will require milder conditions than other well-known pretreatment methods used for lignocellulosics.

Finally it is observed that the arabinose release is low and do not follow the deferuloylation as may have been expected, when the esterase activity catalyses the exposure of terminal substituted arabinoxyls. The lack of arabinose release corroborates the complexity of corn bran as substrate for enzymatic hydrolysis and leads to the considerations that arabinose may not all originate from terminal substitutions. Based on the results obtained here, it can be speculated that arabinose originate from arabinan structures or heterogenous side chains in relation to cell wall proteins or pectic substances and in that case be inaccessible to the arabinofuranosidase activities employed here. Heterogenous side chains may as well originate directly from arabinoxylan as is also described in the introduction. Particularly, side chains with arabinose moieties surrounded by other components will affect the extent of arabinose release.

The following three chapters explore the basic set of hemicellulases compared to others, some of the structural and compositional features of corn bran through the soluble fraction just described and finishes by assessing the presence of cell wall proteins in order to address some of the issues originating from the current data.
Enzymatic Xylose Release from Pretreated Corn Bran Arabinoxylan: Differential Effects of Deacetylation and Deferuloylation on Insoluble and Soluble Substrate Fractions

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In the present work enzymatic hydrolysis of arabinoxylan from pretreated corn bran (190 °C, 10 min) was evaluated by measuring the release of xylose and arabinose after treatment with a designed minimal mixture of monocomponent enzymes consisting of α-L-arabinofuranosidases, an endoxylanase, and a β-xylosidase. The pretreatment divided the corn bran material ∼50:50 into soluble and insoluble fractions having A:X ratios of 0.66 and 0.40, respectively. Addition of acetyl xylan esterase to the monocomponent enzyme mixture almost doubled the xylose release from the insoluble substrate fraction and gave release of 1 mol of xylose/mol of acetic acid released, whereas addition of feruloyl esterase promoted release of only ∼0.4 mol of xylose/mol of ferulic acid released. For the soluble substrate fraction up to 36% of the xylose could be released by the enzymatic treatment. Acetyl xylan esterase addition on top of the minimal monocomponent enzyme mixture resulted in liberation of up to 0.5 mol of xylose/mol of acetic acid released, whereas feruloyl esterase addition released 1 mol of xylose/mol of ferulic acid released from the soluble substrate. The results imply that on the insoluble material the acetyl xylan esterase was more important for the enzymatic degradation than feruloyl esterase, whereas on soluble arabinoxylan the feruloyl esterase seemed to be more important for the release of xylose.

KEYWORDS: Enzymatic hydrolysis; acetyl xylan esterase; feruloyl esterase; arabinoxylan; corn bran; xylose

INTRODUCTION

In 2007 the Food and Agricultural Organization (FAO) of the United Nations reported that the total production of corn in the United States was 330 × 10^6 tons (1). Corn bran is an agro-industrial byproduct resulting from the wet milling step in corn starch processing and consists almost exclusively of the pericarp tissue, testa, and pedicel tip of the corn kernel (2). Considering that a large starch producer in the United States processes about 50000 tons of corn per day (3), with a cautious estimate of 5% (by weight) of this corn becoming corn bran, the yield of corn bran would be approximately 9 × 10^6 tons per year from this producer alone. As a clean and readily available agricultural residue, corn bran may have the potential of becoming a source for new C5 biofuel products or for the manufacture of food ingredients. These novel uses require partial or complete degradation of the biomass to its constituent monomers. The investigation of the chemical composition and the enzymatic degradability of corn bran is therefore an important objective of several current studies.

Corn bran originates from a gramineaceous plant having a primary cell wall that is mainly composed of heteroxylans (approximately 50% by dry weight), notably arabinoxylan (3). Much effort has been put into describing the complexity of arabinoxylan from corn bran and into understanding the interactions between arabinoxylan and other cell wall components (3–6). A simplified sketch of the corn bran arabinoxylan structure is shown in Figure 1. The arabinoxylan backbone in corn bran is composed of a xylan backbone of β-(1→4)-linked D-xylopyranosyl residues. Linkage analysis has suggested that up to 85% of the xylopyranosyl moieties are substituted with various components (4). The main substitutions are α-L-arabinofuranosyl residues linked to the O-2 or O-3 position on monosubstituted xylopyranosyls (40%) or to both O-2 and O-3 on doubly substituted xylopyranosyl units (20%). Arabinofuranosyl substitutions have also been suggested to occur as short side chains. As much as 40% of the total arabinofuranosyl substitutions have been reported as nonterminal (4). It has also been suggested that xylan is further substituted with xylopyranosyls by a (1→3)-linkage and that the arabinofuranosyls can be further decorated with xylopyranosyls or even 1-galactopyranosyls (5, 6). Xylan may furthermore be directly substituted with ß-galactopyranosyl and ß-glucuronosyl residues, which may each account for approximately 3–5% of the biomass dry weight (3, 6).

Besides glycosidic linkages, arabinoxylan is also substituted by esterifications. Acetic acid is found to constitute 3–5% of the corn bran dry matter; this acetic acid is esterified directly to the

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xylan backbone in position O-2 or O-3, whereas hydroxycinnamic acids (3–6% of the biomass dry weight) are esterified to arabinoferansyls in position O-5 (5, 7). The hydroxycinnamates are mainly p-coumaric acid, ferulic acid, and dehydrodimers of ferulic acid (5, 8–10). Suggestions have even been made that feruloyl groups can be positioned on arabinoferanse in the previously mentioned short heterogeneous side chains rather than terminally on the arabinoferansyl moity (5). These side chains have commonly been referred to as FAX and FAXG. Also, trimers and tetramers of ferulic acid have been isolated from corn bran (11). It is known that, in particular, diferulates cross-link arabinoxylan molecules, thereby creating covalent intermolecular relations (10, 12, 13). These interactions have been shown to impede the enzymatic degradation of corn bran (14, 15). Finally, corn bran also contains cellulose (~20%), lignin (~10–14%), and structural proteins (~5%). The lignin and structural proteins have been suggested to also participate in intermolecular interactions with arabinoxylan through diferulate cross-linking, giving rise to a highly complex network of heterogeneous molecules (6, 15, 16, 17).

With respect to enzymatic digestibility, corn bran has been acknowledged as a recalcitrant substrate with little release of monosaccharides despite the application of several mixed enzyme activities at high dosage (18, 19). To achieve reasonable enzymatic hydrolysis, hydrothermal pretreatment has been applied with success (19, 20). The recalcitrance to degradation has been ascribed to be a consequence of the highly branched structure of the arabinoxylan, and indeed feruloylation has been held responsible (21, 22). Remarkably, acetyl substitution also occurs in corn bran to the same extent as feruloylation, but the role of acetylation has been given very little attention in relation to enzymatic degradation of corn bran, even though early studies with other substrates such as wheat and aspen xylans have shown that enzymatic digestibility of both cellulose and xylan increased remarkably with increasing chemical deacetylation (23). A direct correlation between the enzymatic release of xylose and concomitant deacetylation of xylan has been reported for purified beech- and birchwood xylans (24, 25). Hence, we hypothesized that deacetylation could be important for the overall enzymatic digestibility of xylan. The purpose of the present work was to assess and compare the overall release of xylose from corn bran arabinoxylan using relevant monocomponent enzyme activities and thereby to obtain indications as to the significance of deacetylation for enzymatic xylose release from corn bran arabinoxylan.

MATERIALS AND METHODS

Substrate. Raw corn bran was provided by Archer Daniel Midlands Co., Decatur, IL. The material was milled, enzymatically destarched, freeze-dried, and milled again before use. Initial wet milling was performed at 2% dry matter (DM), and destarching was carried out using a thermostable α-amylase (Termamyl SC, dosed at 7560 KNU-S/kg of corn bran) incubated at pH 6 and 95 °C for 0.5 h, followed by incubation with amyloglucosidase (Spirizyme Plus FG, dosed at 69000 AGU/kg of corn bran) at pH 5 and 60 °C for 1 h. After the enzymatic treatment, the material was washed in water and decanted to remove liberated glucose. The washing was performed three times, resulting in a free glucose concentration in the last volume of washing water below the detection limit of the HPAEC. Freeze-drying was done over 5 days, and the biomass was finally milled again to reduce the particle size to <1 mm. The destarched material was stored at −18 °C.

Enzymes. All enzymes were provided by Novozymes A/S, Bagsværd, Denmark.

Table 1. Enzymes Used for Hydrolysis of Pretreated Corn Bran[a]

<table>
<thead>
<tr>
<th>enzyme</th>
<th>microorganism</th>
<th>family/hype</th>
<th>EC no.</th>
<th>ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>endoxylanase</td>
<td>Humicola insolens</td>
<td>GH10</td>
<td>EC 3.2.1.8</td>
<td>34</td>
</tr>
<tr>
<td>β-xylosidase</td>
<td>Trichoderma reesei</td>
<td>GH3</td>
<td>EC 3.2.1.57</td>
<td>34</td>
</tr>
<tr>
<td>α-αrabinofuranosidase</td>
<td>Meripilus giganteus</td>
<td>GH51</td>
<td>EC 3.2.1.55</td>
<td>34</td>
</tr>
<tr>
<td>α-αrabinofuranosidase</td>
<td>Humicola insolens</td>
<td>GH43</td>
<td>EC 3.2.1.55</td>
<td>34</td>
</tr>
<tr>
<td>acetyl xylan</td>
<td>Flavobasalia sp.</td>
<td>CE1</td>
<td>EC 3.1.1.72</td>
<td></td>
</tr>
<tr>
<td>feruloyl esterase (FAE)</td>
<td>Aspergillus niger</td>
<td>type A CE1</td>
<td>EC 3.1.1.73</td>
<td>35, 36</td>
</tr>
<tr>
<td>Cellic CTeC</td>
<td>Trichoderma reesei</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

[a] All enzymes were provided by Novozymes A/S, Bagsværd, Denmark.
Pretreatment. Sixty grams of destarched corn bran was suspended in 1 L of water and pretreated at 190 °C with a 10 min holding time. Heating and cooling periods together added up to 6 min. The pretreatment was conducted in a loop autoclave at Risø DTU as described in refs 28 and 29. No chemicals or gases were added to the suspension either before, during, or after the treatment. Afterward, the material was separated by filtration into insoluble and soluble fractions that were characterized and enzymatically hydrolyzed individually.

Biomass Composition. Arabinoxylan and xylose were determined after acid hydrolysis with 0.4 M HCl according to the method given in ref 30. Cellulose, acetic acid, and lignin were determined after sulfuric acid hydrolysis according to the procedure of NREL (29). Sulfuric acid hydrolysis as described by NREL was chosen for acetic acid determination because comparison with alkaline saponification results showed higher release. All samples were analyzed for cellulose and acetic acid content, whereas lignin was determined only in the insoluble samples. Lignin was determined (on nonextracted samples) as Klason Lignin (29).

Enzymatic Hydrolysis. Berberine, acetic acid, and four of the dehydrodimers of ferulic acid, namely, 8-O-4', 8,5'-benz, 5,5', and 8,5' dehydrodimers of ferulic acid, other forms of dehydrodimers of ferulic acid, including 8,8'-diferulic acid, were not identified. nd, not determined; <dl, below detection limit. Estimated loss is calculated values (the values are the relative losses for each component and do therefore not add up to 100%).

Table 2. Monomeric Biomass Composition of Destarched Corn Bran (DCB) and Insoluble and Soluble Fractions of Pretreated Corn Bran

<table>
<thead>
<tr>
<th>arabinopyranosyl</th>
<th>arabinoxylan</th>
<th>arabinoxylan</th>
<th>cellulose</th>
<th>lignin</th>
<th>protein</th>
<th>ferulic acid</th>
<th>diferulic acid</th>
<th>acetic acid</th>
<th>weight distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCB (g/kg of DM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>insoluble (%)</td>
<td>17.5</td>
<td>29.5</td>
<td>73.4</td>
<td>76.4</td>
<td>57.4</td>
<td>21.4</td>
<td>24.7</td>
<td>23.8</td>
<td>45.6</td>
</tr>
<tr>
<td>soluble (%)</td>
<td></td>
<td>free</td>
<td>bound</td>
<td></td>
<td>18.0</td>
<td>3.4</td>
<td>3.4</td>
<td>33.9</td>
<td>5.8</td>
</tr>
<tr>
<td>estimated loss (%)</td>
<td>35.3</td>
<td>33.2</td>
<td>21</td>
<td>23.6</td>
<td>29.2</td>
<td>41.2</td>
<td>73.4</td>
<td>20.3</td>
<td>13.9</td>
</tr>
</tbody>
</table>

Table 3. Ratios of Different Substitution Groups of Corn Bran Arabinoxylan before and after Pretreatment

<table>
<thead>
<tr>
<th></th>
<th>DCB</th>
<th>insoluble</th>
<th>soluble</th>
</tr>
</thead>
<tbody>
<tr>
<td>arabinose/xylose</td>
<td>0.71</td>
<td>0.42</td>
<td>0.66</td>
</tr>
<tr>
<td>acetic acid/xylose</td>
<td>0.26</td>
<td>0.21</td>
<td>0.42</td>
</tr>
<tr>
<td>ferulic acid/arabinose</td>
<td>0.08</td>
<td>0.10</td>
<td>0.09</td>
</tr>
<tr>
<td>diferulic acid/arabinose</td>
<td>0.03</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>total substitution on xylan</td>
<td>0.97</td>
<td>0.63</td>
<td>1.08</td>
</tr>
</tbody>
</table>

All substitution ratios were calculated on the basis of the hydrated values for all components, because all components were measured as hydrated molecules.

Combined with the arabinose substitution, this gave a total substitution on the xylan backbone of almost 100%, meaning that hypothetically each xylopyranosyl residue held one substitution. This value may be overestimated if arabinofuranosyls as proposed previously (4) were present in short chains instead of as terminal arabinofuranosyl substitutions. Ferulic acid and dehydrodimers of ferulic acid were also found in DCB and constituted ~4.4% of the biomass (Table 2). The hydroxycinnamates were presumably esterified to arabinofuranosyl, resulting in approximately 10% of the arabinofuranosyl units being substituted with either feruloyl or diferuloyl units. All glucose was assumed to originate from cellulose, making cellulose the other
major polysaccharide component (21%) in the destarched corn bran.

The presence of lignin (12%) and structural proteins (~9%) completed the impression that corn bran was made up of large complex polymers.

**Influence of Pretreatment on DCB.** Table 2 also contains the relative composition and mass distribution between each of the two fractions (insoluble and soluble) after pretreatment. The pretreatment resulted in solubilization of arabininoxylan to a certain extent and furthermore altered the composition of the remaining insoluble arabininoxylan. Due to the nature of the pretreatment process, not all material was retrieved from the reactor, causing a certain loss of dry matter (pretreatment process, not all material was retrieved from the remaining insoluble arabinoxylan). Due to the nature of the pretreatment, most of the acetic acid was found on the solubilized arabininoxylan. In the soluble material, 0.42 of the xylopyranosyl residues were substituted with acetyl, whereas this number was 0.21 for the insoluble remains (Table 3).

All in all, this characterized the solubilized xylooligosaccharides as extremely highly substituted with a molar ratio of arabinose and acetic acid to xylose of > 1. This extent of substitution was similar to that of the xylan from the original corn bran (0.96, Table 3). Several xylopyranosyl moieties must therefore have been doubly substituted, or arabinose may have occurred in short chains on the xylan backbone. The remaining insoluble xylan was merely substituted up to 0.63. Hence, the pretreatment drastically changed the arabinoxylan composition, and further work was evaluated on this basis, treating each fraction as an individual substrate for enzymatic hydrolysis.

**Enzymatic Hydrolysis.** Arabinose. The yields of arabinose tended to increase with addition of the auxiliary enzymes, especially for the insoluble fraction, but the arabinose increases were less pronounced than the xylose increases (Table 4). Only minor differences in the arabinose release were seen even when ferulic acid was released to a large extent, and there seemed to be hardly any effect of the simultaneous release of acetic acid either. This trend pointed toward the α-L-arabinofuranosidases not being dependent in their catalytic activity on either deacetylation or deferuloylation or alternatively that the debranching in the case of the insoluble remains was sufficient to allow access for the relatively large ACβ-L-arabinofuranosidases (65–70 kDa) (34). However, the arabinose release in the solubilized material was almost three times as high as that of the insoluble material (Table 4), most likely caused by increased enzyme accessibility. The maximal release was in all cases achieved within the first 4–6 h of incubation (data not shown).

**Xylose.** Enzymatic release of xylose was affected by the addition of acetyl xylan esterase and/or feruloyl esterase. In particular, xylose release in the insoluble fraction (Figure 2A; Table 4) was promoted by the presence of acetyl xylan esterase and cellulases. When the 24 h data points were compared, it was evident that feruloyl esterase was not responsible for any additional release of xylose compared to the hemicellulases alone, whereas the acetyl xylan esterase caused release of approximately twice the amount of xylose compared to the hemicellulases alone.
In the experiment combining the two types of esterases, the xylose release was not higher than when compared to the acetyl xylan esterase incubated alone with the hemicellulases. Furthermore, the cellulase preparation induced additional release of xylose, and even though the *T. reesei* complex contains β-xylosidase activity (27), the increased xylose release was most likely a consequence of simultaneous cellulose degradation.

The effect of acetyl xylan esterase versus feruloyl esterase was not pronounced to the same degree for the solubilized fraction (Figure 2B), indicating that the accessibility for the hemicellulases was not restricted in the same manner as on the insoluble fraction. In addition, the yield of xylose in the solubilized material was higher (up to 36% of the fraction maximum, 10% of the original amount of xylose) than in the insoluble material (approximately 15%, 4% of the original amount of xylose), meaning that the enzymatic accessibility had indeed improved by the solubilization. Despite the low content of cellulose in the soluble fraction (Table 2), addition of the cellulase preparation improved the xylose release significantly (Figure 2B). Hence, this effect could be due to the particular hydrolysis-boosting protein present in the *C. cellulare* preparation or, alternatively, less likely, a result of synergistic interactions between the enzymes in the full blend (Cellic CTec; minimal cocktail, AXE, and FAE). Appropriate controls of esterases incubated alone did not show any release of xylose (data not shown).

**Ferulic Acid and Acetic Acid.** Table 4 also shows the release of acetic and ferulic acid after enzymatic hydrolysis. The acetyl xylan esterase was capable of releasing acetic acid equivalent to approximately 20 and 30% of that present in the insoluble and soluble material, respectively. The corresponding releases of ferulic acid by the feruloyl esterase were approximately 65 and 91%. In these experiments no release of diferulic acid was detected, even though the feruloyl esterase from *A. niger* has been known to release especially the 5,5′ and 8-O-4′ dehydrodimers from other substrates such as brewer’s spent grain and wheat arabinoxylan (37, 38). However, only high-dosage experiments (10 times) showed detectable release of the 5,5′ dehydrodimer from the soluble and insoluble fractions (data not shown). The acetic and ferulic acid release seemed relatively unaffected by the presence of enzymes other than the esterases. At first, it seemed that the ongoing catalysis by the hemicellulases and cellulases could not provide more substrate for the esterases, but changes in esterase activity might also be the cause of stagnating release, for instance, as a consequence of prolonged incubation at an elevated temperature. Indeed, Figure 3 shows the result of the heat stability study on AXE and specifies that prolonged incubation at 50 °C significantly reduced the activity of the enzyme. Actually, the enzyme activity was down to

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**Figure 2.** Xylose release from enzymatic hydrolysis of pretreated corn bran in percent of maximum with minimal cocktail (Mini, consisting of endoxylanase, β-xylosidase, and two α-L-arabinofuranosidases), acetyl xylan esterase (AXE), feruloyl esterase (FAE), and cellulase preparation Cellic CTec (CTec): (A) insoluble fraction; (B) soluble fraction.

**Figure 3.** Heat stability of AXE, determined as an activity measurement on pNP acetate after 0–24 h of incubation at 50 °C.
approximately 7% of the original after 24 h of incubation. Appropriate controls incubating acetyl xylan esterase alone or in combination with the hemicellulases did not show any release of ferulic acid. Neither did feruloyl esterase treatment alone or in combination with hemicellulases lead to the release of acetic acid (data not shown).

When the acetic acid and ferulic acid release were compared to the respective xylose release (Table 5), it was clear that deacetylation promoted the xylose release to a greater extent than deferuloylation in the insoluble material. Here the reaction ratio was 1:1, leading to the release of 1 mol equiv of xylose for every released mole equivalent of acetic acid. The corresponding release of ferulic acid was 0.39 mol of xylose released for every mole of ferulic acid (Table 5). This fact implied that catalysis by the endoxylanase was entirely dependent on the degree of substitution directly on the xylan backbone rather than on the removal of extended branching. Furthermore, these data support that reactions catalyzed by these types of enzymes (glycosyl hydrolases) could be promoted even on insoluble substrates if the appropriate activities were present and accompanying each other.

The opposite effect was observed for the solubilized oligosaccharides. Here the release of xylose compared to ferulic acid release was 1:1, whereas deacetylation caused only 0.5 mol equiv of xylose/mol equiv of acetic acid. In this case it would have been expected that the arabinose release would have increased equally, but this was not the case. As pointed out previously, arabinose release was affected only marginally by the presence of either of the esterases. As discussed later, this result has several implications, one of them being that the enzymes may be highly dependent on the overall substrate structure rather than just the presence of the bonds to be hydrolyzed.

**DISCUSSION**

The total release of the different components from both the soluble and the insoluble fractions is summarized in Table 6 and gives an indication of the remaining material to be hydrolyzed. Addition of esterases to the hemicellulases improved the overall degradation of the corn bran, with addition of AXE showing the highest increase. The cellulase blend further increased the release and therefore the full combination of enzymes released up to 15% of the xylose and 20% of the arabinose present in the original DCB. Assessment of the loss of individual components as a result of the pretreatment was difficult and is based on estimations, but would probably lie in the range of 30–35% for both xylose and arabinose when the total loss of material and the heat lability of these two monosaccharides, in particular, are considered. Taking the loss into consideration 50–55% of the xylose and 45–50% of the arabinose were still not released. A higher dosage of enzymes might further increase the yield as seen in ref 19, and a test with a 10 times higher dosage as compared to the results reported was performed. The increased dosage did release more arabinose and xylose from the soluble material; however, the effect on the insoluble fraction was negligible (data not shown). In the case of the soluble material, it might be relevant to operate at higher enzyme dosages, but this would have less industrial relevance and might, therefore, not be a feasible option. The pretreatment caused loss of valuable monosaccharides, so avoiding pretreatment would be preferable, but previous studies have shown that enzymatic hydrolysis on untreated corn bran can be exceptionally difficult (18), even though relevant activities were applied. The pretreatment used in the experiments presented here was based upon the findings of ref 19. Here thermal pretreatment promoted the release of xylose at temperatures above 180 °C by significantly solubilizing the biomass and rendering the insoluble residue more accessible to enzymatic attack. Loss of constituents in ref 19 was in the same range as that obtained in the experiments presented here. The relatively large losses of feruloyl (~41%) and diferuloyl (~73%) substituents caused by the pretreatment are of particular relevance to these experiments (Table 2). The loss was somewhat disproportional to the loss of other components and could be due to heat lability. The experimental setup and the data obtained did not allow us to provide any firm conclusions about the reason and origin of the ferulic acid losses. Despite the good results obtained so far, a more favorable pretreatment method appears to be desirable to make the biomass less recalcitrant to enzymatic attack without destroying valuable components.

The results showed that pretreating the substrate positively affected the enzyme accessibility on both fractions of the biomass. Of exceptional interest was the finding that acetyl xylan esterase could promote the xylose release from the insoluble corn bran to such a high degree as compared to the promotion induced by feruloyl esterase. As pointed out previously, the effects of deacetylation indicated that the endoxylanase activity toward the insoluble substrate was highly dependent on the exposure of the xylan backbone even when some arabinofuranosyl substituents remained. The relatively little effect of exposing arabinofuranosyls by deferuloylation could be the result of either low arabinose content in general or the fact that the deferuloylation in the insoluble material did not cause sufficient opening of the substrate for the relatively large arabinofuranosidase molecules (34). Usually it is recognized that feruloyl substitutions and cross-linkings hinder enzymatic (14) attack, but the experiments presented here and previously (25) speak to the fact that acetyl substitution plays an even more important role in the resistance toward enzymatic xylose release from insoluble xylan. Acetyl substitutions have for a long period of time been neglected in the literature, but AXE could be included with success in the battery of enzyme activities necessary to open and degrade corn bran.

The opposite effect of AXE and FAE activity in depolymerizing the solubilized oligosaccharides without the concomitant release of arabinose clearly envisions the diversity of the enzymatic attack performed by the same enzymes. The solubilized oligosaccharides represent a completely different substrate both with respect to substitution pattern, degree of substitution, and most probably also the degree of polymerization (DP) as

| Table 5. Xylose Yield per Released Substituent (Acetic Acid or Ferulic Acid) a |
|-------------------------|-------------------------|-------------------------|
| AXE (mol of xylose/mol | FAE (mol of xylose/mol |
| of acetic acid)        | of ferulic acid)       |
| insoluble              | 1.06                    | 0.39                    |
| soluble                | 0.51                    | 1.01                    |

| Table 6. Total Conversion of Pretreated Corn Bran after 24 h of Enzymatic Hydrolysis a |
|-------------------------|-------------------------|-------------------------|
|                        | xylose                 | arabinose              | acetic acid       | ferulic acid     |
| mini                   | 8.6                     | 19.0                    |
| mini + AXE             | 12.4                    | 19.4                    | 23.5              |
| mini + FAE             | 10.9                    | 18.6                    | 48.1              |
| mini + AXE + FAE       | 13.3                    | 20.1                    | 21.6              | 48.2              |
| mini + CTEC + AXE + FAE| 15.0                    | 20.2                    | 23.3              | 48.2              |

"a Yield expressed as mole equivalents of xylose released per mole equivalent of acetic acid/ferulic acid released when acetyl xylan esterase or feruloyl esterase is added to the minimal cocktail AXE, acetyl xylan esterase; FAE, feruloyl esterase.

The opposite effect of AXE and FAE activity in depolymerizing the solubilized oligosaccharides without the concomitant release of arabinose clearly envisions the diversity of the enzymatic attack performed by the same enzymes. The solubilized oligosaccharides represent a completely different substrate both with respect to substitution pattern, degree of substitution, and most probably also the degree of polymerization (DP) as
The increased release of xylose occurring with deferylolation without the simultaneous release of arabinose could indicate that the feruloyl substitutions hindered the endoxylanase and/or β-xylosidase action differently from steric hindrance alone. The endoxylanase used here was from the GH10 family of xylanases known to attack close to substitutions (9), and even though the study in question focused on glucuronoxylans, this particular catalytic capability of GH10 xylanases was also shown on heteroxylans and acetylated xylans. However, hydrophobic interactions, such as feruloylation, could possibly influence how close to arabinofuranosyl substitutions the GH10 xylanase would attack the substrate, and deferylolation would therefore remove this obstacle for xylose release. Combined with the presumed low DP of the substrate, this could perhaps make it possible for the endoxylanase to associate with the substrate closer to arabinofuranosyl substitutions compared to the distance necessary on the insoluble substrate. The lack of arabinose release further brings into question the significance of the present α-L-arabinofuranosidases. However, if arabinofuranosyl substitutions were to some degree made up of short chains rather than entirely of single-moiety substitutions (4, 6), ferulic acid may be positioned along these short chains. The release of ferulic acid from this position would then in turn not render the arabinofuranosyl substitutions available for the α-L-arabinofuranosidases, and therefore simultaneous release of arabinose would not occur. It could also be speculated that ferulic acid was not solely esterified to arabinose but also to xylose in the same manner as acetyl residues. The release of ferulic acid would then open the xylan backbone for endoxylanase attack. Yet no previous suggestions toward this argument have been reported in the literature, and it therefore seems less plausible.

These results also signified that the degree of substitution on the poly-/oligomeric substrates was not necessarily a hindrance for the release of ferulic acid as postulated previously (21, 22). In these experiments it has been evident that the highly substituted material was more accessible to both esterases. However, the accessibility was most likely related to the solubility of the substrate rather than the degree of substitution. Even though the extent of substitution had declined in the insoluble arabinoxylan as a result of pretreatment, it might have only resulted in a limited boost in enzymatic accessibility. Findings have shown that longer stretches of unsubstituted xyloid could precipitate as a result of hydrogen bonding (24) and thereby leave the substrate enzymatically unavailable. Furthermore, the results here clearly show that the general enzymatic degradation was higher on the soluble substrate and that the initial reaction rate of AXE was also highest when the substrate was soluble (data not shown). The results demonstrate that the tight cooperation of different enzyme activities on polymeric substrates can make even the smallest, seemingly insignificant, activity the link that sustains the continued action of all the other enzymes and that substrate solubility is a vital factor for enzyme accessibility. Future work will have to focus on matching new enzymes to the present recognized battery of arabinoxylan-degrading enzymes and work toward increasing the substrate solubility without the loss of valuable components.

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4 Exploring endo-xylanases, feruloyl esterases and arabinosidases

The lesson from the initial results in Paper 1 is first and foremost that the overall hydrolysis of arabinoxylan is limited. Therefore it becomes vital to evaluate the performance of the basic set of hemicellulases and the following chapter concerns the choice of endo-xylanase and arabinofuranosidases. Both unpretreated DCB and the soluble corn bran fraction obtained after hydrothermal pretreatment in Paper 1 are used as substrates.

4.1 Endo-xylanases and feruloyl esterases

Two potential candidates of endo-xylanases has been chosen and evaluated against the performance of the GH10 endo-xylanase from H. insolens. These two both originate from A. aculeatus and are available under the commercial names Shearzyme (GH10) and BioFeed Wheat (GH11) from Novozymes A/S. These two are chosen because they have been well-described in literature (Rantanen et al., 2007, Vegas et al., 2008) as efficient xylanases. They are applied in a purified form. GH10 endo-xylanases are known to catalyse hydrolysis of the xylan backbone close to substituents, whereas GH11 preferably catalyse the hydrolysis of bonds in a further distance from substitutions (Kormelink et al., 1993). Initial evaluation of their performance is done by incubating the three xylanases individually at enzyme dosages of 0.5 mg/g DM with unpretreated DCB in a 2% DM suspension for 24 hours at pH 5, 50 °C. Hereafter the amount of solubilized dry matter and the concentration of reducing ends has been measured and compared to a blank sample containing no enzyme and the results are shown in figure 10.

![Figure 10: Solubilization of DCB by endo-xylanases measured as solubilized dry matter (DM, primary axis) and reducing ends (secondary axis) after 24 h incubation, pH 5, 50 °C at 2% DM concentration.](image)

The general impression is that neither of the endo-xylanases is capable of solubilizing noteworthy amounts of material, even though the GH10 from H. insolens seems to have larger impact than the other two. The results of GH10 and GH11 from A. aculeatus are not different from the control. Notably, enzyme
loadings are made according to enzyme protein concentration, but comparison of specific activity reveal that GH10 and GH11 from *A. aculeatus* both have higher specific activity towards AZO-Wheat arabinoxylan than the GH10 from *H. insolens*. GH10 from *H. insolens* has a specific activity of 2 U/mg/min, whereas GH10 and GH11 from *A. aculeatus* have 4 and 16 U/mg/min respectively at vendor’s assay specifications (Megazyme, 1% w/v, 40 °C, pH 4.5 for 10 min). These results again prove the recalcitrance of corn bran to enzymatic hydrolysis and further evaluation of the endo-xylanases is performed on the soluble corn bran fraction.

**Figure 11**: Xylose release from soluble corn bran fraction (Agger et al., 2010 Paper 1) after 5 hours incubation, pH 5, 50 °C, 2% DM with endo-xylanases and β-xylosidase.

The three endoxylanases were tested alone and in combination with each other. All experiments also contained β-xylosidase and the effect of the endo-xylanases is evaluated indirectly by xylose release after 5 hours incubation at pH 5, 50 °C in a 2% solution of the soluble corn bran. Endo-xylanases and β-xylosidase are dosed at 0.5 mg EP/g DM each. Figure 11 shows that GH10 is most efficient on this particular substrate. There seems to be only little difference between the catalysis by GH10 from *H. insolens* and *A. aculeatus* and furthermore, no significant effect of combining GH10 and GH11. The latter result could indicate that the xylan backbone is substituted in a manner so the additional specificities of GH11 are needless or that the DP of the xylan structures is lower than what GH11 prefers as substrate.

Due to possible synergistic effects between endo-xylanases and feruloyl esterases an additional test also including all three endo-xylanases is made. In particular, a purified feruloyl esterase type B from *H. insolens* has been available for my work and is a promising candidate for obtaining increased diferulic acid and xylose release. Previous results have proven diferulate release using a *H. insolens* preparation (Ultraflo™ L) for digesting brewer’s spent grain (Faulds et al., 2002+2004). This particular preparation shows prevalent feruloyl esterase type B activity and is therefore particularly unusual and interesting, since type B esterases are commonly known not to release diferulates and also to have a preference for
feruloyl substitutions in the O-2 position on arabinosyls or the O-6 position on galactosyls. FAE-B was tested in combination with FAE-A from *A. niger* (FAE-III) and the three endo-xylanases on the soluble corn bran fraction in a setup like the one described above. Feruloyl esterases were loaded at 1 mg EP/g DM each. Figure 12 generally shows that FAE-A from *A. niger* is superior in catalyzing the release of ferulic acid from the soluble corn bran. FAE-B for *H. insolens* only catalyses limited release in comparison and generally no synergistic or promoting effects from the endo-xylanases exist. This is in direct contrast to the otherwise common conception that endo-xylanases boost the catalysis by feruloyl esterases (Faulds and Williamson 1994, Faulds and Williamson 1995, Bartolomé and Gómez-Cordovés 1999, Faulds et al., 2002). However, the fact that the substrate is fully soluble most probably causes the feruloyl esterases to be less dependent on concomitant solubilization/disassociation of the substrate, as the feruloyl groups are positioned freely for the enzymes to react with.

![Ferulic acid release from soluble corn bran fraction with treated with feruloyl esterase FAE-A and FAE-B alone and in combination with endo-xylanases after 5 h incubation, pH 5, 50 °C at 2% DM concentration.](image)

With respect to diferulic acids; in these experiments only FAE-A from *A. niger* is capable of releasing diferulic acid from soluble corn bran, and the two configurations detected are the 8-O-4′- and the 5,5′-form. FAE-A releases up to 1.6 g/kg DM of the 5,5′-form and 0.9 g/kg DM of the 8-O-4′-form which corresponds to approx. full release and 50% release respectively. The concentration of dehydrodimers of ferulic acid is relatively low in this particular substrate and based on the available data, it is not possible to say, whether FAE-A is capable of releasing the diferulic acids from the mono- or the diester form. As is the case for ferulic acid release, no promoting effects of endo-xylanases are observed on the diferulic acid release (data not shown). This can again be related to the apparent availability of the substrate when in a soluble form. Previous investigations of FAE-A activity on the native DCB showed no release of diferulic acids.

No release of diferulic acids are observed in samples with purified FAE-B from *H. insolens* and ferulic acid release is poor compared to the release promoted by FAE-A from *A. niger* (figure 12). This is contradictory
to previous findings (Faulds et al., 2002) with two possibly explanations. Either the FAE-B purified for these studies is not the same as the one causing the observed effects of Faulds et al., 2002, or the effects observed in 2002 was not caused by a type B esterase even though the preparation showed major type B activity. Minor type A side activities may have caused the diferulic acid releases observed in those studies. Otherwise, the differences in catalytic activities observed here between FAE-A and FAE-B is in good correlation to the common conception of feruloyl esterase specificities, where type B esterases has limited activity towards the O-5 positioned feruloyl groups.

Based on the findings here it is concluded that the GH10 endo-xylanase from H. insolens is in fact among the best available candidates and therefore used further on.

4.2 Arabinosidases

Due to an incomplete release of arabinose from both native corn bran, but indeed also from the soluble corn bran fraction, the specificity of the two α-L-arabinofuranosidases (GH43 from H. insolens and GH51 from M. giganteus) are somewhat questioned. It is known from previous studies on wheat arabinoxylan that the GH43 from H. insolens is capable of releasing the O-3 positioned arabinofuranosyls from doubly substituted xylose moeties, whereas the GH51 from M. giganteus releases the O-2 and on the O-3 positioned arabinofuranosyls on single substituted xylose (Sørensen et al., 2006). In this manner, complete arabinose release should be possible, if these two arabinofuranosidases would catalyse in a similar manner on corn bran and if the positioning of arabinofuranosyls are comparable to wheat bran.

To test if arabinosyls may somehow be positioned as oligomeric structures and not solely as terminal substitutions, an endo-acting arabinosidase activity was tested on the soluble corn bran fraction. The soluble corn bran was chosen over the native corn bran for these experiments both because arabinose content is high and because other factors like substrate inaccessibility may have created false negative results. From A. nidulans a GH43 endo-α-(1,5)-arabinosidase (AN3044.2, Bauer et al., 2006) expressed by P. pastoris in house has been tested in combination with the two known arabinofuranosidases. If the arabinosyls are indeed presents as α-1,5-arabinan structures this may in turn improve the xylose release. Therefore, the arabinosidases are incubated with endo-xylanase GH10 from H. insolens, β-xylosidase, feruloyl esterase FAE-A and acetyl xylan esterase for 24 hours, pH 5, 50 °C in a 2% DM solution. The results show (figure 13) that the GH43 of A. nidulans is not capable of releasing any arabinose from the soluble corn bran fraction. The fact that this arabinosidase do not catalyse any immediate reactions in soluble corn bran implies either that α-1,5-arabinan is not present or that the reaction is not detected due to lack of exo-activity for the release of arabinose. It does not necessarily prove that arabinan structures are not present. However, xylose release is not affected by the presences of this endo-arabinase and this implies that arabinan structures associated with xylan is either not of an α-1,5-kind or not present at all. If arabinan structures are present they may be more prevalent in other configurations for instance as α-1,2- or even β-arabinan.
The effects of leaving out either of the two α-L-arabinofuranosidases is that interestingly enough, GH43 from *H. insolens* is not capable of releasing any arabinose by itself, but boost the arabinose release when in combination with GH51 from *M. giganteus*. This indeed suggests that the mechanisms for these two arabinosfuranosidases are similar to that observed for wheat arabinoxylan (Sørensen et al., 2006). Xylose release is also positively affected by the combined action of the two.

Figure 13: Results of testing arabinosidases on soluble corn bran fraction when added in combination with endo-xylanase, β-xylosidase, acetyl xylan esterase and feruloyl esterase after 24 h incubation, pH 5, 50 °C at 2% DM concentration.

In addition to the GH43 and GH51 already applied, two α-L-arabinofuranosidases from *A. nidulans* belonging to the GH54 (AN1571.2) and GH62 (AN7908.2) family have been tested (Bauer et al., 2006), following the idea that possible hemicellulose binding domains in these families and the action of yet other kinds of arabinofuranosidases may target the arabinosyl substitution on soluble corn bran better, and thereby increase the yields (Miyanaga et al., 2006, Hashimoto et al., 2011). However, neither of the two arabinofuranosidases is able to enhance the release of arabinose when combined with the known GH43 and GH51 (data not shown).

All in all the results presented here indicate that the basic set of four hemicellulases, endo-xylanase GH10 from *H. insolens*, β-xylosidase GH3 from *T. reesei*, α-L-arabinofuranosidase GH43 from *H. insolens* and GH51 from *M. giganteus* is a suitable set of enzymes for targeting corn bran arabinoxylan, even though the way to complete hydrolysis is still long. Furthermore, the choice of FAE-III from *A. niger* as a suitable feruloyl esterase is also established. In order to improve the hydrolysis, it seems necessary to target the arabinose release, as this will most probably induce an extended xylan hydrolysis. Therefore, clues to understanding why arabinose release at present is limited are of great importance.
5 Exploring soluble corn bran arabinoxylan

The soluble corn bran fraction from the hydrothermal pretreatment in Agger et al., 2010 Paper 1 is an interesting substrate, exactly because it features one of the most desirable physical properties, namely solubility. It is therefore additionally motivating to try to comprehend why these structures are recalcitrant and in that sense the soluble corn bran fraction has been further subjected to analysis by linkage analysis and mass spectrometry.

5.1 Materials and methods

A sample for linkage analysis was sent to M-Scan (in Wokingham, UK) how performed the analysis by permethylation with NaOH/methyl iodide for 2 hours, followed by purifying extraction in chloroform. Hereafter the sample was hydrolysed by 2 M TFA for 2 hours, 120 °C and reduced with NaBD₄ in 2 M NH₄OH for 2 hours at room temperature. Hereafter washed with 3 additions of methanol in glacial acetic acid (90:10) followed by lyophilization. The sample was then acetylated by acetic anhydride for 1 hour at 100 °C and purified by chloroform extraction. Finally, the partially methylated alditol acetates were analysed by GC-MS (Perkin Elmer Turbomass Gold mass spectrometer coupled to an autosystem XL gas chromatograph) and compared to a mixture of standards. The GC column was a 30 m x 0.32 mm silica capillary with DB-5MS coating with 1.5 ml/min helium as carrier gas. The run was 1 min held at 40 °C, 25 °C/min up to 100 °C, 8 °C/min up to 290 °C and ended by holding at 290 °C for 5 min. The MS was operated with ionization voltage of 70 eV in a scanning acquisition mode within a mass range of 50-500 Da. Results were provided by MScan as a spectrogram.

Mass spectrometry data has been obtained after MALDI-TOF executed at Univeristy of Southern Denmark, Department of Biochemistry and Molecular Biology, where 1 µl of sample and 0.5 µl of matrix solution (20 mg/ml 2,5-dihydroxybenzoic acid, 70% acetonitrile and 0.1% trifluoroacetic acid/water) was added to an Opti-TOFTM 384 well plate. For fast crystallization the sample was dried under a lamp and a further 0.5 µl of matrix solution added. The samples were analyzed on a 4800 Plus MALDI TOF/TOFTM (AB SCIEX) mass spectrometer. The instrument was operated in reflector, positive ion mode. Acceleration voltage was 20kV. Depending on the sample analyzed, laser intensity and number of laser shots were varied to obtain optimal spectra. The mass range was set to 100-2000 Da. The MS data were exported as text files using DataExplorer (version 4.6) and each spectrum were smoothed labeled and analyzed manually employing M/Z (Genomic Solutions®).

Before analysis by MALDI-TOF a sample of the soluble corn bran has been partly purified by reverse phase chromatography on ODS-L Optimal (250 x 4.6 mm, 5 µm) from Capital HPLC Ltd., Scotland using a standard elution profile (Agger et al., 2010 Paper 1). Figure 14 shows the chromatography profile of the fractionation run and collection was done from 3 to 12 min as marked by punctured lines. The spectral data from this part of the chromatographic run indicate occurrence of feruloylated species in the eluting compounds, especially in peak at 7 min and 10 min (data not shown).
Finally, a sample of soluble corn bran has also been analysed by high-preformance size exclusion chromatography using P680 HPLC column, an ASI-100 sample injector and an RI-101 refractive index detector (Dionex Corp, Sunnyvale, CA) equipped with a Shodex SB-G guard column (50 x 6 mm) from Showa Denko K.K. (Tokyo Japan). Elution was done isocratically for 30 min with water at a flow rate of 0.5 ml/min (Rasmussen and Meyer 2010).

5.2 Results and discussion

The results of linkage analysis in figure 15 and table 4 confirm the general understanding of the soluble corn bran since pentoses generally dominate the spectrogram. The linkage analysis is not directly quantifiable even though peak intensities do represent a certain estimate of the abundance. In table 4 a summary of all the observed linkages is given ordered according to apparent prevalence. The analysis is capable of distinguishing between pyranose and furanose forms, but not when the pyranose involves in an O-4-linkage and when the furanose involves in an O-5-linkage. They most often have the same retention time and annotation of structural origin is therefore based on an assessment. The most prevalent linkage confirmation (table 4) is a 1,4-linked pyranose (or 1,5-linked furanose). Based on the knowledge that xylose constitutes the majority of the pentoses and is probably mostly confined to the xylan backbone, it is reasonable to judge that this compound (RT 13.64 min) is indeed evidence of the 1,4-β-linked xylan backbone. Furthermore, RT 13.54 min contains the same possible combination of linkages, so it is likely in this case, that it actually represents a 1,5-linked arabinose (possibly esterified arabinosyl substitution on the xylan backbone). Certainly, this linkage could also indicate α-1,5-linked arabinan, but the relatively high intensity of the peak makes it less likely, and speaks more of the possibility of O-5 esterified arabinosyls.

It is also evident that terminal arabinose is the major form of arabinose (RT 11.7 min). At the same time there is a slight overrepresentation of the O-3 substituted xylose (RT 14.74 min) compared to the O-2
substituted xylose (RT 13.08 min). This does not inform whether arabinosyl substitutions are more prone to the O-3 position than the O-2 because acetylations will also contribute significantly to this substitution pattern. Besides, the O-2 linkage on xylose may also originate from xylose in heterogeneous side chains as those observed by Allerdings et al., 2006 and Saulnier et al., 1995a.

Interestingly enough the compound with retention time 14.77 min indicate arabinose with O-5 esterification while being at the same time linked by the O-2 position to another unit and proves the very existens of other configurations of arabinose. If in fact the O-5-linked compound is an esterification, it indicates that it is arabinosyls from arabinoxylan and not pectin derived arabinan, since an esterification will then be located at the O-2 position. Indeed, the linkage analysis also provides information of a 1,3-linked pyranose, which may originate from yet another heterogeneous side chain on arabinoxylan or from a substitution on the xylosyl unit in the non-reducing end. Fully linked pyranose and/or furanose are generally low in abundance.
Table 4: Summary of linkage analysis of soluble corn bran with possible structural origin. Assigning structural evidence is based on an assessment of the most abundant confirmation. Text in paranthesis indicate the lesser abundant/likely structural origin. *confirmed by M-Scan that both configurations are present in the same peak.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Retention time</th>
<th>Peak intensity</th>
<th>Structural evidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,4-linked pentose (pyranose)</td>
<td>13.64</td>
<td>+++</td>
<td>Xylan backbone</td>
</tr>
<tr>
<td>1,5-linked pentose (furanose)</td>
<td>11.7</td>
<td>+++</td>
<td>Terminal arabinose substitutions</td>
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<tr>
<td>Terminal pentose (furanose)</td>
<td>14.74</td>
<td>+++</td>
<td>O-3 substituted xylose</td>
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<td>1,3,4-linked pentose (pyranose)</td>
<td>1,27</td>
<td>++</td>
<td>Non-reducing end of xylan backbone</td>
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<td>1,3-linked pentose (furanose)</td>
<td>13.08</td>
<td>++</td>
<td>O-3 substituted arabinose (confirmed)*</td>
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<tr>
<td>1,2-linked pentose (pyranose)</td>
<td>13.08</td>
<td>++</td>
<td>O-2 substituted xylose (confirmed)*</td>
</tr>
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<td>13.54</td>
<td>++</td>
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<td>Terminal galactose substitution (or non-reducing end of galactan)</td>
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<td>+</td>
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<td>14.08</td>
<td>+</td>
<td>Non-reducing end of glucan (or terminal glucose substitution)</td>
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<td>+</td>
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<td>+</td>
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<td>+</td>
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<td>Fully substituted xylose (or arabinose)</td>
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<td>1,3,6-linked galactose</td>
<td>17.22</td>
<td>+</td>
<td>Galactan branch point (Type II)</td>
</tr>
</tbody>
</table>

Galactose seems to be the most dominating hexose, possible as terminal substitutions on arabinoxylan (RT 14.41 min). Glucose is also present as terminally positioned but only in minor amounts and this may originate from the non-reducing end of cellulose or β-glucan. Indeed, both 1,3- and 1,4- linkages to glucose is present (RT 15.29 and 15.41 min). Different linkages to galactose indicate that arabinogalactans of both type I and type II exist in corn bran and this is probably related to either pectinaceous substances or even
to glycosylations of structural proteins (AGPs). The latter will be further addressed in the following chapter.

Figure 16: Mass spectrum of soluble corn bran fraction up to ~1200 Da. * Indicate dehydrated series.

The linkage analysis data has been supplemented by MALDI-TOF data. Figure 16 and the identified peak series in table 5 and 6 show that pentose series dominate the spectrum with respect to diversity, but hexose series are dominating with respect to intensity. However, the latter must not necessarily be interpreted as a measure of concentration as it merely illustrates which molecules respond best to ionization. This seems plausible in comparison to the low abundance of hexoses in the linkage analysis and in the monosaccharide composition in general (Agger et al., 2010 Paper 1). The general observation is that series with acetylations are common and the presence of hexoyl substitutions on the pentose backbone is also common. The m/z values in table 5 for unsubstituted pentose series (POx Na) cover a whole range of possible combinations of these, as the data does not distinguish between arabinose and xylose and does not inform about substitution pattern. The acetylated species may also display a certain variety, even though the size of the base peak for for instance POxHO1 Ace indicates that the acetylation and the hexoyl group are both positioned at the same pentose unit. The same is the case for the base peak of the double acetylated species proving that double acetylated xylose moieties do occur, whereas base peak for the species with three acetylations corresponds to two pentose units.
On the contrary, feruloylated species are observed far less in the spectrum, despite the fact that both hydrolysis and total saponification experiments show that they are present. There is only one signal from such a series and it is low in intensity and may not even represent a true response. In this context it is also important to emphasise that annotating feruloyl groups to the mass of 176 may be over interpretation since uronic acids eg. glucuronic acid also weighs 176 in a dehydrated form. The reason for the low detection of feruloylated species can be related to lower susceptibility to ionization for these compounds compared to some of the others. Interestingly enough a dehydrated counterpart to the hydrated feruloyl series (m/z 463, 595, 727) is observed. Neither of the other pentose series is observed in a dehydrated form, and the dehydration may be an artifact from the hydrothermal pretreatment and may not be a true representation of the native substrate.

It should also be noted that some m/z values appear more than one time in table 5 and 6, e.i. 509, 519 and 833. This indicates that one peak may originate from more than one component because the mass fits in several series.

**Table 5: MS data for pentose series in soluble corn bran fraction. First m/z value in column indicate base peak.**

<table>
<thead>
<tr>
<th>Pentose series</th>
<th>m/z</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>POx</td>
</tr>
<tr>
<td></td>
<td>Na</td>
</tr>
<tr>
<td>PO1</td>
<td>(173)</td>
</tr>
<tr>
<td>PO2</td>
<td>305</td>
</tr>
<tr>
<td>PO3</td>
<td>437</td>
</tr>
<tr>
<td>PO4</td>
<td>569</td>
</tr>
<tr>
<td>PO5</td>
<td>701</td>
</tr>
<tr>
<td>PO6</td>
<td>833</td>
</tr>
<tr>
<td>PO7</td>
<td>965</td>
</tr>
<tr>
<td>PO8</td>
<td>1097</td>
</tr>
<tr>
<td>PO9</td>
<td>1229</td>
</tr>
</tbody>
</table>

*indicate peaks with low intensity.

**Mass also fits with dehydrated pentose serie as sodium adduct. First m/z value in column indicate base peak.
The fact that several hexose series (table 6) appears in the spectrum is somewhat unexpected, in particular since the major hexose series is dehydrated and up to a 7-mer. Also unknown hexose series and a series with an additional 176 appear informing that cellulose is not the only hexose-derived polymer structure to consider in this substrate. Compared to the recently described linkage analysis, this unknown structure may be related to galactan an in this case it is plausible that the mass equivalent of 176 stems from a feruloyl group on this galactan. However, as already mentioned the peak intensities in these MS spectra can not be directly translated into a measure of content and hexose series are most probably not as abundant as they appear in figure 16.

In order to improve the signal from feruloylated species a partial purification of the soluble corn bran is performed prior to MALDI-TOF (figure 14) with the aim of removing some of the intense responses from unsubstituted pentoses and hexoses. MS data show (table 7, figure 17 and 18) that the signals with masses matching feruloylated species have indeed improved and hexose series has virtually disappeared from the spectrum. At the same time, DAD spectral data from the chromatographic elution profile (figure 14) indicate that feruloyl groups is present in the sample and therefore it is believed that mass equivalents of 176 represent feruloyl groups. Furthermore, one series with difenuloyl substitution is also observed notably with a base peak of two dehydrated pentoses (m/z 655). It is not possible based on these data to say, whether some of the difenuloyl related peaks with higher m/z value represent actual cross-linked oligomers, but it seems plausible that the peaks at least represent a mixture of mono- and diester-linked difenuloyls.

### Table 6: MS data for hexose series in soluble corn bran fraction. First m/z value in column indicate base peak

<table>
<thead>
<tr>
<th>Hexose series</th>
<th>Dehydr. HOx Na</th>
<th>HOx + 176</th>
<th>Unknown</th>
<th>Unknown</th>
</tr>
</thead>
<tbody>
<tr>
<td>HO1</td>
<td>-</td>
<td>357</td>
<td>529</td>
<td>403</td>
</tr>
<tr>
<td>HO2</td>
<td>347</td>
<td>519</td>
<td>691</td>
<td>565</td>
</tr>
<tr>
<td>HO3</td>
<td>509</td>
<td>681</td>
<td>853</td>
<td>727</td>
</tr>
<tr>
<td>HO4</td>
<td>671</td>
<td>843</td>
<td>-</td>
<td>889</td>
</tr>
<tr>
<td>HO5</td>
<td>833</td>
<td>1005</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HO6</td>
<td>995</td>
<td>1167</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HO7</td>
<td>1157</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 7: MS data for soluble corn bran fraction after partial purification by RP-HPLC.

<table>
<thead>
<tr>
<th>Pentose series</th>
<th>m/z POx Na</th>
<th>POx Ace Na</th>
<th>POx Fer Na</th>
<th>POx Fer* Na*</th>
<th>POx DiFA Na</th>
<th>POx HO1 Na</th>
<th>POx HO1 Unknown</th>
<th>Unknown</th>
<th>Unknown</th>
<th>Unknown</th>
</tr>
</thead>
<tbody>
<tr>
<td>PO1</td>
<td>173</td>
<td>-</td>
<td>349</td>
<td>309</td>
<td>-</td>
<td>511</td>
<td>335</td>
<td>351</td>
<td>-</td>
<td>365</td>
</tr>
<tr>
<td>PO2</td>
<td>305</td>
<td>374</td>
<td>481</td>
<td>441</td>
<td>655</td>
<td>643</td>
<td>467</td>
<td>483</td>
<td>493</td>
<td>497</td>
</tr>
<tr>
<td>PO3</td>
<td>437</td>
<td>479</td>
<td>613</td>
<td>-</td>
<td>787</td>
<td>775</td>
<td>599</td>
<td>615</td>
<td>625</td>
<td>629</td>
</tr>
<tr>
<td>PO4</td>
<td>569</td>
<td>611</td>
<td>745</td>
<td>-</td>
<td>919</td>
<td>-</td>
<td>747</td>
<td>-</td>
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<tr>
<td>PO5</td>
<td>701</td>
<td>743</td>
<td>877</td>
<td>-</td>
<td>1051</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PO6</td>
<td>833</td>
<td>875</td>
<td>1009</td>
<td>-</td>
<td>1183</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PO7</td>
<td>965</td>
<td>1007</td>
<td>1141</td>
<td>-</td>
<td>1315</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PO8</td>
<td>1097</td>
<td>1139</td>
<td>1273</td>
<td>-</td>
<td>1447</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>PO9</td>
<td>1229</td>
<td>1271</td>
<td>1405</td>
<td>-</td>
<td>1579</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>PO10</td>
<td>1361</td>
<td>1403</td>
<td>1537</td>
<td>-</td>
<td>1711</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PO11</td>
<td>1493</td>
<td>1535</td>
<td>1669</td>
<td>-</td>
<td>1843</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PO12</td>
<td>1625</td>
<td>1667</td>
<td>1801</td>
<td>-</td>
<td>1975</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PO13</td>
<td>1757</td>
<td>1799</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PO14</td>
<td>1889</td>
<td>1931</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Dehydrated

Partially purifying the sample also improved the signal for the larger molecules, and the largest oligosaccharides (m/z 1889-1931, table 7) correspond to up to a 14-mer even though it is likely that this represents branched structures of arabinoxylan and not a linear xylan backbone, owing to the high content of arabinose. Furthermore, the spectrum in figure 17 also shows an array of other peaks (as exemplified in figure 18) that do not seem to originate from any particular series and some peaks where it has not been possible to annotate a certain mass. In order to pursue the peak annotation, MS/MS was applied to certain peaks (m/z 335, 377, 479, 509, 641, 773 and 905). However, the results turned out poor and provided no additional information, because one peak seldom represents a single compound (data not shown).

Also in the purified samples the data shows that acetylations are common. The frequent appearance of different configurations of acetyl substituted pento-oligosaccharides in the MS-data also stresses the importance of targeting this specific group in an enzymatic hydrolysis, as is also observed in Agger et al., 2010 Paper 1. On the other hand, it is somewhat surprising that feruloyl substituted species are less common in the MS spectra, since it is also concluded from Paper 1, that deferuloylation is particularly important in the soluble fraction. This can again be related to relatively low ionization during the MS analysis and may not actually represent the true distribution and variety between acetylated and feruloylated species.
Figure 17: Mass spectrum of soluble corn bran fraction after partial purification by RP-HPLC.

Figure 18: Mass spectrum for soluble corn bran fraction after partial purification by RP-HPLC. Zoom of figure 17 (m/z ~500-750).
Diferuloylated species are rare in the MS spectra and only appear after purification by reverse phase chromatography. Biomass compositional analysis also point to the fact that diferulic acid content is low in the soluble fraction, possibly due to degradation during the pretreatment process. The relatively low occurrence of these species also suggests that continued resistance towards enzymatic hydrolysis is not originating from cross-linking/substitution by diferuloyls, but possibly more related to the heterogeneous nature of the carbohydrate-related substitutions.

In that context speculations as to the structure of clean pento-oligosaccharides resistant to degradation can be as that in figure 19. Configuration no. 1 illustrates the possibility that arabinose is positioned as short side chains instead of only as terminal substitutions. These are resistant to the attack by the α-L-arabinofuranosidases used in these experiments and the hydrolysis of substitutions in the neighboring area may also be affected. Even though the two employed arabinofuranosidase activities together are capable of catalyzing hydrolysis of doubly substituted arabinosyls, close proximity of these may also pose a barrier for extended hydrolysis as may be the case in conformation no. 2. Finally, if side chains are of a more heterogeneous nature, enzymatic hydrolysis may also be restricted as for configuration no. 3 in figure 19.

Figure 19: Possible configurations of enzyme resistant pento-oligosaccharides corresponding to the mass of PO14

With respect to the size of the oligosaccharides in the soluble corn bran, the MS data provide evidence of up to 2 kDa, but a size exclusion chromatography profile reveals that even larger substances are present (figure 20). The profile shows a major peak in the lower range of 40-17 kDa but larger molecules start to elute up to 10 min before the biggest standard of 110 kDa and seems to peak around 18-20 min. This indicates that larger molecules are present. The actual size of these can not be directly determined, but the profile indicates molecular sizes bigger than 400 kDa (Rasmussen and Meyer, 2010).

Figure 20: SEC profile for soluble corn bran, red curve. Standards of 17.2 (purple), 40 (blue) and 110 (green) kDa included. Major peak at approx. 24 min represents eluent interference peak and do not originate from the samples.
All in all the linkage and MALDI-TOF analysis of soluble corn bran has revealed that it is a highly diverse mixture of heterogeneous structures and as expected mostly dominated by arabinoxylans of varying sizes. The MALDI-TOF furthermore confirms the presences of doubly acetylated xylosyls. Based on these data it is possible to speculate configurations of arabinoxylan that the applied set of hemicellulases will have difficulties degrading thus confirming that the structural heterogeneity is possibly a major limitation to the extent of hydrolysis in the soluble substrate. In turn these structures will require that the debranching enzyme activities are exceptionally close shaving and not inhibited by close sitting side chains possibly of varying length and hydrophobicity (in the case of acetylations and feruloyl groups). The data also confirms the presence of hexoyl structures, mostly dominated by galactans and possibly arabinogalactan.
6 Hydroxyproline rich glycoproteins in corn bran

The following chapter concerns the abundance and composition of structural, glycosylated proteins and other residual proteins in corn bran. This work is done in order to further investigate the hypothesis that especially arabinose and galactose in corn bran may originate from other structures than arabinoxylan as both indicated by MALDI-TOF and linkage analysis on soluble corn bran. In turn, this may explain some of the incomplete enzymatically catalysed release of monosaccharides from corn bran (Paper 1, 2 and 3). Furthermore, the presence of polymeric interactions between polysaccharides and proteins may also inhibit the overall enzymatic hydrolysis and this issue has been addressed as well. First, an introduction to cell wall associated structural and storage proteins is given based on a literature review and hereafter own results on the matter is presented.

6.1 Structural proteins in general

Structural proteins are described by the generic term hydroxyproline rich glycoproteins (HPRGP) and are generally recognized by their content of hydroxyproline and significant degree of glycosylations. Hydroxyproline stems from a posttranslationally modified proline residue, present in the protein backbone of HPRGP and it is unique for plant cell wall proteins. It was first proved by Lamport and Northcote in 1960. Still, in order to categorize a protein as hydroxyproline rich, the content has to be higher than 5% (Sommer-Knudsen et al., 1998). Another common feature for HPRGPs is the glycosylation pattern, which is almost exclusively made up by arabinosyl and galactosyl moieties, O-linked to hydroxyproline or serine (Fry, 1988). Due to the nature of HPRGPs it is not possible to uncover the proteins solely based on DNA sequencing because their unique trademarks are expressed after translation. Historically this has made it significantly difficult to identify the HPRGPs. Furthermore, the pattern of glycosylation has also proven complicated to establish, as the O-linkages are hard to break and therefore hydrolysis experiments result in partial degradation of the peptide backbone leaving a puzzle of partly glycosylated pieces behind. However, with the latest development in bioinformatics techniques it was recently shown that 166 new HPRGPs from Arabopsis thaliana could be identified by BLAST and sequence alliances based on certain common features (Showalter at al., 2010). Still, it is only partly possible to predict the glycosylation pattern.

Hydroxyproline rich glycoproteins are among the best studied type of plant proteins and main emphasis has been put into describing dicot HPRGPs as they are more abundant than monocot HPRGPs (Kieliszewski et al., 1992). The most common amino acids found in HPRGPs are glycine, alanine, valine, leucine, proline, hydroxyproline, serine, threonine, glutamine, lysine, histidine, tyrosine and tryptophane. The structural proteins are mostly confined to the primary cell walls probably due to their physiological roles, however other localizations has also been reported. Generally speaking, the HPRGPs can be divided into four groups (Harris 2005, Showalter 2001, Kieliszewski and Shpak, 2001, Kieliszewski 2001, Sommer-Knudsen et al., 1998), one of which is not strictly a HPRGP but still comparable. Only two of these
groups, namely extensins and AGPs (arabinogalactan proteins) will be touched upon here, but also proline-rich (PRP) glycoproteins and glycine-rich (GRP) proteins belong to the HPRGPs.

6.2 Extensins

Extensins have several well-defined repetitive motifs with Ser-(Hyp)₄ being the most dominating one and they are typically basic glycoproteins due to a large content of lysine. The amino acid composition of the protein backbone is unusual because it contains high amounts of hydroxyproline (approx. 30-40 %mol), serine (approx. 12 %mol), lysine (approx. 10 %mol) and tyrosine (approx. 10 %mol). Histidine is also present in vast amounts in some extensins but virtually absent in others (Fry 1988). The glycosylation pattern varies between species but the degree of glycosylation is generally higher in dicots than in monocots. Typically almost all Hyp-residues carry arabinosyl glycosylation from one to four units. These arabino-oligo side chains are typically linked by a combination of α-1,3- and β-1,2-linkages, with α-1,3- as the terminal linkage. Serine can be glycosylated with single galatosyls by α-linkages from carbon atom number one (Fry, 1988). Total glycosylation for extensins have been reported to lie in the range of 50-75% of the total protein mass (Sommer-Knudsen et al., 1998 and Showalter, 1993). Figure 21 shows a schematized overview of the common features of extensins.

Extensins are produced as soluble monomers but instantly become insoluble when deposited in the cell wall presumably caused by intra- and intermolecular cross-linking. It is known that ether linkages between tyrosine residues can create an iso-dityrosine intramolecular linkage (Fry, 1982, Epstein and Lamport, 1984), but no direct evidence of such cross-linkings between protein molecules have yet been fully established in vivo (Showalter, 1993) even though it becomes more and more accepted that they do exist (Biggs and Fry, 1990). It is believed that the ultrafast insolubilization of extensins is a cell response to wounding and fungal attacks as it strengthens the cell wall possibly by creating an offset for lignification (Sommer-Knudsen et al., 1998). It has been difficult to decisively determine covalent interactions between cell wall polysaccharides and extensins (Mort and Lamport, 1977) but it has been proposed that ionic interactions between pectin and extensins occur, where the positively charged lysine residues in extensin interact with negatively charged galacturonic acid residues in pectin hereby “zipping” together three to four pectin molecules. The extent of these ionic interactions can be controlled by changes in cell wall pH or by the level of Ca²⁺ thereby controlling the properties of the cell wall (Showalter 1993 and Sommer-Knudsen et al., 1998).
Repetitive motif

Ser | Hyp | Hyp | Hyp | Hyp

Glycosylation pattern

Gln | Trp | Ser | Ser | Hyp | Hyp | Hyp | Val

Galp | Galp | Araf | Araf | Araf | Araf | Araf | Araf

α-1,3 | β-1,4 | β-1,2 | α-1,3 | β-1,2 | α-1,3 | β-1,2 | α-1,3

Total glycosylation 50-75% of mass
Highly basic.
Insoluble when deposited in the cell wall. May be soluble as mono-/oligomeric molecules
Intra-/intermolecular linkages by iso-dityrosine cross-links

Figure 21: Stylized structure of extensin, repetitive motif and glycosylation pattern. Galactosyls exclusively O-linked to serine residues, not all serine residues glycosylated. Arabinosyls exclusively O-linked to hydroxyproline residues with chain lengths varying from terminal to four units, most hydroxyproline residues glycosylated.

6.3 Arabinogalactan proteins

Arabinogalactan proteins (AGPs) are a class of HPRGPs that are notably different from the others due to their heavy glycosylation with arabinogalactan side chains. AGPs are highly soluble. The “classical” core protein of AGPs is rich in alanine, serine, glycine and threonine in addition to hydroxyproline, however “non-classical” AGPs have also been reported and may include cysteine rich AGPs, hydroxyproline poor AGPs and asparagine rich AGPs (Showalter, 2001). The repetitive motifs of AGPs are Hyp-Ala, Ala-Hyp or Ser-Hyp and the carbohydrate moieties in AGPs can account for as much as 98% of the molecular mass mainly composed of arabinose and galactose (Sommer-Knudsen et al., 1998). In contrast to other HPRGPs, the glycosylation pattern is of a polysaccharide nature rather than single or short side chain substitutions and the size varies from 30 to 150 units (Showalter, 2001).

The glycosylations resemble type II arabinogalactan structures with a β-1,3-linked D-galatosyl backbone, substituted by side chains of β-1,6-branched D-galactosyls again substituted by terminal α-1,3-linked arabinose. Other less-abundant monosaccharides might also be present along with arabinose side chains.
similar to those in extensins, and these monosaccharides include rhamnose, mannose, xylose, glucose, fucose and uronic acids (Showalter, 2001 and Sommer-Knudsen et al., 1998). As for the extensins the carbohydrates are attached to the protein core by O-glycoside linkages to hydroxyproline and serine and possibly also to threonine, as unknown glycosidic linkages to threonine in AGPs have been established (Showalter, 2001). Figure 22 shows a schematic overview of the AGP structure with repetitive motifs and typical glycosylation pattern.

A certain Hyp-contiguity hypothesis has been stated for AGPs saying that Hyp-residues placed in continuous clusters are all substituted by arabino-oligos whereas single non-continuous Hyp-residues are glycosylated with polysaccharides. By this hypothesis the glycosylation pattern for AGPs can in general be predicted based on the amino acid sequence of the protein core (Kieliszewski and Lamport, 1994, Kieliszewski 2001). Studies carried out so far on this predictive method have supported the hypothesis and have to some extent been shown also to apply to other HPRGPs like extensins (Kieliszewski and Shpak, 2001). The physiological role of AGPs is more unclear than that of extensins and is speculated to have little structural importance. Rather they seem to function as signaling molecules for cell expansion and development, reproductive growth, programmed cell death and cell adhesion (Showalter, 2001).

It appears that the diversity of AGP functions are great and complex. AGPs are found both extracellular and bound to the cell wall or plasma membrane by GPI anchoring, which again adds to the complexity of functions. The carbohydrate moieties of AGPs seem to be of great importance to their versatile functions as oligosaccharides often play an important role in many signal transduction pathways. Enzymatic modifications of AGP glycosylations are hereby potential signaling processes predicted to participate in a wide array of biochemical reactions (Showalter, 2001).

In relation to the Hyp-contiguity hypothesis, the term glycomodules are introduced by Kieliszewski 2001 and refers to the fact that specific, simplified repetitive amino acid motifs with specific patterns of glycosylations are characteristic for the different classes of HPRGPs. In principle this means that the plant may 'mix and match' their structural proteins based on the sequence and combination of these glycomodules, hereby obtaining specific structural proteins with specific properties. Since the glycosylations are generally substantial, they largely dominate the molecular 3D-structure and thereby also completely determine the molecular properties. This gives rise to principally different proteins from a simple set of building blocks (Kieliszewski 2001, Kieliszewski and Shpak, 2001), especially evident from the diverse properties, location and functions of AGPs.
Repetitive motif

Hyp — Ala — Ser — Hyp — Ala — Hyp

Glycosylation pattern

Total glycosylation 90-98%

Highly soluble

Glycosylated with Type II arabinogalactan and arabinooligo side chains. Arabinogalactan glycosylation exclusively to Hyp in non-contiguous Hyp stretches. Arabinooligo glycosylation to Hyp residues in contiguous stretches.

Figure 22: Stylized structure of arabinogalatan proteins, repetitive motifs and glycosylation pattern. Single galactose units attached to serine, galactans attached to hydroxyproline. Arabinan structures present only on hydroxyproline residues.

6.4 Special features for monocot structural proteins

Hydroxyproline rich glycoproteins are less abundant in monocots than in dicots (Carpita 1986) but all four classes of proteins have been confirmed in monocots, however with somewhat different appearances. Firstly, the extensins in monocots have slightly different repetitive motifs and secondly they are less glycosylated than dicot extensins. Corn extensins have been the focus of some studies which show that they include threonine-hydroxyproline rich glycoproteins (THPRGPs) with approx. 25 mole% threonine and histidine-hydroxyproline rich glycoproteins (HHPRGP), which are also rich in alanine (Showalter,
The THPRGP has been subjected to the most studies, which show that it does have similarities to dicots extensin but only contains approx. 30% glycosylation, exclusively with arabinosylated Hyp-residues and mostly dominated by arabinosylation with terminal arabinosyls or trimers (Kieliszewski and Lamport, 1987). It has not been possible to point directly to a specific function of the monocot extensins different from those proposed for dicots, so the structural differences might merely be a result of differentiating evolution. However, one theory as to why the content of monocot extensins is low is that cereals respond differently to wounding and infections than dicots, namely by accumulating cell wall phenolics rather than accumulating extensins (Li and McClure, 1990, Carpita and Gibeaut, 1993). In support of this theory is that studies of wounding barley and oat primary leaves do not show a significant change in the expression of extensin, but rather induce enhanced peroxidase activity related to enhanced phenolic cross-linking (Li and McClure, 1990). However, the exact opposite results have been shown for wounding corn coleoptiles and young leaves (Ludevig et al., 1990), where the expression of HPRGPs is induced. Whether one theory is more correct than the other is unclear. However, it is possible that both hypotheses carry a certain truth as the data is based upon analysis of different tissue from different species. Therefore, the studies might not have been specific enough to target the same HPRGPs leading to contradictory conclusion. Different interactions between monocot and dicots extensins with other cell wall components have also been proposed by Kieliszewski et al., 1990, where HF treatment solubilized the vast majority of THPRGP in contrast to results obtained for dicots HPRGPs, suggesting that the two types of extensin networks are different. However, no reportings on specifically what the differences might be have been made.

AGPs have also been found in monocots and recently the first pure AGP from wheat was obtained by Yariv precipitation and described with respect to amino acid sequencing and glycosylation pattern (Göllner et al., 2010). It seems that the AGP of wheat has glycosylations quite similar to dicots with type II-like arabinogalactan glycosylations however deviating by the presence of terminal α-1,5-linked arabinofuranosyls and complete lack of uronic acids. From corn, a putative extensin rich in histidine has also been recognized as an AGP since the galactose content in glycosylations was substantial (Kieliszewski et al., 1992).

### 6.5 Storage proteins

The storage proteins are not confined to the plant cell wall in the living plant but most often to seeds and other endosperm tissues. Nevertheless, particularly in the case of corn bran, storage proteins are also central to recognize due to the wet milling process flow, where residual proteins from corn gluten meal may contaminate the corn bran fraction (figure 2, Introduction).

To the plant seed storage proteins function as energy and nitrogen reserve for germination and other developmental stages and their synthesis largely depend on the nutritional conditions. In the mature seeds the storage proteins are often deposited in discrete bodies called protein bodies and are made up of several different kinds of storage proteins (Shewry et al., 1995). Plant storage proteins are among the
earliest proteins to have been characterized and the first wheat gluten was isolated back in 1745. In 1924 Thomas Burr Osborne classified the plant seed storage proteins into four groups based on their extraction and solubility. This classification is still accepted today and categorizes the storage proteins into albumins (soluble in water), globulins (soluble in dilute salts), prolamins (soluble in aqueous alcohol) and glutelins (soluble in alkaline or acid buffers). However, the glutelins are today most often categorized with the prolams (Shewry et al., 1995). For corn, α-zein belonging to the prolams is the dominating type of storage protein where it constitutes up to 70% of the kernel endosperm protein content (Lawton 2002).

Prolamin storage proteins are characterized by having a high content of proline and glutamine and in contrast to the other storage proteins they are exclusively found in monocot grasses. Here they constitute the vast majority of all storage proteins. Within the prolams important proteins like β and γ-hordeins from barley, γ-secalin from rye and α- and γ-gliadins from wheat are found all belonging to the S-rich group of prolams and forming a superfamily of prolams (Shewry et al., 1995). An important exception from the prolamin superfamily is α-zein from corn (β- γ- and δ-zein all belong to the superfamily), which is distinct from the others by being rich in leucine and alanine besides proline and glutamine (Shewry et al., 1995 and Tatham et al., 1993). α-zein has a molecular weight of either 19 kDa or 22 kDa depending on whether it has nine or ten repeats of the repetitive motif and is soluble in 95% aqueous ethanol (Cook et al., 1996). Today commercial zein is mainly composed of α-zein and is extracted from corn gluten meal (figure 2) and used for the production of coatings, films, inks, fibers and adhesives (Lawton, 2002).

The last sections of this chapter contain the results of removing proteins from DCB prior to enzymatic hydrolysis with hemicellulases. The aim of the work has been to evaluate whether proteins associated to the cell wall may interfere with the enzymatic hydrolysis of arabinoxylan, either by creating a hydrophobic interface or other steric barriers. Secondly, it is also an important goal to gain knowledge about the nature of these proteins and evaluate whether they may carry significant amounts of glycosylations, which could be inaccessible to the enzyme array generally applied in this work. As already described linkage analysis indicates the presence of arabingalactan type II which can originate from AGPs. Also the presence of arabinan-structures is indicated by the linkage analysis.

6.6 Materials and methods

Substrate
The substrate used in the following experiments is the native destarched corn bran with the basic monosaccharide composition presented in table 1 (section 3.1).

Amino acid composition
The amino acid composition and total protein content has been analysed according to the method described in Barkholt and Jensen 1989, which includes a 6 M HCl hydrolysis step followed by separation of amino acids by ion exchange chromatography. With this method glutamine and asparagine is converted into glutamic acid and aspartic acid respectively and therefore analysed as such. Tryptophane is degraded
and can not be determined. Serine and threonine are slowly degraded during the hydrolysis with up to 10 and 5% loss respectively. Isoleucine and valine are released slowly and might therefore be underestimated and methionine is sensitive to oxidation, however less than 10% loss.

**Extraction**

Corn bran has been extracted by two different means in order to target different kinds of protein. Firstly, a CaCl₂ extraction was carried out by incubating DCB at room temperature over night in a 0.2 M CaCl₂ buffer containing 0.1% SDS (Hood et al., 1991). Hereafter, the suspension was centrifuged and separated into a protein enriched supernatant and a solid residue. The solids were washed three times in water and freeze dried for further use. In the other case, DCB was extracted with 80% ethanol according to Cook et al., 1996, specifically trying to target zein. In this case DCB was pre-wetted with water and 95% ethanol added to a final concentration of 80%. The suspension was heated to 60 °C and pH adjusted to 5.9 before incubation for 1 hour. The suspension was centrifuged into a protein enriched supernatant and a solid residue. The solids were washed three times in water and freeze dried. An aliquot of the two extract supernatants were also freeze dried for further hydrolysis.

**Pre-digestion by proteases**

Destarched corn bran was pre-digested with six different proteases before hydrolysis with hemicellulases. The proteases were bromelain, papain, α-chymotrypsin, pepsin and two proteases from Novozymes A/S (table 8). These proteases have different pH and temperature optimum conditions and therefore individual pre-digestion setups were done for each protease (according to table 8). Reaction conditions were based on vendor specifications for activity testing. Proteases were loaded at two different concentrations, E/S of 1:1000 and 1:100, assuming 10% protein content in DCB and incubated for 1 hour. After pre-digestion, protease activity was inactivated at 100 °C for 10 min, samples centrifuged and the supernatant removed to the largest extent possible. Hereafter a 0.1 M succinate buffer pH 5 was added to each sample to a final substrate concentration of 2% DM for further hydrolysis.

<table>
<thead>
<tr>
<th>Protease</th>
<th>Supplier</th>
<th>Origin</th>
<th>Product #</th>
<th>pH</th>
<th>Temperature</th>
<th>Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bromelain</td>
<td>Sigma-Aldrich</td>
<td>Pineapple Stem</td>
<td>B4882-10G</td>
<td>4,5</td>
<td>25</td>
<td>0.01 M succinate</td>
</tr>
<tr>
<td>Papain</td>
<td>Sigma-Aldrich</td>
<td>Carica papaya</td>
<td>76218</td>
<td>6,2</td>
<td>25</td>
<td>0.01 M succinate</td>
</tr>
<tr>
<td>Protease 1 (P1)</td>
<td>Novozyme A/S</td>
<td>N/A</td>
<td>N522103</td>
<td>5,0</td>
<td>40</td>
<td>0.01 M succinate</td>
</tr>
<tr>
<td>Protease 2 (P2)</td>
<td>Novozymes A/S</td>
<td>N/A</td>
<td>N522104</td>
<td>5,0</td>
<td>40</td>
<td>0.01 M succinate</td>
</tr>
<tr>
<td>α-chymotrypsin</td>
<td>Sigma-Aldrich</td>
<td>Bovine pancreas</td>
<td>C4129-250MG</td>
<td>8,5</td>
<td>37</td>
<td>0.01 M succinate</td>
</tr>
<tr>
<td>Pepsin</td>
<td>Sigma-Aldrich</td>
<td>Porcine gastric mucosa</td>
<td>P7000-25G</td>
<td>2,0</td>
<td>37</td>
<td>None - HCl adjusted</td>
</tr>
</tbody>
</table>

**Solubilised protein**

Solubilised protein in the extract supernatants and in the protease pre-digested hydrolysates were assayed by Pierce® BCA Protein Assay Lit (Thermo Scientific, Product # 23225) using bovine serum albumin as an external standard and measured spectrophotometrically at 562 nm.
Hydrolysis

All solid residues after extraction and pre-digestion respectively was enzymatically hydrolysed by minimal enzyme cocktail as that employed in Agger et al., 2011 (Paper 2), consisting of the basic set of four hemicellulases together with FAE-III from A. niger, acetyl xylan esterase from Flavovolaschia sp and Cellic™ CTEc. Samples were incubated for 24 hours at 50 °C, pH 5 and inactivated for 10 min at 100 °C. Freeze dried supernatant fractions from extraction experiments were hydrolysed for 2 hours at 100 °C using 2 M HCl to release any associated monosaccharides. All hydrolysates (enzyme and acid hydrolysates) were analysed for monosaccharides arabinose, galactose, glucose and xylose using HPAEC-PAD, BioLC Dionex with a CarboPac™ PA1 analytical column according to Sørensen et al., 2003.

6.7 Results and discussion

The amino acid profile of destarched corn bran (figure 23) indicates a high content of glutamine/glutamic acid, proline, alanine and isoleucine, typical for α-zein. It is therefore likely, that the majority of the total protein in corn bran originates from endosperm storage proteins rather than structural cell wall proteins. However, hydroxyproline is also detected, evidence of structural proteins. Other amino acids typical for structural proteins are serine and glycine, both constituting a relatively high percentage in the total amino acid profile. Furthermore, threonine is also present and based on previous findings of threonine rich glycoproteins in corn (Kieliszewski et al., 1990) it can be speculated that some of the threonine originates from this. Therefore, the amino acid composition supports the presence of structural glycoproteins even though they most likely contribute a minor part of the total protein content.

Figure 23: Amino acid composition of proteins in destarched corn bran. Total protein content of approx. 89 g/kg DM
The amino acid profile does not inform directly about the glycosylation and for that purpose the extracted proteins are acid hydrolysed to determine whether any carbohydrates are associated with the proteins. The CaCl\(_2\) extraction method solubilized 69.2% of the protein originally present in DCB, whereas the ethanol extraction dissolved 27.4%. It was also observed that the solubilized material in the ethanol extract, precipitated if the ethanol concentration was decreased below 40%, whereas the CaCl\(_2\) extract after freeze drying was readily soluble in water. This indicates that different kinds of protein residues had solubilized by the two different extractions. HPLC analysis of the extracted residues show that after HCl hydrolysis primarily arabinose but also minor amounts of galactose, glucose and xylose are released from the CaCl\(_2\) extract and that galactose is released from the ethanol extract. Neither of the two extracts contains any uronic acids. A cautious estimate is that galactose in the ethanol extract amounts approx. 2.6 g/kg DM, whereas arabinose from the CaCl\(_2\) extraction is approx. 0.6 g/kg DM. This content of galactose and arabinose represents only minor amounts of that present in DCB, however it suggests that proteins in DCB are glycosylated and that these glycosylations are of an arabinan/galactan nature as also indicated by linkage analysis. Due to the possible presence of galactan structures, enzymatic hydrolysis with two different β-galactanases, endo-β-1,6-galactanse from *N. crassa*, NCU09102.7 and GH53 endo-β-1,4-galactanase from *A. nidulans*, AN5727.2 (Bauer et al., 2006) expressed by *P. pastoris* in house has been tested to evaluate the effect of galactan hydrolysis on the overall hydrolysis of arabinoxylan, however no effect was observed (data not shown).

Introducing a pre-digestion with proteases is done in order to evaluate whether the removal of proteins prior to enzymatic hydrolysis with the hemicellulases affects the degree of hydrolysis in a positive way. Zein in particular is recognized as a hydrophobic protein and the presence of this in the substrate may interfere by preventing enzyme/substrate interactions due to hydrophobic/hydrophilic obstructions. Furthermore, partial degradation of other associated proteins may leave the substrate more available for the hemicellulases and in that sense boost the hydrolysis. The hydrolysates after protease pre-digestion is analysed for solubilized protein (corrected for protein loading of proteases) and show that only papain, Protease 2, α-chymotrypsin and pepsin are capable of solubilizing protein from the substrate (figure 24).
Figure 24: Protein solubilization by protease pre-digestion. Results given as percent solubilized protein compared to the total protein content in DCB. Bromelain and Protease 1 were not capable of solubilizing protein from DCB.

Figure 25 shows the results after enzymatic hydrolysis with hemicellulases of the pre-digested DCB. In most cases no difference is observed between the samples that have been pre-digested with protein. It is observed that the highest release of xylose and arabinose occur in the samples incubated with pepsin, but the corresponding blank sample result in the same level of release. The increase in hydrolysis is therefore merely a result of the acidic conditions applied during pepsin digestion and not caused by the higher removal of proteins. Despite the relatively low solubilization of proteins caused by Protease 2 and no measurable solubilization by Protease 1, these pre-digestions are the only ones with slight indications of having a positive effect on the overall arabinoxylan degradation. However, the effects are very limited and no conclusions can be drawn based on this.

The fact that pre-digestion with proteases do not have any effects on the overall arabinoxylan degradation suggest that proteins in general do not associate with the carbohydrate structures and therefore their removal is not significant for arabinoxylan hydrolysis. However, the explanation can also be that the proteases employed here are not specific enough for target the proteins in DCB, or that glycosylation on certain proteins hinder protease activity. There is also the possibility, that the reaction conditions for the proteases employed here are suboptimal. Such matter may be investigated further.
Figure 25: Xylose release (top) and arabinose release (bottom) after enzymatic hydrolysis of protease pre-digested DCB. Results given as percent of xylose and arabinose respectively originally present in DCB.

Whether glycosylated proteins represent a true barrier for enzymatic hydrolysis of corn bran remains to be answered. However, the results indicate that both structural cell wall proteins and storage proteins are present in corn bran, and that at least some arabinan or galactan related glycosylation may be present. Further elucidation of the possible protein/polysaccharide interactions is an interesting subject and seems to be an area of opportunities for those mastering the analytical techniques. Especially, the search for enzymes capable of hydrolyzing the O-linkage between the protein backbone and glycosylations will be valuable and truly represents new and groundbreaking research. Corn bran may however not be the
optimal substrate for such studies as the occurrence and availability of structural proteins are probably achieved better elsewhere.

The last three chapters have explored the use of basic hemicellulases, the composition of certain parts of arabinoxylan originating from corn bran and the possible influence of proteins in hindering the enzymatic hydrolysis. The first major conclusion is that the basic enzymes are applicable and most probably among the best known today. Moreover, structural proteins do exist but are not immediately among the major reasons why native corn bran is so recalcitrant.

Corn bran features a highly diverse composition of mainly arabinoxylan and not even solubilization of these structures are at present enough to make them completely hydrolysable. Therefore, physical properties of corn bran are addressed in the following two papers. According to hypothesis 3, these are probably of major importance and should therefore receive appropriate attention. Also hypothesis 1 regarding diferulic acid cross-linkings must be targeted differently if the hypothesis is to be evaluated.
Main issues

In order to obtain significant enzymatic hydrolysis of corn bran, it is at present necessary to employ a pretreatment strategy. However, considering the relatively heat, acid and alkali-labile linkages and components in corn bran it is also important to identify the minimum requirements for use of energy and chemicals in the pretreatment. In the light of already obtained data on the efficiency of heat pretreatment (Agger et al., 2010 Paper 1), we here want to study the effects of altering pH prior to pretreatment on the expense of lowering the pretreatment temperature. The aim of this study is therefore to investigate the interactive effects of employing medium temperatures, alternating pH conditions and extended time intervals on a subsequent enzymatic hydrolysis for enhanced xylose release. It is hypothesized that optimal pretreatment conditions can be obtained. Furthermore, a newly designed pretreatment reactor optimized to mix the biomass slurry during pretreatment is also tested (Pedersen 2010). The experiments were designed so that statistic modeling of the data is possible and hereby extrapolations to the effects of pretreatment time, temperature and pH can be made.

Finally, the study aim to enlighten the effects of chemical removal of diferulic acid cross-linkings prior to enzymatic hydrolysis, since it is expected that alkaline pretreatments cause hydrolysis of major parts of the cross-linkings. When including both acidic and alkaline pretreatments this study gives a unique opportunity to study and compare the effects of pretreatments at both ends of the pH scale within the same experimental conditions. It is therefore also the aim of this study to either support or reject hypothesis 1, namely that removal of diferulic acids will render the arabinoxylan less recalcitrant to degradation.

Main results

The work was carried out in an iterative manner starting out by setting the experimental constraints to be temperature (100-150 °C), initial pH (2-12) and holding time at the specified pretreatment temperature (10-120 min). Hereafter, the total slurry is enzymatically hydrolysed with the minimal blend of hemicellulases, esterases and cellulases. The general observation is that acidic pretreatments are more effective in boosting the overall enzymatic hydrolysis of arabinoxylan yet at the same time causes higher release of free monosaccharides during the pretreatment. Especially arabinose is released in vast amounts during the acidic pretreatments causing limited enzymatically catalysed release. Modeling the results of enzymatic xylose and glucose release show that optimal conditions were not yet found within the initial parameter setup and therefore a second set of acidic pretreatments were investigated. A pH range from 1 to 2 was investigated along with changing pretreatment time (from 10 to 65 min) at a maintained
temperature of 150 °C. With these pretreatment conditions arabinose release occur only during the pretreatment and hence no enzymatically catalyzed release is seen. On the contrary, enzymatic glucose release is enhanced and optimal conditions are found to be pH 1.5 for 45 min at 150 °C, which result in 68% glucose release. The increasingly acidic conditions also cause the xylose release during pretreatment to increase. Consequently, the release obtained by enzymatic hydrolysis decrease. Looking to the total xylose release, the optimal conditions are found at pH 1.3 for 50-55 min reaching approx. 61% of the original xylose content. However, the enzymatic xylose release do not find an optimum within these pretreatment conditions but point towards initial conditions of pH 2, 65 min at 150 °C as close to the optimal pretreatment.

The pretreatments also induce solubilization of dry matter and when this is compared to the extent of enzymatic hydrolysis (figure 26) it is evident that increasing solubilization has a positive effect on the enzymatic hydrolysis until a certain point. This observation corroborates hypothesis 3 namely that enzymatic hydrolysis is linked to the solubility and the physical appearance of the substrate. The curves bend off because increasing pretreatment severity causes increasing chemical hydrolysis of particularly arabinoxylan.

It is also apparent from figure 26 that enzymatic glucose release is not limited by chemical hydrolysis and possibly reaches a maximum with the given pretreatment conditions, corresponding to approx. 68% of the total glucose content. The glucose release most probably originates from cellulose, since the pretreatment with hydrochloric acid as a catalyst has very limited capacity to release glucose.
The yields after pretreatment and enzymatic hydrolysis still indicate incomplete hydrolysis and in order to evaluate the mass balances more carefully, the loss induced by the pretreatment is assessed for certain acidic and alkaline experiments. After acidic pretreatments xylose and arabinose content in the solid fraction are in the range of 70-120 and 10-25 g/kg DM respectively, and compared to the original amounts in DCB it is clear that acidic pretreatments induce a loss of approx. 60% xylose and 40-50% arabinose. Corresponding losses after alkaline pretreatments show that approx. 40% xylose and 55% arabinose is lost. Therefore, when the yields of enzymatic hydrolysis are evaluated against these new levels, complete enzymatic hydrolysis has been achieved especially when DCB has been exposed to acidic pretreatment. But it is on the expense of vast structural changes to the substrate and loss of approx. half of the valuable pentoses. From an academic point of view this sort of pretreatment makes it difficult to target and control the enzymatic hydrolysis. This is exceptionally evident with the very low enzymatic arabinose release, where no more of the original substrates for the α-L-arabinofuranosidases are present. If higher understanding of the substrate composition is to be achieved and thereby indirectly higher enzymatic yields, it is not enough to just chemically hydrolyse the substrate beyond recognition.

However, as expected alkaline pretreatments induce removal of diferulic acids but otherwise alkaline pretreatment do not cause as much promotion of the hydrolysis as the acidic. This questions the validity of hypothesis 1. It is also illustrated by the correlation plot between xylose release and diferuloyl removal in figure 27B. This plot can be interpreted as how the enzymatic xylose release is related to the total removal of diferulic acids and can be compared to how it is related to total arabinose release. The plots in figure 27 strongly question the hypothesis that enzymatic degradation of arabinoxylan is significantly hindered by the presence of diferulic acid cross-links. In theory, there may be a tendency that enzymatic xylose release is related to the diferulic acid content, but the very presence of two remote lying data points stress the fact that other parameters in the arabinoxylan composition has a much stronger influence on the enzymatic hydrolysis of arabinogalactan.

These results are not a matter of sidelining the effects of arabinose removal on the enzymatic xylose release to the effects of diferulic acid removal. Considering the basic structure and composition of arabinoxylan, arabinose removal is of course vital to enzymatic xylose release. The results are merely a way of illustrating that the effects of diferulic acid removal are limited and may not deserve the attention sometimes given (Grabber et al., 1998a+b). Based on these observations we believe that hypothesis 1 is less likely to be true. Still it is interesting to observe the effects of enzymatic removal of the diferulic acids, if this ever really happens, because it will give the opportunity to observe the pure effects of this, and not a response induced by random chemical modifications where other structural changes most probably also influence the data. The results do not allow us to completely dismiss the idea that diferulic acids play a structural role in the cell wall, but they are not a significant hindrance to enzymatic hydrolysis.
Figure 27: Correlation plots: A: Total release of arabinose after pretreatment and enzymatic hydrolysis of DCB versus the enzymatically catalyzed release of xylose. Linear regression fit $R^2=0.96$. B: Remaining diferulic acid content after pretreatment versus enzymatically catalyzed release of xylose. Linear regression fit with $R^2=0.02$. Results are given as % of that originally present in DCB.

**Conclusion**

Even though pretreatment seems indispensable at present it is exceptionally unsatisfying to lose large amounts of monosaccharides and to evaluate results based on disrupted structures of arabinoxylan. Corn bran represents a type of substrate different from other lignocellulosic materials and thereby responds stronger to pretreatment. Finetuning the pretreatment conditions continue to be a challenge until more
efficient enzyme reactions are available. Balancing the costs and benefits of pretreatment will have to rely on individual processing requirements, but for scientific work with corn bran, pretreatment should be kept at a minimum and not include chemical catalysts. For the ongoing work in this project it is therefore decided only to study the unpretreated DCB and the heat pretreated DCB fractions similar to that used in Paper 1, bearing in mind that the overall yields will then decrease. The general impression from the heat pretreated material used in Paper 1 is that, the degree of chemical hydrolysis is much lower than observed here and thereby the substrate will resemble the native structures more.
pH catalyzed pretreatment of corn bran for enhanced enzymatic arabinoxylan degradation

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Corn bran is mainly made up of the pericarp of corn kernels and is a byproduct stream resulting from the wet milling step in corn starch processing. Through statistic modeling this study examined the optimization of pretreatment of corn bran for enzymatic hydrolysis. A low pH pretreatment (pH 2, 150°C, 65 min) boosted the enzymatic release of xylose and glucose and maximized biomass solubilization. With more acidic pretreatment followed by enzymatic hydrolysis the total xylose release was maximized (at pH 1.3) reaching ~50% by weight of the original amount present in destarched corn bran, but the enzyme catalyzed xylose release was maximal after pretreatment at approx. pH 2. The total glucose release peaked after pretreatment of approx. pH 1.5 with an enzymatic release of approx. 68% by weight of the original amounts present in destarched corn bran. For arabinose the enzymatic release was negatively affected by the acidic pretreatment as labile arabinosyl-linkages were presumably hydrolysed directly during the pretreatment. A maximum of 60% arabinose release was achieved directly from the optimal (acidic) pretreatment. The total content of diferulic acids, supposedly involved in the cross-linking of the arabinoxylan polymers, decreased by both alkaline and acidic pretreatment pH, with the loss by alkaline pretreatments being highest. No direct correlation between the enzymatic release of xylose and the content of diferulic acids in the substrate could be verified. On the contrary the enzymatic release of xylose was significantly correlated to the total release of arabinose, indicating that the degree of arabinosyl-substitutions on the xylan backbone is an essential parameter for enzymatic hydrolysis of corn bran arabinoxylan.

Introduction

Corn bran consists of the pericarp tissue, testa and pedicel tip of corn kernels [1] and is a byproduct resulting from the wet milling step in corn starch processing. Corn bran is mainly made up of polysaccharides and is particularly rich in pentoses, that is, arabinose and xylose. These C5 monosaccharides have the potential of being a resource for the production of bioethanol, xylitol, and value added platform chemicals like furans, formic acid, and levulinic acid [2,3]. The arabinoxylan may even be upgraded to functional nanoparticle structures or bioactive food ingredients [4,5]. Arabinoxylan comprises up to 56% of the corn bran biomass dry matter [6] – with the rest of the dry matter being mainly made up of starch, cellulose and protein [7], in addition to 10% by weight of lignin [6]. Arabinoxylan from corn bran has proven recalcitrant to enzymatic degradation [8–10], a trait that has been attributed to its structural complexity, that is, notably the various substitutions on the xylan backbone. A major hypothesis is that diferulic acids impede enzymatic access to the xylan backbone [1,11] by cross-linking of the arabinoxylan chains and by participating in inter-polymeric reactions through radical cross coupling to lignin and structural proteins [12–15].

A form of hydrothermal pretreatment of corn bran before enzymatic hydrolysis seems indispensable at present and may
be justified as long as the energy, chemicals, and process expenses do not exceed the final value of the corn bran products. However, the majority of studies reported on corn bran pretreatment have employed harsh pretreatment conditions with respect to temperature and chemical use leading to special process and equipment requirements. Experimental thermal pretreatments, followed by enzymatic hydrolysis using either culture broths or relevant hemicellulose enzyme preparations aiming to release furanic acid and monosaccharides from corn bran, have thus made use of hot water cooking at high temperatures of up to 160–210 °C [16, 17]. The best of these pretreatments resulted in dissolution of 80% of the available neutral sugars [17] and specifically 90% saccharification of glucose and 80% of xylose and arabinose, respectively [16]. In the quest for attaining enzymatic accessibility to corn bran arabinosylan, solubilization appears to be a vital parameter. However, corn bran arabinosylan solubilization rarely comes without a loss of biomass. While glucan recovery has been found to range from 82% to 121%, the recovery and solubilization of hemicelluloses after pretreatment have varied from 29% to 82% [18]. Similarly, our own previous results gave arabinosylan recovery in the range of 65–70% and approximately 50% solubilization [6]. Very high temperature pretreatments (above 220 °C) causing almost complete hemicellulose solubilization are accompanied by high glucose degradation [19]. A few experiments with alkaline pretreatment [20, 21] have been reported, but with divergent outcome. Saha and Bothast [21] did not succeed in releasing more than negligible amounts of xylose while Leathers and Gupta [20] achieved release of 67% of the available xylose after alkaline hydrogen peroxide pretreatment. Lastly, several studies have employed pretreatment with dilute sulfuric acid at different conditions of temperature, time and acid loading [7, 21, 22]. A common feature has been that glucose was readily released by enzymatic hydrolysis after sulfuric acid pretreatment; the glucose being liberated either from residual starch or from cellulose. It has also generally been observed that arabinose is readily released during acidic pretreatments as a consequence of the heat and acid labile arabinosylan glycosidic linkages [7]. However, an overview of the exact influence of different pretreatment parameters (pH, temperature, and pretreatment time), and notably a quantitative understanding of their interactions to allow the design of mild, but efficient pretreatment of corn bran is lacking.

In the present work, we hypothesized that an optimal pretreatment method could be developed by investigating the influence of pH, temperature and pretreatment time on the enzymatic accessibility of arabinosylan, in particular evaluated through the assessment of the enzymatic release of xylose. The objective of the present study therefore was to test this hypothesis and at the same time assess the influence of the pretreatment parameters for subsequently achieving maximal enzymatic arabinosylan hydrolysis.

Materials and methods

Substrate

Raw corn bran was obtained from Archer Daniel Midlands Company, Decatur, IL, USA. The material was milled and enzymatically destarched with α-amylase and amyloglucosidase as previously described [6]. The destarched substrate will be referred to as destarched corn bran (DCB) and results will be stated as a percentage by weight of each individual monosaccharide present in the original DCB before pretreatment. The DCB was composed of (in g/kg DM) arabinose: 267, xylose: 374, glucose: 233, lignin: 120, protein: 89.1, ferulic acid: 27.3; diferulic acids: 17.0, and acetic acid: 38.5 as analysed and reported previously [6]. When added up the mass sum is higher than 1000 g/kg DM; this is because the monosaccharides, furanic and acetic acids were quantified in their hydrated form. In the native substrate, components are bound in polymeric structures and therefore an anhydrous adjustment has to be made. In short, arabinose and xylose content was assessed by 0.4 M HCl hydrolysis for two hours at 100 °C according to Sørensen et al. [23]. Glucose, lignin and acetic acid contents were determined after 72% H2SO4 hydrolysis at 37 °C for one hour followed by 4% H2SO4 hydrolysis at 121 °C for two hours according to the standard procedure of the U.S. National Renewable Energy Laboratory (NREL) [24, 25]. Protein content was determined as total amino acid quantification by 6 M HCl hydrolysis for 24 h and quantified by ion exchange chromatography [26]. Furanic acid and dehydromers of furanic acids were determined as described below.

Pretreatment experimental layout

Initial pretreatment experiments were set up as a Box–Behnken response surface modeling design with three factors using the statistical software Modde 7.0.0.1 (Umetrics AB., Umeå, Sweden) as an aid for the statistical design and the multivariate analyses of data. Factors were defined as temperature (100–150 °C), pH (2–12) and pretreatment time (10–120 min). Responses were defined as xylose and glucose release, respectively, after enzymatic hydrolysis (see below), content of diferulic acids after total saponification of the pretreated material (analytical method described below), and total release of arabinose (as the sum of free arabinose after pretreatment and enzymatic hydrolysis). The experimental design resulted in 12 different experimental pretreatments with three repetitions of the center point (125 °C, pH 7, 65 min). Intensive low pH pretreatment experiments were statistically designed with offset in the initial experiments. The low pH pretreatment set was a full CCF (central composite face centered) response surface modeling design. Temperature was maintained at 150 °C and factors defined as pH (1–2) and pretreatment time (10–65 min) resulting in eight different pretreatment experiments and three repetitions of the center point (pH 1.5, 35 min). Besides the statistically designed pretreatment experiments, selected additional experiments were performed to investigate the influence of prolonged pretreatment time. Pretreatment at 150 °C, pH 2 was prolonged to 120, 180 and 210 min and pretreatment at 125 °C, pH 12 was prolonged to 240 min. These latter pretreatment experiments were not included in the multiple linear regression modeling.

Statistical test of data correlation (F-test)

Evaluation of the linear correlation fits was done by applying a correlation F-test on the ratio between the mean square regression and the mean square error of the residuals with the null hypothesis that the data were linearly correlated [27].

Pretreatment

The pretreatment was performed in a custom built pretreatment reactor consisting of a circular pipeline with an inner diameter of 2.2 cm and a total length of 160 cm, giving a total reactor volume
of approx. 600 ml. The reactor was equipped with a progressive cavity pump operated at 150 rpm. Pretreatment was done at a dry matter concentration of 2% by weight in an aqueous nonbuffered suspension. pH was adjusted initially to the required level using either HCl or NaOH. The investigated time span was set to the holding time at the specified temperatures. After each experiment the pH was adjusted to pH 5 to normalize pH for the enzymatic hydrolysis. The total slurry and solubilized dry matter level were determined for each experiment.

**Enzymes**

All enzymes were provided by Novozymes A/S, Bagsværd, Denmark as mono component preparations except Cellic™ CTec (Table 1). The latter is a commercially available cellulase mixture based on the *Trichoderma reesei* cellulase complex (exo-glucanase, endo-glucanase, and β-glucosidase activities) with particular additional β-glucosidase and glycoside hydrolyase family 61 hydrolysis boosting proteins [28].

Enzyme activities are given as units (U) per milligram of total protein (Table 1) as determined by the use of the following activity assays: endo-1,4-β-xylanase activity was assayed on AZO-wheat arabinoxylan (Megazyme International, Wicklow Ireland) at pH 4.5, 40 °C for 10 min with the degree of hydrolysis quantified photometrically at 590 nm as described by a standard Megazyme procedure. β-xylanase activity was assayed on *p*-nitrophenyl β-xylopyranoside (pNPX) and α-1-arabinofuranosidase activity on *p*-nitrophenyl α-1-arabinofuranoside (pNPA) at pH 5 (0.125 M sodium acetate), 50 °C for 15 min, in both cases using a substrate concentration of 37 mM (both substrates obtained from Sigma–Aldrich, Chemie GmbH, Steinheim Germany). In each case the reaction was terminated by the addition of 1 M Na2CO3 and the absorbance of the liberated *p*-nitrophenyl was measured at 410 nm [29]. Acetyl xylan esterase activity was assayed principally in the same way on *p*-nitrophenyl acetate (pNPAc) (Sigma–Aldrich, Chemie GmbH, Steinheim Germany) at pH 5, 50 °C for 10 min. One unit (U) of β-xylanase, α-1-arabinofuranosidase, or acetyl xylan esterase activity was defined as the amount of enzyme catalyzing the hydrolysis of 1 μmol min⁻¹ of pNPX, pNPA, or pNPAc, respectively. Feruloyl esterase was assayed on methyl 4-hydroxy-3-cinnamic acid (Alfa Aesar, Karlsruhe Germany) at pH 5, 50 °C for 10 min and quantified as free ferulic acid by RP-HPLC analysis as described below. Finally, Cellic™ CTec activity was assayed as total filter paper units (FPU) as determined according to the standardized filter paper assay procedure provided by NREL [30].

### Enzymatic hydrolysis

Enzymatic hydrolysis was performed on each individual pretreatment experiment in triplicate. Enzymes were dosed according to their enzyme protein concentration (EP) and the exact dry matter content of each experiment. The dry matter (DM) content was in all cases approx. 2% (w/w). Endoxylanase, β-xylosidase and both of the α-1-arabinofuranosidasases were dosed at 0.25 g EP/kg DM each. Acetyl xylan esterase (AXE) and feruloyl esterase (FAE) were dosed at 0.5 g EP/kg DM each and the Cellic™ CTec preparation at 4 g EP/kg DM. All enzyme hydrolysis experiments were performed at pH 5, 50 °C for 24 h and inactivated at 100 °C for 10 min immediately after the incubation.

### Total diferulic acid determination

From each pretreatment experiment a fraction of the suspension was freeze dried for total diferulic acid determination. The content of 8-O-4', 5,5'-, 8,5'- and 8,5'-benzofuran dehydrodiferulic acid was determined after alkaline saponification with 2 M NaOH at 25 °C, performed in two successive steps, followed by ethyl acetate extraction and evaporation as described by Andreasen et al. [31].

### Analyses (monosaccharides and diferulic acid)

Monosaccharides were analyzed using HPAEC-PAD, BioLC Dionex with a CarboPac™ PA1 (analytical 4 × 250 mm) column from Dionex according to Sørensen et al. [23]. Dehydrodimers of ferulic acid were analyzed using RP-HPLC with DAD detection, Chemstation 1100 series, Hewlett Packard and an ODS-L Optimal (250 × 4.6 mm, 5 μm) column from Capital HPLC. The chromatographic profile consisted of solvent A (5% acetonitrile (CH3CN), 1 mM TFA) and solvent B (100% acetonitrile) starting with 20% B, increasing the gradient over 35 min reaching 40% B, then further up to 100% B during another 3 min. The column was regenerated to 20% B for 2 min. Column temperature was maintained at 40 °C. Identification and quantification were done as previously described [6].

### Results and discussion

**Effect of pretreatment factors on monosaccharide release and enzymatic hydrolysis**

The pretreatment conditions and the corresponding release of arabinose, xylose and glucose both from the pretreatments and from the subsequent enzymatic hydrolysis showed that the most drastic effects seemingly occurred in the pretreatment when pH was low (experiments 1–15, Table 2). Thus the largest release of all

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**TABLE 1**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Microorganism</th>
<th>Activity</th>
<th>Family/type</th>
<th>EC number</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endo-1,4-β-xylanase</td>
<td><em>Humicola insolens</em></td>
<td>1.73 U/mg EP</td>
<td>GH10</td>
<td>EC.3.2.1.8</td>
<td>[35]</td>
</tr>
<tr>
<td>β-Xylanase</td>
<td><em>Trichoderma reesei</em></td>
<td>0.06 U/mg EP</td>
<td>GH3</td>
<td>EC.3.2.1.37</td>
<td>[35]</td>
</tr>
<tr>
<td>α-1-Arabinofuranosidase</td>
<td><em>M. giganteus</em></td>
<td>1.89 U/mg EP</td>
<td>GH51</td>
<td>EC.3.2.1.55</td>
<td>[35]</td>
</tr>
<tr>
<td>α-1-Arabinofuranosidase</td>
<td><em>Humicola insolens</em></td>
<td>0.04 U/mg EP</td>
<td>GH43</td>
<td>EC.3.2.1.55</td>
<td>[35]</td>
</tr>
<tr>
<td>Acetyl xylan esterase (AXE)</td>
<td><em>F. sp.</em></td>
<td>0.59 U/mg EP</td>
<td>CE1</td>
<td>EC.3.1.1.72</td>
<td>[6]</td>
</tr>
<tr>
<td>Feruloyl esterase (FAE)</td>
<td><em>A. niger</em></td>
<td>0.07 U/mg EP</td>
<td>Type A, CE1</td>
<td>EC.3.1.1.73</td>
<td>[36,37]</td>
</tr>
<tr>
<td>Cellic™ CTec</td>
<td><em>Trichoderma reesei</em></td>
<td>0.36 FPU/mg EP</td>
<td>–</td>
<td>–</td>
<td>[6]</td>
</tr>
</tbody>
</table>

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The remaining content of diferulic acids was primarily affected by alkaline pH where down to approx. 51% (equivalent to approx. 13 g/kg DM) of the original amount was found (Table 2). The fact that high pH pretreatments decreased the content of diferulic acid was not unexpected because ester linkages are labile under alkaline conditions.

The pretreatments caused solubilization of dry matter and an estimate showed that most material was solubilized during the low pH pretreatments (Table 2). This finding was in accordance with the observation that the highest enzymatic release of monosaccharides also occurred after the low pH pretreatment conditions. Solubilization will be addressed again later.

For experiments 6 and 10 an acid hydrolysis (HCl hydrolysis) of the solid fraction after pretreatment showed that 70–120 g/kg DM of xylose and only 10–25 g/kg DM arabino- l released by the subsequent enzymatic hydrolysis, in particular arabinose, xylose and glucose release and remaining content of diferulic acid. Results given as % of that present in the original DCB. DCB refers to destarched corn bran not subjected to pretreatment. Combined severity factor (log(R/C0)) given as log(R/C0) = log(10 - pH × e(T - 100/14.75)). Center points (13–15) expressed as average of three experiments with a coefficient of variance from 1% to 9%.

### Table 2

Overview of initial of pretreatment experiments on destarched corn bran (DCB). Pretreatment conditions and the corresponding effects of the pretreatment and the following enzymatic hydrolysis, in particular arabinose, xylose and glucose release and remaining content of diferulic acid. Results given as % of that present in the original DCB. DCB refers to destarched corn bran not subjected to pretreatment. Combined severity factor (log(R/C0)) given as log(R/C0) = log(10 - pH × e(T - 100/14.75)). Center points (13–15) expressed as average of three experiments with a coefficient of variance from 1% to 9%.

<table>
<thead>
<tr>
<th>Pretreatment conditions</th>
<th>Effects of pretreatment</th>
<th>Effects of enzymatic hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Arabinose release (%)</td>
<td>Xylose release (%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Glucose release (%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Remaining diferulic acid (%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Solubilization (%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Arabinose release (%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Xylose release (%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Glucose release (%)</td>
</tr>
<tr>
<td>Temp. (°C)</td>
<td>pH</td>
<td>Time (min)</td>
</tr>
<tr>
<td>100</td>
<td>6.7</td>
<td>10</td>
</tr>
<tr>
<td>150</td>
<td>5.9</td>
<td>10</td>
</tr>
<tr>
<td>100</td>
<td>7.0</td>
<td>120</td>
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<tr>
<td>150</td>
<td>4.9</td>
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<td>150</td>
<td>1.9</td>
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<tr>
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<td>100</td>
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<td>1.8</td>
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<tr>
<td>150</td>
<td>1.9</td>
<td>180</td>
</tr>
<tr>
<td>150</td>
<td>2.3</td>
<td>210</td>
</tr>
<tr>
<td>DCB*</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

n.d.: none detected. Triplicate determination of each data point with a coefficient of variance from 0.6% to 15%.

*Experiments not included in the multiple linear regression models.

Significant amounts of arabinose were released during the acidic pretreatments and this resulted in low release by the subsequent enzymatic hydrolysis (Table 2), presumably because the pretreatments had scavenged the available substrate for the α-L-arabinofuranosidas. Labile glycosidic linkages to arabinosyl substitutions have also been reported before [7]. After alkaline pH pretreatment, release of monosaccharides was only obtained after enzymatic hydrolysis and not as a result of the pretreatments alone. The effects of alkaline pretreatment were not as prominent as for the acidic pH pretreatments, except for the release of arabinose (Table 2). The highest enzymatic release of arabinose resulted in liberation of ~26% of that originally present in DCB, and was obtained after two hours of alkaline pretreatment – a pretreatment that did not in itself release any detectable arabinose (experiment 12, Table 2).

The table shows the results of the pretreatments and enzymatic hydrolysis. The table includes the effects of pretreatment on the release of arabinose, xylose, and glucose, as well as the remaining content of diferulic acid. The table also includes the solubilization of dry matter. The remaining content of diferulic acids was primarily affected by alkaline pH where down to approx. 51% (equivalent to approx. 13 g/kg DM) of the original amount was found (Table 2).
enzymatically released monosaccharides reported in Table 2 are percentage values as compared to the starting material. If the enzymatic release was related to the content actually remaining in the solid fractions, the enzymatic release after acidic pretreatments actually caused a full release of the remaining xylose and arabinose. Correspondingly, approx. 17% of xylose and 55% of arabinose were released from the alkaline pretreatment. Indeed this implied that the acidic pretreatments were more effective in rendering the material more susceptible to enzymatic hydrolysis as compared to the alkaline, but the loss ought to be considered substantial from both pretreatment forms. Interestingly, even though no free monosaccharides were observed after the alkaline pretreatments the loss was almost as significant as that of the acidic pretreatments. However, only minor amounts of 2-furfuraldehyde after pretreatments were detected (data not shown) and could not explain the gap and it was therefore concluded that the pentoses degraded to other components.

**Prolonged pretreatment**

The experimental conditions applied in experiment 1–15 (Table 2) did not result in maximum release of xylose, arabinose or glucose. Consequently, the most extreme acidic pretreatment (pH 2, 150°C) was prolonged to up to 210 min to obtain a pretreatment method for higher xylose release. It was evident that increasing the pretreatment time resulted in decreased enzymatic release of xylose and arabinose, even though the total release of these two monosaccharides increased (Fig. 1 and Table 2). Apparently, the increasing unspecific hydrolysis promoted during prolonged pretreatment decreased the enzymatic availability by causing a loss of substrate for the enzymes, but in total resulted in release of up to approx. 60% (corresponding to ~158 g/kg DM) of the available arabinose and 54% (~163 g/kg DM) of the available xylose (highest for experiment 19, Table 2). This indicated that the pretreatments affected the arabinoxylan substrate structure to a degree so that the subsequent enzymatic hydrolysis of arabinoxylan was negatively influenced. By contrast, glucose release from the pretreatments was limited, but increasing severity affected the enzymatic glucose release positively with a maximum of 69% release after prolonged pretreatment (Table 2). With the aim of optimizing the pretreatment for highest enzymatic release of xylose the best pretreatment was therefore obtained by maintaining the pretreatment time in the mid range level around 65 min (Fig. 1). The content of diferulic acids was also affected by prolonging the pretreatment time (Table 2) and for comparison, an alkaline pretreatment (125°C, pH 12) was also prolonged to 240 min. Both the prolonged acidic and alkaline pretreatments strongly affected the dehydro-dimer content leaving only 12–14% (~2 g/kg DM) of the original amount of dimers in the biomass (Table 2).

**Modeling the release of monosaccharides**

The significance parameters and regression coefficients for fitting a linear regression model to the release of monosaccharides showed that the best model fit within a 95% confidence interval was obtained by that in Table 3 mentioned combinations of factors, giving model fits (R²) in the range 0.839–0.889. Despite good model fits, model predictability (Q²) and model validity showed poor fitting, indicating that the models would not be adequate for predicting the outcome of experiments with factor limits beyond the ones employed in the present experiment. This is completely in accordance with the finding that none of the monomer releases reached a maximum under the applied circumstances – as also visualized in the 3D response surface plot exemplified by enzymatic xylose release (Fig. 2) (the similar, corresponding plots for total arabinose and enzymatic glucose release can be found in the supplementary material). Despite the lack of predictability, the model did reveal the significance of all three pretreatment factors pH, temperature, and time; the pretreatment pH was in all cases the most prominent factor, whereas time generally seemed to have the least effect on monomer release (Table 3). The pH regression coefficients were in all cases negative because decreasing pH had an increasing effect on the monomer release (Table 3). The regression coefficients and significance values for temperature and time were numerically similar for arabinose and xylose release indicating that the effects of these two factors were comparable (Table 3). The interactive effects of factors revealed that pH-pH and temperature-pH were significant in all cases. The interaction between pH and time was not significant but the significance only fell just outside the confidence interval (a 0.05). The low significance value of factor interaction pH-pH and the large regression coefficient stressed the importance of pH during the pretreatment experiments (Table 3). The change in sign and the numerical size showed that the effect of pH-pH was large and twisted around the center point towards the low pH value (also illustrated in Fig. 2). In the case of combining pH with temperature and time, respectively, the regression coefficients were negative indicating that pH had the strongest effect (Table 3).

**Modeling diferulic acid content**

Modeling of diferulic acid content after pretreatment revealed a more complex response. It was evident that pH had a significant effect on the remaining content, but from the saddle-like shape of the surface plot it was clear that also low pH levels had a decreasing effect on the content (Fig. 3a). Removal of diferulate thus occurred both during alkaline and acidic pretreatment conditions (Fig. 3a...
and b). Furthermore, Fig. 3a suggests that there was an interactive effect of time and pH as the surface is twisted. Temperature was not significant (Table 3), which is also illustrated by the surface plot being only slightly tilted along the temperature axis (Fig. 3b). The plot (Fig. 3a) underlines that further lowering of the pH during pretreatment will also negatively influence the content of dimers, as the surface plot reaches a maximum and bends off. Modeling these data (Table 3) to a suitable model was done with $R^2$ equal to 0.897. The pH was highly significant but because both high and low pH had a decreasing effect, the regression coefficient was relatively small (Table 3, Fig. 3). It was negative due to a stronger pull towards the effects of high pH than those of low pH. Only the interactive effect of time and pH was significant (Table 3).

**Correlations**

The model fittings showed that pretreatment pH was significant for the diferulic acid content and for changing the enzymatic accessibility to corn bran. However, opposite effects were observed with low pH pretreatment having an increasing effect on the enzymatic release of particularly xylose, whereas high pH pretreatments caused removal of the largest amounts of diferulic acids. To test if and how there were any correlations between the responses a correlation test was performed. For this purpose, correlation plots seen in Fig. 4 show the enzymatic release of xylose related to total release of arabinose and to the remaining diferulic acid content, and includes the linear regression fitting for each dataset ($R^2$). Testing the correlation between total arabinose release and enzymatic xylose release clearly showed that the data were highly correlated in turn signifying that arabinose removal was a vital parameter for maximizing enzymatic xylose release (Fig. 4a). It can be noticed that two apparently remote lying datapoints were included in this dataset. They represent two of the experiments set out by the statistical experimental design and are therefore valid data points. They originate from two of the low pH pretreatment experiments (experiments 6 and 10 in Table 2) and significant release of arabinose occurred in these experiments, as already mentioned. Even though this hydrolysis represented unspecific acid catalysed arabinose hydrolysis it still caused the enzymatic xylose release to be larger than in the other experiments. These data points therefore do not represent statistical outliers and cannot be excluded from the data set (even if they are excluded the positive linear correlation is still statistically significant, but the $R^2$ of the fit is lower). The plot should be interpreted as a relation between total removal of arabinose and how this affected enzymatic xylose release.

**TABLE 3**

Significance parameters ($P$) and regression coefficients for fitting multiple linear regression models to total arabinose release, enzymatic release of xylose and glucose after pretreatment and remaining content of diferulic acid after pretreatment of destarched corn bran. Confidence interval 95%. $P$ values below 0.05 indicate significant factors. –, factor combination not included in optimal model fit.

<table>
<thead>
<tr>
<th>Factor combination</th>
<th>Total arabinose release</th>
<th>Enzymatic xylose release</th>
<th>Enzymatic glucose release</th>
<th>Diferulic acid content*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Regression coefficient</td>
<td>$P$</td>
<td>Regression coefficient</td>
<td>$P$</td>
</tr>
<tr>
<td>Constant</td>
<td>51.80</td>
<td>$&lt;0.001$</td>
<td>20.35</td>
<td>0.003</td>
</tr>
<tr>
<td>Temperature</td>
<td>12.73</td>
<td>0.028</td>
<td>10.87</td>
<td>0.046</td>
</tr>
<tr>
<td>Time</td>
<td>12.52</td>
<td>0.030</td>
<td>10.04</td>
<td>0.061</td>
</tr>
<tr>
<td>pH</td>
<td>$-20.21$</td>
<td>0.003</td>
<td>$-11.63$</td>
<td>0.036</td>
</tr>
<tr>
<td>pH pH</td>
<td>28.32</td>
<td>0.004</td>
<td>24.74</td>
<td>0.006</td>
</tr>
<tr>
<td>Temperature pH</td>
<td>$-19.46$</td>
<td>0.020</td>
<td>$-16.23$</td>
<td>0.038</td>
</tr>
<tr>
<td>Time pH</td>
<td>$-14.35$</td>
<td>0.065</td>
<td>$-14.96$</td>
<td>0.051</td>
</tr>
</tbody>
</table>

Model fit ($R^2$) 0.886 0.839 0.889 0.897
Model predictability ($Q^2$) 0.446 0.211 0.435 0.444
Model validity 0.04 $-0.2$ $-0.2$ 0.927
Reproducibility 0.994 0.997 0.999 0.716

*$P$ values below 0.05 indicate significant factors. –, factor combination not included in optimal model fit.
By contrast, there was no statistical correlation between the diferulic acid content and the enzymatic xylose release (Fig. 4b). On this basis, the alleged hypothesis that diferulic acid cross-linking in corn bran arabinoxylan is a significant barrier for enzymatic degradation [1,11] therefore seems less likely. However, at present it cannot be completely ruled out that diferulic acid content and cross-linking may have hindered the enzymatic degradation. In favor of this was also the fact that alkaline pretreatments showed a tendency to leave the substrate more accessible to the action of $\alpha$-L-arabinofuranosidases; however, no such correlation could be substantiated (data not shown). The prolonged pretreatments resulted in a large removal of diferulic acids, but at the same time a lowering of the enzymatic xylose release (Fig. 1 and Table 2). As already mentioned, these pretreatments probably introduced excessive changes to the substrate.

**Intensive low pH pretreatments**

Because the data obtained in the initial pretreatments did not reach a maximum for release of monosaccharides, a second set of intensive low pH pretreatments were investigated. The pretreatment conditions were defined based on the findings in the initial setup, with conditions of experiment 6 (pH 2, 150°C, 65 min) as a corner point in the new setup. Because pH was generally the most significant factor, the hypothesis was that an optimum release was to be found with a lowering of pH and the interval investigated was therefore pH 1–2. Temperature was the second most significant factor but due to equipment limitations higher process temperature was not an option and temperature was maintained at 150°C for all experiments. Finally, as pretreatment time was the least significant factor we hypothesized that it would be possible to scale down the time frame to 10–65 min.
Looking initially to the models describing parameters for total release of arabinose and xylose (Table 4; $R^2$, $Q^2$, validity and reproducibility) these were all significantly better than the previous models (Table 3). This is related to the fact that a maximum in total release was reached within the given parameter ranges which is also realized from Fig. 5a and b. Hence, according to these models, a maximum release of both arabinose and xylose can be obtained at 150°C, pH 1.3, 50–55 min. Here, approx. 61% (~163 g/kg DM) of arabinose and 50% (~187 g/kg DM) of xylose will be released (Fig. 5a and b).

Modeling enzymatic xylose and glucose release showed good model fitting parameters as well (Table 4). No enzymatic release of arabinose was observed (data not shown). pH was still the most significant single factor but quite contrary to the previous results, increase in pH now had an increasing effect on the enzymatic xylose release. Lowering pH too much simply had a negative effect on the enzymatic xylose release (Fig. 5c). A comparison of the total xylose release and the enzymatic xylose release (Fig. 5b and c) point to the fact that acidic pretreatment at these specific severities will alter the native structure of the substrate to a degree that renders enzymatic hydrolysis a secondary process and not the main route to xylose hydrolysis.

Modeling of the enzymatic release of glucose showed enhancement by the acidic pretreatment; however, pH as a single factor was not significant. Instead the interactive effect of time and pH was highly significant. This was the complete opposite situation compared to the initial modeling of enzymatic glucose release and showed that it can be intricate to formulate interpretations based on inadequate model fitting as that in Table 3. The optimal enzymatic release of glucose is illustrated in Fig. 5d and will occur after pretreatment at 150°C, pH 1.5 and 45 min and result in approx. 68% release of glucose.

The model for remaining diferulic acid content shows, in accordance with the model in Table 3, that pH was the only significant factor and that lowering pH had a decreasing effect on the content of diferulic acids (Table 4, Fig. 5e). The regression coefficients were generally low because the spatial distribution of the dataset was relatively narrow. Minimum contents of diferulic acids were seen at the most extreme conditions, pH 1, 65 min with approx. 40% (~7 g/kg DM) remaining. Maximal enzymatic release of xylose occurred when approx. 72% (~12 g/kg DM) diferulic acids were remaining.

In these experiments temperature was maintained at the highest possible level causing time to become a significant factor. Therefore future experiments might pursue even higher pretreatment temperatures if shortening of pretreatment time is to be achieved. Exactly which temperature and time conditions that would provide the best basis for optimal pretreatment might however also depend on balancing economical, practical and environmental aspects such as energy and chemical expenditure, process time and equipment facilities.

### Combined severity factor

For pretreatment of lignocellulosic material it is common practice to compare pretreatment effects through a severity factor [32,33]. Inclusion of the end pH in the severity factor results in the combined severity factor:

$$\log(R_s) = \log(10^{-\text{pH}} \cdot t \cdot e^{(T-100)/(14.75)})$$

where $t$ is the holding time in min, $T$ is the pretreatment temperature, and 14.75 is a fitted value of the arbitrary constant $\omega$ based on the activation energy for lignocelluloses when assuming first order kinetics [33]. The $\log(R_s)$, with the constant 14.75, has also been used for assessing xylan degradation [34]. When relating $\log(R_s)$ to the enzymatic release of arabinose, xylose and glucose it was evident that the highest severity factors caused highest enzymatic release of xylose and particularly of glucose (Fig. 6; the lines in Fig. 6 only indicate a trend in the data). On the contrary, arabinose release was negatively affected (Fig. 6). Alkaline pretreatment, and thus a low negative severity factor, showed little effect on the content of diferulic acids (Table 4, Fig. 5e). The regression coefficients were generally low because the spatial distribution of the dataset was relatively narrow. Minimum contents of diferulic acids were seen at the most extreme conditions, pH 1, 65 min with approx. 40% (~7 g/kg DM) remaining. Maximal enzymatic release of xylose occurred when approx. 72% (~12 g/kg DM) diferulic acids were remaining.

| TABLE 4 | Significance parameters ($P$) and regression coefficients for fitting multiple linear regression models to total arabinose release, total and enzymatic release of xylose, enzymatic release of glucose and remaining content of diferulic acid after pretreatment of destarched corn bran after intensive low pH pretreatment experiments. Confidence interval 95%. $P$ values below 0.05 indicate significant factors. –, factor combination not included in optimal model fit |
|----------------|-------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------|
| **Total arabinose release** | **Total xylose release** | **Enzymatic xylose release** | **Enzymatic glucose release** | **Diferulic acid content** |
| **Regression coefficient** | **P** | **Regression coefficient** | **P** | **Regression coefficient** | **P** | **Regression coefficient** | **P** | **Regression coefficient** | **P** |
| **Constant** | 157.83 | 0.001 | 198.71 | 0.001 | 62.90 | 0.001 | 158.84 | 0.001 | 8.97 | 0.001 |
| **Time** | 16.23 | 0.001 | 39.65 | 0.004 | 6.80 | 0.077 | 16.25 | 0.001 | 0.02 | 0.958 |
| **pH** | –7.73 | 0.042 | –20.24 | 0.035 | 29.56 | 0.001 | 0.16 | 0.932 | 1.95 | 0.001 |
| **Time pH** | –11.62 | 0.046 | –22.35 | 0.097 | –23.78 | 0.006 | –14.06 | 0.003 | 0.64 | 0.161 |
| **Time pH** | –11.62 | 0.046 | –22.35 | 0.097 | –23.78 | 0.006 | –14.06 | 0.003 | 0.64 | 0.161 |
| **Model fit ($R^2$)** | 0.897 | 0.941 | 0.983 | 0.972 | 0.909 |
| **Model predictability ($Q^2$)** | 0.624 | 0.372 | 0.555 | 0.876 | 0.406 |
| **Model validity** | 0.753 | 0.544 | 0.356 | 0.350 | 0.483 |
| **Reproducibility** | 0.893 | 0.991 | 0.999 | 0.995 | 0.970 |
FIGURE 5
Response surface model plots for intensive low pH pretreatments of DCB. Results given as % of that originally present in DCB. (a) Total arabinose release after pretreatment and enzymatic hydrolysis. (b) Total xylose release after pretreatment and enzymatic hydrolysis. (c) Enzymatic xylose release after pretreatment. (d) Enzymatic glucose release after pretreatment. (e) Remaining diferulic acid content after pretreatment.
on xylose release, but tended to result in the highest enzymatic arabinose release (Fig. 6). These correlations have to be interpreted with caution as the use of the combined severity factor for describing and comparing the impact of pretreatment (on lignocellulosic substrates) favors pretreatments performed at acidic pH [33]. This is also evident when inspecting the data in Table 2. Nevertheless, the results indicated that substrate matrix opening apparently did occur during alkaline pretreatments inducing enzymatic hydrolysis, yet the effects were not as pronounced as those introduced by the acidic pretreatments. Clearly, the effects of alkaline pretreatments are not understood to the same degree as acidic pretreatments, especially not, when applied to non-lignified or low-lignified plant materials like corn bran.

Solubilization of dry matter

Solubilization has been recognized as an important factor for increasing enzymatic accessibility [18,19]. The amount of dry matter solubilization was determined after the pretreatments and expressed as weight % soluble dry matter of total DCB dry matter concentration and ranged from 0% to 52% across the different pretreatments (Table 2). Figure 7 illustrates the correlation between solubilization and enzymatic release (lines only indicate a trend in the data). As solubilization increased the enzymatic release of all three monomers also increased, but at a certain point the release ceased and ultimately decreased for xylose and arabinose (Fig. 7). The reason for the decrease was most probably related to the extensive chemical hydrolysis caused by the increasingly severe pretreatments as already described. About 20% (200 g/kg) of the total dry matter could be enzymatically released at both relatively low and relatively high extents of solubilization (Fig. 7). However, as seen from Fig. 7, approx. 25% of the total dry matter (250 g/kg DM) could be enzymatically hydrolysed to its constituent carbohydrate monomers at high solubilization. It is known that arabinoxylan and cellulose together constitute up to 75% of the total dry matter [6]. However, when adding the release of arabinose and xylose from the pretreatments a maximum of approx. 46% of the dry matter was released as monosaccharides, which leaves approx. 30% of the total biomass dry weight as unhydrolysed or lost. This observation corroborated the finding that 40–60% of arabinose and xylose was unaccounted for after certain pretreatments.

Conclusion

Firstly, the results inform that to obtain increased enzymatic accessibility of corn bran arabinoxylan after pretreatments, acidic pretreatment is the most effective route to follow. The low pH pretreatment generated an unspecific acidic prehydrolysis thus leaving the substrate open for enzymatic attack. However, the results also showed that large amounts of valuable monosaccharides were lost during the pretreatments and that increasingly severe acidic and alkaline pretreatments significantly altered and disrupted the native structure of the substrate. A maximum of 60% by weight of the available arabinose and 50% by weight of the available xylose was released leading to the question as to why this was the apparent upper limit. For the low pH pretreatments, the answer could solely be explained by the loss of monosaccharides during pretreatments, because full enzymatic release of xylose and arabinose from the remaining arabinoxylan was actually achieved. For the alkaline pretreatments loss combined with insufficient enzymatic hydrolysis seems to be the issue. Nevertheless, the substantial loss of xylose and arabinose in the range of 40–60% by extreme pH pretreatments would be unacceptable in commercial utilization of corn bran arabinoxylan.

Secondly, pretreatment was optimized for total release of monosaccharides through multiple linear regression modeling of the data with enzymatic xylose release as the major evaluation criterion. Within the methods tested here, the results indicated that the optimal pretreatment method for maximal enzymatic release of xylose would be close to operating at 150°C, pH 2 for 65 min., whereas lower pH during pretreatment, that is, pH 1.3, resulted in higher total xylose release because of increased xylose liberation during the pretreatment. Pretreatment pH of 1.3–1.5 also resulted
in maximal total glucose liberation, but as a result of increased enzyme catalyzed cellulose hydrolysis after the more acidic pretreatment. Finally, no direct correlation between diterulic acid content and enzymatic xylose release could be established by showing that the partial elimination of diterulic acid was not enough to cause sufficient increase in enzymatic degradability as compared to effects of low pH pretreatments, such as arabinose release. It cannot be dismissed that difluent cross-linking obstructs enzymatic degradation of arabinoxylan, but the data clearly envisioned that other aspects of the arabinoxylan structure, like arabinose substitution are more important to target to fundamentally overcome the complexity of corn bran recalcitrance. Hydrolysis of arabinoxylan from corn bran continues to be a demanding task and obtaining better hydrolysis stresses the importance of understanding the polysaccharide composition and molecular interactions.

Comprehension of the biomass complexity is achieved better through enzymatic reactions than through physiochemical pretreatments that will randomly attack and destroy the material. Besides obtaining scientific valuable knowledge, replacement of technical prospects.

Acknowledgement

The work presented here has been co-financed by NovoZymes A/S and the Food Graduate School Denmark to whom we are very thankful.

Appendix A. Supplementary data


References

35. Sørensen, H.R. et al. (2007) Enzymatic hydrolysis of wheat arabinoxylan by a recombinant “minimal” enzyme cocktail containing β-xylanase and novel endo-1,4-β-xylanase and α-L-arabinofuranosidase activities. Biotechnol. Prog. 23, 100–107
Main issues

Substrate availability is a central issue when assessing enzymatic hydrolysis in the sense, that all relevant enzyme activities can be present in a reaction without any hydrolysis occurring, exactly if the substrate is unavailable. According to hypothesis 3 the physical appearance of the substrate is important and so is the total substrate surface area. Increasing the total substrate surface area will in turn increase the substrate availability and can be achieved by mechanical milling of the biomass. The major advantages of this pretreatment form compared to the previously applied ones (Paper 1 and Paper 2) are the absence of chemical catalysts and high heat energy input. However, no significant solubilization should be expected from such a pretreatment, thus rendering the substrate in a state quite similar to the native, but probably also limited in the enzymatic yields compared to other methods.

Initially, particle size reduction of the material is intended as a means to increase the yields of enzymatic hydrolysis without having to alternate the substrate composition. DCB is therefore sorted into different particle size fractions, but upon doing so it is observed that the biomass composition between size fractions is not uniform. This preliminary observation has interesting perspectives because it indicates that the original bulk DCB is not of uniform conformation. In turn this may be a valuable observation for directing the enzymatic hydrolysis. The purpose of this study therefore is to verify differences in biomass composition and structure between particle size fractions. If indeed this is the case, different particle sizes of DCB may represent structurally different substrates for enzymatic reactions. It is therefore also a goal of this study to investigate the effects of reducing the substrate particle size on enzymatic hydrolysis with the observation in mind that biomass composition varies. The hypothesis for this particular work is that the distribution and composition of arabinoxylan and cellulose in corn bran is not uniform and that these differences affect the enzymatic hydrolysis. If in fact so, these particular differences will become visible when the substrate is milled and sieved into different substrate fractions.

As already mentioned substrate availability is a central issue, but another one is also the fact that native corn bran is insoluble whereas the enzymes are soluble. This creates yet another obstruction by mass transfer across a solid/liquid barrier, which in fact is only one of several physical conditions that may affect the enzymatic hydrolysis. While working with the results of this study, it became desirable to somehow describe the probability of successful enzymatic reactions together with the efficiency of reactions and thereby understand some of the limitations to enzymatic hydrolysis.
In order to do so, some of the fundamental parameters affecting the reactions will have to be considered and quantifiable to a certain extent. This is not a well-defined task and many parameters influence hydrolysis like enzymes kinetics, mode of action, substrate/enzyme ratio, diffusion, affinities etc. Some of these parameters are controllable, while others are not. Particularly in the case of hemicellulase and cellulase enzymes systems, complete substrate hydrolysis is dependent on concomitant reactions from several types of enzymes causing yet another noticeable obstacle for successful reactions. However, in the work presented here, the offset for describing reaction limitations and boundaries are taken in the differences in substrate surface area and we try to explore in more general terms the efficiency of the reactions and hereafter compare it to the actual observed data.

**Main results**

The work involves milling and sieving of the raw DCB fraction into four different particle size fractions. Also the solid residue after hydrothermal pretreatment (Agger et al., 2010 Paper 1) is milled and sieved. Figure 28 and 29 include the results of biomass compositional analysis in a standardized form, where the content of xylose, arabinose and glucose in each particle size fraction is related to the content in the total non-sieved bulk. For DCB (figure 28) it is evident, that monosaccharide content is not constant between particle size fractions, since especially xylose and glucose content vary. Arabinose content is on the other hand relatively similar between fractions. This leads to the conclusion that not only do the total contents vary, but also the composition of especially arabinoxylan will have to be different in the different particle size fractions. The general picture of the different particle size fractions is that the largest particles are richer in cellulosic glucose and in relatively unsubstituted arabinoxylan, whereas the smaller particles have a higher content of highly substituted arabinoxylan. Figure 29 shows the equivalent standardized composition of pretreated DCB and indeed shows the same tendency, except with less variation between sizes compared to the native corn bran.

These results indicate proof of the part of the hypothesis that concerns biomass composition between particle sizes, namely that milling and sieving of the substrate create a sorting of the substrate into differently composed fractions. It is also shown by additional milling and sieving of the largest particle size fraction from DCB that the material after second round of milling is indeed more similar between fractions than after the first milling and sieving. In principle this implies that repetitive milling and sieving will create substrate fractions of perfectly similar composition of the heterogeneous xylans and cellulose. The fact that the material in the first place divides into differently composed fractions indicate that the cell wall is originally composed of regions of polysaccharides of different compositions and possibly with different properties for the mechanical forces applied during milling. This is actually already indicated in the heat pretreated fractions obtained in Agger et al., 2010 Paper 1 where two distinctively different fractions appeared. For this to happen, the native corn bran must inevitably have consisted of differently composed regions of polysaccharides, giving rise to different physical properties.
Figure 28: Standardized monosaccharide composition of sieved particle size fractions of DCB relative to non-sieved DCB. Level of each component set to 100 in the non-sieved fraction.

Figure 29: Standardized monosaccharide composition of sieved particle size fractions of pretreated DCB relative to non-sieved pretreated DCB. Level of each component set to 100 in the non-sieved fraction.

Reducing the particle size is significantly affecting the yields of hydrolysis as seen from table 4 in the manuscript for Paper 3. The difference between particle sizes are more pronounced for DCB than for pretreated DCB, but markedly enough, the yield differences of arabinose and xylose between particle size fractions of pretreated DCB are much smaller than for glucose, indicating that reducing the particle size is
not equally affecting the degradation of arabinoxylan and glucose. Correlated to the fact that biomass composition in the pretreated DCB is more similar between fractions compared to the raw DCB leads to the thought that not only particle size is influencing the enzymatic hydrolysis. These additional effects may very well be related to differences in biomass composition. Especially the observation, that pretreatment reduce the effect of particle size reduction has been reported before (Pedersen and Meyer, 2009, Chundawat et al., 2007, Zeng et al., 2007) but these observations have mostly just been acknowledged without any further explanations.

Quantifying the effects of reducing the substrate particle size is intriguing and we therefore tried to estimate and compare some of the physical parameters in order to evaluate if the enzymatic reactions has obvious limitations. For this matter a set of basic assumptions are made, and the system is greatly simplified. There are three major assumptions, namely that

1. All successful enzymatic reactions will lead to the release of one average monosaccharide
2. All enzymes will react independent of each other
3. Substrate mass and surface appearance will not change during the reaction.

It is of course necessary to evaluate the validity of these assumptions, and unfortunately neither of them will be entirely true. Firstly, the enzymes in the reaction are a mixture of processive and endo-acting enzymes, why only some of them will obey the first assumption. Secondly, exactly because they have different modes of action, they do not react independently of each other in an extended process. Lastly, the estimations do not account for mass changes during the reaction, but it will definitely change and as the mass decreases the enzymatic reaction rates will also decrease.

Despite these simplifications we fell that the estimations bring some clarification to the events in an enzymatic reaction and how changes to substrate surface affects the number of successful collision occurring in a reaction.

Comparing estimations of total available surface area (table 6 Paper 3) to the total enzyme load of 1500 pmol indicate that the enzyme to substrate ratio is not immediately limiting. In order to mimic the physical conditions in the reaction, it is important to consider, that collisions between enzymes and substrate occur on an “individual” basis, meaning that even though a substrate particle holds many moles of substrate for the enzymes, individual enzyme molecules will collide with the entire substrate particle. In order to evaluate the collision probability it will therefore be necessary to compare the number of individual enzyme molecules to the number of individual substrate particles. In the light of that, the total enzyme loading in the samples account to approx. $9 \times 10^{14}$ individual enzyme molecules, which should be compared to approx. $30-8400$ substrate particles. It seems obvious directly from the difference in order of magnitude, that the probability of enzyme/substrate collision is high, why this is probably not the limiting factor. Instead it seems that the rare and limiting events in the reaction are that a collision is successful.
Based on a theoretical example of total substrate conversion it is possible to derive an extrapolating expression for the extent of enzymatic hydrolysis if this is only dependent on changes to the particle size. Figure 30 shows the results of should extrapolations when they are compared to the experimentally determined conversions. The extrapolations take the offset in the hydrolysis of the largest particles and the degree of hydrolysis of the second largest particles actually completely followed the extrapolated conversion. However, for the two particles sizes, 355 and 250 µm the conversions observed in the experiments are higher than those estimated by the extrapolations and it there seems plausible that other parameters than the particle size influence the hydrolysis. In these two particular cases other conditions actually promote the hydrolysis. Lastly, the conversion in the smallest particle size is lower than anticipated and in this case informing that factors are inhibiting the extent of hydrolysis. The latter conclusion could not be directly drawn based on the experimental hydrolysis data, since the absolute hydrolysis is largest in the smallest particle size fraction.

Figure 30: Conversion of biomass as a function of particle size comparing extrapolated and experimental data.

These estimations provide insight to the influence of particle size on enzymatic hydrolysis and thereby lead us to conclude that even though the physical effects of changing the substrate availability is prominent they can not encounter the entire differences occurring. In some cases hydrolysis is larger than what could be ascribed to particle size reduction and in other cases it is the opposite situation. These estimations can not be interpreted as a direct quantification of influence of particle size, because the numbers do not make sense on an individual basis. Only by comparison between each other do these numbers make sense as that would equal all other parameters. Unfortunately, the estimations do not provide information as to what causes these differences, but differences in biomass composition is a reasonable explanation. These estimations corroborate the second part of the hypothesis to this particular
paper, namely that not only differences in particle size, but also differences in biomass composition influence the hydrolysis.

Conclusion

This work is important because it indicates certain features of biomass composition of corn bran and because it has allowed us to evaluate and access the enzymatic reactions by an alternative theoretical approach. Related to the biomass composition it is important exactly because it opens the possibility of sorting the material into more similar fractions and thereby directing the enzymatic hydrolysis even more. The theoretical estimations allow us to “think out of the box” by considering if other parameters may determine the reactions, which are normally not accounted for in the usual methods for evaluation of performance of enzymatic reactions. The data presented here clearly indicate that hypothesis 3 has a strong influence on the overall hydrolytic reactions, possibly because physical appearances and properties are exceptionally more important in reactions with insoluble substrates.
Biomass composition in response to changing substrate particle size and the consequences for enzymatic hydrolysis of corn bran

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Abstract

Corn bran is a by-product from corn starch processing. This work examined the effects of changing substrate particle size on enzymatic hydrolysis of raw and pretreated, destarched corn bran, respectively. The biomass composition of the corn bran varied between particle size fractions: The largest particles ([1000;710]µm) were richer in cellulose and arabinoxylan with relatively low degree of arabinofuranosyl substitutions, whereas the smaller particles ([250;150]µm) contained less cellulose, but arabinoxylan with higher arabinofuranosyl substitution. Enzymatic hydrolysis yields improved with decreasing substrate particle size, particularly for raw corn bran. The increased enzymatic yields with decreasing substrate particle sizes were related to the increased substrate surface area but also by the biomass composition. Theoretical estimations of enzymatic reaction efficiency supported that biomass composition affected the reaction yields and provided new insight into the impact of substrate particle size on enzymatic biomass hydrolysis.

Keywords: destarched corn bran, arabinoxylan, particle size, enzymatic hydrolysis, biomass, composition
1. Introduction

Corn bran is as a co-processing product from the corn starch wet-milling process and constitutes a readily available agro-industrial fibrous residue. Corn bran represents the tough outer layer of corn kernels and is rich in C-5 and C-6 carbohydrates and ought to be upgraded to food and fuel products [1,2]. The milled corn bran mainly consists of primary cell walls from the pericarp of the corn kernel and possibly also the pedicel tip of the kernel, testa and some residual starch. The non-cellulosic polysaccharides of corn bran are mainly heteroxylans that have proved very complex and diverse in structure and composition [2-5] and exceptionally recalcitrant to enzymatic degradation [1,6-8]. Besides arabinoxylan, cellulose and starch make up the main constituents of corn bran [1-3]. A trademark of arabinoxylan is the extent and positioning of α-L-arabinofuranosyl substitutions on the β-D-(1→4) linked xylan backbone; for corn bran arabinose to xylose ratios in the range of 0.6-0.7 have been reported [1,9]. Corn bran arabinoxylan may furthermore be decorated with L- and D-galacto-pyranosyls, acetyl, coumaryl, feruloyl and diferuloyl residues, the latter even cross-linking arabinoxylan polymers [4,10-11]. Glucuronic acid is also known as a common component in arabinoxylan and recently also galacturonic acid has been claimed to be associated with the complex xylan oligomer fractions in corn bran [2]. Since efficient enzymatic hydrolysis is dependent upon the amenability of the substrate to enzymatic attack most cellulosic biomasses are pretreated either mechanically, physically, chemically or by a combination of these to enhance enzymatic conversion [12,13]. Several studies have examined the effects of diminishing substrate particle size prior to pretreatment or enzymatic hydrolysis [14-18]. Increased enzymatic degradation in response to biomass particle size diminution has normally been suggested to be related to increases in substrate surface area by creating higher enzymatic accessibility of the substrate.
However, differences in biomass composition among varying particle sizes have been observed for different cellulose/xylan substrates and cellulose and xylan degradation are not affected equally by particle size reduction [17, 19]. It has also been shown that different particles sizes might inherently sort the material to originate from different plant tissues [15]. Also Sinitsyn et al. (1991) [20] found that enzymatic hydrolysis on crystalline cellulose was not significantly affected by increasing the specific substrate surface area. These types of results have not received appropriate attention since differences in biomass composition as a result of substrate particle size reduction have rarely been addressed.

An overall purpose of our work is to enhance the enzymatic degradation of corn bran to produce high yields of monosaccharides. In this regard the objective of the present work was to describe the monosaccharide composition of different corn bran substrate particle sizes and to evaluate whether particle size and/or any possible compositional differences affected the enzymatic hydrolysis evaluated as enzymatic monosaccharide yields. We hypothesized that the structural composition and content of non-starch polysaccharides in corn bran would not necessarily be uniform in all particle size fractions and that any increasing enzymatic yields with decreasing particle size were not only a result of increased substrate surface area, but also influenced by differences in biomass composition.
2. Materials and Methods

2.1 Substrate

Corn bran was obtained from Archer Daniels Midlands Co., Decatur, IL USA as the by-product from corn starch wet-milling. The material was destarched with α-amylase and amyloglucosidase in a two step process as previously described [1]. The destarched corn bran will be referred to as destarched corn bran (DCB). Pretreated destarched corn bran, i.e. pretreated DCB was obtained by heat treatment of DCB at Risø DTU, National Laboratory for Sustainable Energy, Roskilde, DK as described previously [1]. The pretreatment encompassed heating of a 6% (w/v) aqueous slurry in a loop autoclave at 190 °C for 10 min [17,21]. No other chemicals were added. After pretreatment the pH was 4.2 in the total slurry. After pretreatment, the material was separated into a soluble and an insoluble fraction by filtration and the insoluble residue was washed in Milli-Q water and finally freeze dried.

2.2 Substrate particle size reduction and sieving

Particle size reduction was achieved by processing the material through a Retsch SM 2000 cutting mill with a 0.5 mm screen. Further particle size reduction of DCB fraction ]1000;710] µm was achieved by use of a benchtop cutting mill Retsch ZM 100 again with a 0.5 mm screen. After each particle size reduction step, the material was sieved using analytical sieves with apertures of 1000, 710, 355, 250 and 150 µm (Endecotts, London, UK). Mass distribution of each particle size fraction was obtained by weighing. The sieving resulted in 4 particle size fractions, namely: ]1000;710], ]710;355], ]355;250], and ]250;150] µm.
2.3 Substrate composition

In order to determine the overall monosaccharide composition each particle size fraction was subjected to two different kinds of acidic hydrolysis, either a 0.4 M HCl to facilitate quantification of xylose, arabinose and galactose [22] for 2 hours at 100 °C or the two step H$_2$SO$_4$ hydrolysis process to quantify cellulosic glucose and Klason lignin according to the standard procedure of the U.S. National Renewable Energy Laboratory (NREL) [23]. Klason lignin is corrected for protein content as this will influence the lignin quantification. Protein content was determined according to the methods of Barkholt and Jensen 1986 [24] which includes a complete hydrolysis of proteins to amino acids by a 6 M HCl hydrolysis for 24 hours followed by quantification by ion exchange chromatography.

2.4 Enzymatic hydrolysis

All size fractions of both substrates were hydrolysed enzymatically as previously described [1]. In brief, the enzyme mixture consisted of monocomponent endo-β-xylanase, β-xylosidase, two α-L-arabinofuranosidases, feruloyl esterase, acetyl xylan esterase and a commercial cellulase preparation Cellic™ CTec (Generation 2009). Cellic™ CTec is based on the *Trichoderma reesei* cellulase complex (exo-glucanase, endo-glucanase, and β-glucosidase activities) with further additional β-glucosidase and glycoside hydrolase family 61 hydrolysis boosting proteins [25]. An overview of enzymes and dosages is shown in table 1. All hydrolysis experiments were performed in triplicate at 2% w/v dry matter (DM), incubated for 24 hours, 50 °C at pH 5 in 0.1 M succinate buffer and each reaction was stopped by heating to 100 °C for 10 min.
Table 1: Enzymes used for bench marking of enzymatic hydrolysis of all different particle size fractions of all three substrates. All enzymes provided by Novozymes A/S. Cellic™ CTec is a commercially available mixed cellulase based preparation.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Microorganism</th>
<th>Dosage (mg/g DM)</th>
<th>Family/Type</th>
<th>EC number</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endo-1,4-β-xylanase</td>
<td><em>Humicola insolens</em></td>
<td>0.25</td>
<td>GH10</td>
<td>EC 3.2.1.8</td>
<td>[26]</td>
</tr>
<tr>
<td>β-xylosidase</td>
<td><em>Trichoderma reesei</em></td>
<td>0.25</td>
<td>GH3</td>
<td>EC 3.2.1.37</td>
<td>[26]</td>
</tr>
<tr>
<td>α-L-arabinofuranosidase</td>
<td><em>Meripilus giganteus</em></td>
<td>0.25</td>
<td>GH51</td>
<td>EC 3.2.1.55</td>
<td>[26]</td>
</tr>
<tr>
<td>α-L-arabinofuranosidase</td>
<td><em>Humicola insolens</em></td>
<td>0.25</td>
<td>GH43</td>
<td>EC 3.2.1.55</td>
<td>[26]</td>
</tr>
<tr>
<td>Acetyl xylan esterase (AXE)</td>
<td><em>Flavolaschia sp.</em></td>
<td>0.5</td>
<td>CE1</td>
<td>EC 3.1.1.72</td>
<td>[1]</td>
</tr>
<tr>
<td>Feruloyl esterase (FAE)</td>
<td><em>Aspergillus niger</em></td>
<td>0.5</td>
<td>Type A, CE1</td>
<td>EC 3.1.1.73</td>
<td>[27,28]</td>
</tr>
<tr>
<td>Cellic™ CTec</td>
<td><em>Trichoderma reesei</em></td>
<td>4</td>
<td>-</td>
<td></td>
<td>[1,25]</td>
</tr>
</tbody>
</table>
2.5 Monosaccharide analysis

Monosaccharides arabinose, galactose, glucose, and xylose were analysed and quantified by HPAEC-PAD, on a BioLC Dionex equipped with a CarboPac™ PA1 (analytical 4 x 250 mm) column from Dionex (Sunnyvale, CA). The elution profile consisted of an isocratic pre-run with 25 mM NaOH for 5 minutes, hereafter isocratic elution with 10 mM NaOH for 12 min followed by 7 min regeneration of the column with 500 mM NaOH and 5 min re-equilibration to 25 mM with a flow of 1 ml/min.

3. Results and Discussion

3.1 Biomass composition

Table 2: Relative biomass composition of destarched corn bran and pretreated destarched corn bran after sieving into different particle size fractions: Superscripts a, b, c and d indicate significantly different groups based on ANOVA with pooled standard deviations in a 95% confidence interval. Lower part of table contains relative biomass composition after extra reduction of particle size of fraction [1000;710] µm of destarched corn bran. Lignin content is determined as Klason lignin corrected for protein. Numbers in parenthesis represents relative content compared to non-sieved fraction.
<table>
<thead>
<tr>
<th>Substrate</th>
<th>Fraction (µm)</th>
<th>Mass distrib. (%)</th>
<th>Xylose (g/kg DM)</th>
<th>Arabinose (g/kg DM)</th>
<th>Glucose (g/kg DM)</th>
<th>Protein (g/kg DM)</th>
<th>Lignin (g/kg DM)</th>
<th>A:X</th>
<th>Sum (g/kg DM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Destarched corn bran</td>
<td>[1000;710]</td>
<td>17</td>
<td>476 ±0.4b (127%)</td>
<td>280 ±0.6a (105%)</td>
<td>240 ±11a (103%)</td>
<td>48.8 ±0.0</td>
<td>96.2 ±5.2</td>
<td>0.59</td>
<td>1141</td>
</tr>
<tr>
<td></td>
<td>[710;355]</td>
<td>52</td>
<td>432 ±9.3b (116%)</td>
<td>276 ±7.5a (103%)</td>
<td>219 ±19b (94%)</td>
<td>60.8 ±2.2</td>
<td>116 ±9.8</td>
<td>0.64</td>
<td>1104</td>
</tr>
<tr>
<td></td>
<td>[355;250]</td>
<td>20</td>
<td>296 ±7.0c (79%)</td>
<td>277 ±3.0d (104%)</td>
<td>182 ±19b (78%)</td>
<td>117 ±1.7</td>
<td>103 ±6.6</td>
<td>0.94</td>
<td>975</td>
</tr>
<tr>
<td></td>
<td>[250;150]</td>
<td>9.7</td>
<td>262 ±11d (70%)</td>
<td>265 ±9.2a (99%)</td>
<td>172 ±9.4b (74%)</td>
<td>163 ±0.0</td>
<td>67.4 ±4.1</td>
<td>1.01</td>
<td>929</td>
</tr>
<tr>
<td></td>
<td>Non sieved</td>
<td>-</td>
<td>374 ±0.6</td>
<td>267 ±0.4</td>
<td>233 ±13</td>
<td>89.1 ±0.3</td>
<td>30.9 ±1.8</td>
<td>0.71</td>
<td>994</td>
</tr>
<tr>
<td>Pretreated destarched corn bran</td>
<td>[1000;710]</td>
<td>12</td>
<td>141±3.2b (102%)</td>
<td>59 ±1.6 (91%)</td>
<td>358 ±2.8a (104%)</td>
<td>118 ±2.8</td>
<td>82.6 ±0.5</td>
<td>0.42</td>
<td>759</td>
</tr>
<tr>
<td></td>
<td>[710;355]</td>
<td>53</td>
<td>156±4.4a (112%)</td>
<td>66 ±1.0 (101%)</td>
<td>281 ±22b (82%)</td>
<td>83.6 ±10.0</td>
<td>111 ±9.1</td>
<td>0.42</td>
<td>698</td>
</tr>
<tr>
<td></td>
<td>[355;250]</td>
<td>19</td>
<td>124±3.5c (89%)</td>
<td>61 ±0.3 (93%)</td>
<td>181 ±3.5d (52%)</td>
<td>137 ±0.1</td>
<td>126 ±12</td>
<td>0.50</td>
<td>629</td>
</tr>
<tr>
<td></td>
<td>[250;150]</td>
<td>13</td>
<td>108±2.7d (78%)</td>
<td>56 ±1.0 (86%)</td>
<td>239 ±1.0e (69%)</td>
<td>139 ±12.7</td>
<td>145 ±5.1</td>
<td>0.52</td>
<td>687</td>
</tr>
<tr>
<td></td>
<td>Non sieved</td>
<td>-</td>
<td>139±1.8</td>
<td>65 ±0.9</td>
<td>345 ±1.3</td>
<td>115 ±10.0</td>
<td>94.4 ±4.5</td>
<td>0.47</td>
<td>758</td>
</tr>
<tr>
<td>Extra size reduced DCB [1000;710]</td>
<td>Origins</td>
<td>-</td>
<td>476 ±0.4a</td>
<td>280 ±0.6a</td>
<td>240 ±11a</td>
<td>48.8 ±0.0</td>
<td>96.2 ±5.2</td>
<td>0.59</td>
<td>1141</td>
</tr>
<tr>
<td></td>
<td>[710;355]</td>
<td>47</td>
<td>427 ±2.1b</td>
<td>287 ±2.2a</td>
<td>267 ±26a</td>
<td>38.7 ±2.1</td>
<td>15.2 ±4.0</td>
<td>0.67</td>
<td>1035</td>
</tr>
<tr>
<td></td>
<td>[355;250]</td>
<td>34</td>
<td>377 ±3.7c</td>
<td>287 ±2.3a</td>
<td>257 ±16a</td>
<td>61.8 ±0.0</td>
<td>10.2 ±0.5</td>
<td>0.76</td>
<td>993</td>
</tr>
<tr>
<td></td>
<td>[250;150]</td>
<td>16</td>
<td>371 ±8.1c</td>
<td>281 ±7.2a</td>
<td>274 ±3.8a</td>
<td>62.2 ±1.4</td>
<td>21.8 ±0.6</td>
<td>0.76</td>
<td>1010</td>
</tr>
</tbody>
</table>
The general trend for the relative biomass composition in DCB and in the pretreated DCB was that the polysaccharide content and composition varied with varying particle size (Table 2, significant variations indicated by ANOVA grouping in a 95% confidence interval with pooled standard deviations). The xylose and glucose contents decreased with decreasing particle size whereas arabinose content seemed to be more or less constant in all particle size fractions. Galactose content was low and followed the trend of xylose and glucose (data not shown). The trend in xylose and arabinose content caused the A:X ratio to increase with decreasing particle size, indicating that the extent of arabinofuranosyl substitution of the xylan backbone in the smaller particles was greater than that of the larger particles. Since the material had been destarched prior to the experiments it was assumed that all glucose originated from cellulose and the data thereby signified that the cellulose content was highest in the large particles. Other literature reports on cellulose content in corn bran are in the same range as those reported here [5].

The compositional differences between particle size fractions of pretreated DCB were less pronounced than those observed for native DCB (Table 2). This was not unexpected since previous investigations [1] had shown that approx. 50% of the original DCB biomass was solubilized during hydrothermal pretreatment and that the solubilized material was mainly composed of highly substituted arabinoxylo-oligosaccharides. The insoluble residue after pretreatment was less substituted than the native and richer in cellulose.

The varying biomass composition between particle sizes must inevitably be a result of a relatively heterogeneous starting material. The milling and sieving had apparently caused a sorting of the material into compositionally different substrates originating...
from the same material. In table 2 the relative content of monosaccharides compared to
the original non-sieved material are shown as percentage values and inform that
particularly xylose content differed between particle sizes. Hence the composition of
non-sieved DCB and pretreated DCB was apparently not uniform, but seemed to consist
of regions with alternating contents and compositions of the structural polysaccharides
leading to different physical/mechanical properties for milling. This milling and sorting
into different particle sizes had resulted in each particle size being relatively more
similar than the non-sieved material. The DCB fraction [1000;710] µm was additionally
milled down to smaller particles, sieved again and each size fraction was then
characterized with respect to monosaccharide composition in order to evaluate if the
composition would change again (lower part of table 2). The results showed that the
glucose content was constant across the different particle sizes and similar to the content
in the original [1000;710] µm particles. This was different to the results obtained after
the first round of milling (upper part of Table 2). However, as in the first round of
milling, arabinose content was also constant in the different particle size fractions after
re-milling and in accordance with the level in the original [1000;710] µm fraction. The
xylose content decreased somewhat with decreasing particle size (Table 2) but the
differences in xylose content compared to the original [1000;710] µm fraction were now
markedly smaller (476 to 262 g/kg DM versus 476 to 371 g/kg DM). This implied that
the original [1000;710] µm fraction in itself was heterogeneous but still relatively less
so than the native non-sieved DCB. Again, the additional milling and sieving had
apparently resulted in an organization of the material into three relatively more similar,
heterogeneous fractions. Hence, the data suggest that repetitive milling and sieving can
generate more and more similarly composed fractions of biomass, whereas one round of
milling and particle size sorting will in effect sort the material into differently composed
fractions (of different particle sizes). This conclusion is in complete accordance with previous findings were biomaterials have been found to sort according to origin and composition after milling and sieving [15,19]

3.2 Analytical methodology

Corn bran is a biological material and it is therefore relevant to consider if the data in table 2 represent a generic tendency. One large batch of destarched corn bran (from approx. 15 kg raw material) was used for these studies and it originated from an industrial process. Thus it represented a mixture of corn batches over a certain period of time, which would in turn level out data variations caused by batch differences, consequently making these data more reliable.

The analytical methods used to generate the monosaccharide composition have been carefully chosen to suit this kind of biomass. In that sense, xylose and arabinose has been determined after HCl hydrolysis rather than H₂SO₄ hydrolysis, as the latter was found to grossly underestimate the two components. The principle of H₂SO₄ hydrolysis is to swell and disrupt the cellulose microfibril structure during an initial acid-concentrated step followed by depolymerisation in a dilute acid step, where only the latter includes internal standards [23,29]. The initial acid step would degrade the acid labile pentose from hemicellulose without the chance of estimating a recovery thereby causing underestimation. Furthermore, hydrochloric acid has been found to catalyse less formation of degradation products from xylose and arabinose than sulphuric acid [30].

In the method employed here internal standards were included during the entire HCl hydrolysis. Lignin was determined as Klason lignin after H₂SO₄ hydrolysis and corrected for protein content in each fraction as proteins would create an artificial overestimation of lignin by adding to the insoluble residue after hydrolysis.
The sum of components in table 2, which does not account for the loss of water from monosaccharides when in a polymeric form, tended to overestimate the components in the larger particles and possibly underestimate the content in the smaller particles. This could probably be ascribed to analytical inaccuracies. Particularly in the case of pretreated DCB, the sum do not account for the entire mass (table 2). This might be due to degradation products formed during the hydrothermal pretreatment that could solubilize during the acid hydrolysis and thereby not be a part of the quantified products or residuals. In addition, the ash content of the smaller particles was up to 2-4 times larger than in the large particles in both pretreated and unpretreated DCB (data not shown), explaining the decrease in mass balance closure.
Table 3: Comparison of original analysed composition and calculated weighed average composition of non sieved DCB and pretreated DCB based on the mass distribution and data in table 2. A Balance (%) of 100 represents complete compliance between measured and calculated weighed average. Balance (%) above 100 indicates overestimation by weighed average and Balance (%) below 100 indicates underestimation.

The weighed average calculated as the sum of amounts in each fraction \( (C_{\text{component, fraction}}) \) times the mass distribution \( (X\%) \) from table 2: \( \sum X\% \cdot C_{\text{component, fraction}} \)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Xylose (g/kg DM)</th>
<th>Arabinose (g/kg DM)</th>
<th>Glucose (g/kg DM)</th>
<th>Protein (g/kg DM)</th>
<th>Lignin (g/kg DM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Destarched corn bran</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non sieved original</td>
<td>374</td>
<td>267</td>
<td>233</td>
<td>89.1</td>
<td>30.9</td>
</tr>
<tr>
<td>Weighed average</td>
<td>390</td>
<td>272</td>
<td>208</td>
<td>79.1</td>
<td>104</td>
</tr>
<tr>
<td>Balance</td>
<td>104%</td>
<td>102%</td>
<td>89.1%</td>
<td>88.7%</td>
<td>336%</td>
</tr>
<tr>
<td>Pretreated destarched corn bran</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non sieved original</td>
<td>139</td>
<td>65</td>
<td>345</td>
<td>115</td>
<td>94.4</td>
</tr>
<tr>
<td>Weighed average</td>
<td>137</td>
<td>61</td>
<td>258</td>
<td>103</td>
<td>112</td>
</tr>
<tr>
<td>Balance</td>
<td>98.6%</td>
<td>93.4%</td>
<td>74.7%</td>
<td>89.6%</td>
<td>118%</td>
</tr>
</tbody>
</table>

The components in the different size fractions can be assumed additive and based on the relative mass distribution and the data in table 2, the analytical accuracy can be assessed by comparing a calculated weighed average composition to the original measured composition in non sieved material (table 3). From here xylose and arabinose content in the sieved fractions were in good correspondence with the total content in non-sieved
material, whereas the glucose and protein content in both DCB and pretreated DCB seemed slightly underestimated. However, lignin content in DCB was either vastly overestimated in the sieved fractions or underestimated in the original material. This is most likely related to the nature of Klason lignin determination, as it is defined as the insoluble residue after hydrolysis and thereby not quantified as a specific compound. Also the lignin only made up ~ 40 g/kg DM of the original material, hence even small deviations in the estimations will create large discrepancies in the balance. Generally, the balances in table 3 inform that the hydrolysis methods employed were reproducible and that the variations between biomass compositions thereby represented true variations, even though the methodology has disadvantages. In literature, biomass compositional changes in response to changing particle size are seldom acknowledged even though it has been observed [17-19]
3.3 Enzymatic hydrolysis

Table 4: Release of monosaccharides after enzymatic hydrolysis of different particle size fractions with enzyme mixture. Results are given as a percentage of the content of each monosaccharide component in each fraction.  

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Particle size</th>
<th>Non sieved</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xylose</td>
<td>DCB^a</td>
<td>0.90</td>
</tr>
<tr>
<td></td>
<td>Pretreated DCB^b</td>
<td>15.68</td>
</tr>
<tr>
<td>Arabinose</td>
<td>DCB^a</td>
<td>2.54</td>
</tr>
<tr>
<td></td>
<td>Pretreated DCB^b</td>
<td>24.47</td>
</tr>
<tr>
<td>Glucose</td>
<td>DCB^a</td>
<td>5.45</td>
</tr>
<tr>
<td></td>
<td>Pretreated DCB^b</td>
<td>51.02</td>
</tr>
</tbody>
</table>

Enzymatic hydrolysis of each particle size fraction of DCB and pretreated DCB showed that the release of monosaccharides increased with decreasing particle size (Table 4). The largest relative effect of reducing the particle size was observed for DCB whereas the overall release of monosaccharides was largest for pretreated DCB. This is in complete accordance with other findings where pretreatment promoted enzymatic hydrolysis, but at the same time leveled out differences observed between particle sizes [17,19]. One obvious explanation for the increase in the extent of enzymatic hydrolysis is the resulting total increase in substrate surface area when particle size was reduced, leading to higher substrate availability. However, the impact of reducing the substrate particle size on hydrolysis yields for DCB was somewhat larger than anticipated, inspiring the though that other parameters than substrate surface area were influencing
the extent of enzymatic hydrolysis. The differences in biomass composition between particle sizes were also larger for DCB than for pretreated DCB thus suggesting a correlation between the extent of hydrolysis and biomass composition.

Evaluation of increasing surface area could also be done by comparing the arabinose release to glucose release from pretreated DCB (Table 4). For arabinose the yields were more similar between particle sizes as compared to especially glucose release, thus indicating that arabinose release from pretreated DCB was less dependent on changes in surface area. It would be expected that if changes in surface area were the major limiting factor for enzymatic hydrolysis, the observed effects of all three components would be equally affected, but this was not the case. At the same time especially arabinose content was relatively constant between size fractions. Therefore it seems plausible that the increasing enzymatic yields with decreasing particle size were caused not only by increase in surface area but were also dependent on the particular biomass composition in each fraction. This idea is new compared to previous conclusions drawn from promotion of enzyme catalysis by particle size reduction where traditionally only available surface area and pore volumes have been addressed [16-17,31, but it ought not to be a surprise that biomass composition influences enzymatic hydrolysis.

The implications of this idea is also that the increasing complexity of arabinoxylan by the increasing A:X ratio is not necessarily retarding the enzymatic hydrolysis. This contrasts the conception that increasing complexity of arabinoxylan poses a hindrance to enzymatic hydrolysis [32]. However, in the present work, enzymes specifically targeted to catalyse the removal of arabinosyl residues from both singly and doubly substituted xyloses were used (Table 1, [26,33]) and might therefore have overcome the barriers of extensive arabinosyl substitutions.
Cellulose composition should be somewhat comparable between particle sizes but the content and possible interactions with arabinoxylan might still be different (Table 4). Comparing glucose release from DCB to glucose release from pretreated DCB a total increment by a factor of 1.4 from the largest to the smallest particles were observed as compared to a factor of approx. 5.5 in DCB glucose release. If only substrate surface area was affecting the extent of enzymatic hydrolysis, increase factors of comparable numerical sizes would be expected between particles sizes. Indeed, these results showed that yield differences between substrate particle sizes reduced when pretreatment was introduced to the material. However, any real explanation to this phenomenon has not been proposed in literature, but it seems likely that it could be related to biomass compositional differences, exactly because these were also reduced when DCB was pretreated.

The only exception from the overall trend was the differences in glucose yield obtained for particle size fractions [355;250] and [250;150] µm for pretreated DCB (Table 4). The discrepancy was caused by the relatively low glucose content in size fraction [355;250] µm (Table 2) resulting in a false overestimation of the yield. The raw data did not show a higher degree of hydrolysis in this particular substrate fraction. As a result of these considerations, we believe that the obtained data represent an effect of true differences in biomass composition on top of increased surface area effects.

The fact that distribution of biomass into portions of more similar composed materials will affect the enzymatic hydrolysis opens the possibility of targeting the enzymatic hydrolysis even more. Still the generally applied enzymatic techniques here and elsewhere are not sufficient to degrade the polysaccharide structure of corn bran, despite that all seemingly relevant enzyme activities were present. Obtaining more similarly
composed fractions of substrate simplify the picture and might thereby help to understand and break down the puzzle of the cell wall structure.

3.4 Theoretical assessment of successful enzyme-substrate collisions

3.4.1 Available substrate surface and collision probability

With the results presented here it is intriguing to attempt an assessment of the significance of surface area changes. This can be done by estimating the number of successful enzyme-substrate encounters, i.e. resulting in reaction, between substrate particles and enzyme molecules.

Table 5: Equations used to estimate results in table 6 and 7. Reactions are assumed to occur during 24 hours incubation at conditions equivalent to those stated in materials and methods. It is assumed that substrate particles are dense spheric structures that will pack as close as possible in a cubic closest packed face centered cell [34] defining a unit cell of 4 particles. It is furthermore assumed that substrate particle density is $1 \cdot 10^6 \text{ g/m}^3$ and that the substrate particle is completely made up of polysaccharides. Finally it is assumed that the product of every successful enzymatic reaction is a monosaccharide with equal chance of this being either a hexose or a pentose so that an average molecular weight of 168 g/mol can be used.
<table>
<thead>
<tr>
<th>Eq.#</th>
<th>Equation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>[ V_{\text{unit\ cell}} = (l_{\text{unit\ cell}})^3 \cdot 10^{-12} = (r \cdot \sqrt{8})^3 \cdot 10^{-12} ]</td>
</tr>
</tbody>
</table>
| | \( V_{\text{unit\ cell}} \) is volume of unit cell [ml]  
\( l_{\text{unit\ cell}} \) is length of one edge in the unit cell [µm]  
r is particle radius [µm] |
| 2 | \[ A_{\text{particle}} = 4 \cdot \pi \cdot r^2 \cdot 10^{-12} \] |
| | \( A_{\text{particle}} \) is surface area of spheric particle [m²]  
r is particle radius [µm] |
| 3 | \[ n_b = C_b \cdot \frac{4}{V_c} = C_b \cdot \frac{4}{(\sqrt{8})^3 \cdot 10^{-12}} \] |
| | \( n_b \) is number of substrate particles per sample [substrate particles/sample]  
\( C_b \) is substrate concentration [2% w/v DM]  
r is particle radius [µm] |
| 4 | \[ m_{\text{particle}} = \rho \cdot \frac{4}{3} \cdot \pi \cdot r^3 \cdot 10^{-18} \] |
| | \( m_{\text{particle}} \) is substrate particle mass [g]  
r is particle radius [µm] |
| 5 | \[ M_{\text{sample}} = X_k \cdot C_{\text{DM}} \] |
| | \( M_{\text{sample}} \) is total mass conversion during hydrolysis per sample [g]  
\( X_k \) is conversion in the \( k \)’th particle size [g/kg DM]  
\( C_{\text{DM}} \) is total sample DM [20\cdot10^{-6} kg] |
| 6 | \[ n_e = \frac{m_e \cdot N_A}{\text{MW}_c} \] |
| | \( n_e \) is number of enzyme molecules per sample [enzyme molecules/sample]  
\( m_e \) is enzyme mass loaded to each sample [12 mg]  
\( N_A \) is Avogadro’s number [6.022\cdot10^{23} /mol]  
\( \text{MW}_c \) is average molecular weight of an enzyme [80000 g/mol] |
| 7 | \[ N_b = \frac{m_{\text{particle}} \cdot n_b \cdot N_A}{\text{MW}_c} = \frac{\rho \cdot \frac{4}{3} \cdot \pi \cdot r^3 \cdot C_b \cdot 4 \cdot N_A}{\text{MW}_c \cdot (\sqrt{8})^3 \cdot 10^{-12}} = \frac{\rho \cdot \frac{16}{3} \cdot \pi \cdot C_b \cdot N_A}{\text{MW}_c \cdot (\sqrt{8})^3 \cdot 10^{-12}} \] |
| | \( N_b \) is number of successful hits per substrate particle [hits/substrate particle]  
\( \text{MW}_c \) is the average molecular weight of a carbohydrate monomer |
| 8 | \[ N_{SA} = \frac{N_b}{A_{\text{total}}} \cdot \frac{N_b}{n_b \cdot A_{\text{particle}}} = \frac{m_{\text{particle}} \cdot n_b \cdot N_A}{n_b \cdot A_{\text{particle}} \cdot \rho \cdot \frac{4}{3} \cdot \pi \cdot r^3 \cdot 10^{-18} \cdot N_A} = \frac{\rho \cdot r \cdot N_A}{3 \cdot \text{MW}_c \cdot 10^{-12}} \] |
| | \( N_{SA} \) is total number of successful hits per particle surface area [hits/m²] |
| 9 | \[ X_i = X_k \cdot \frac{r_i^2}{r_k^2} \cdot \frac{n_b}{n_{b,k}} \] |
| | Extrapolation of conversion from the conversion in the \( k \)’th particle (\( X_k \)) to the \( l \)’th particle (\( X_l \)) [g/kg DM]  
r\( i \) is radius of the \( l \)’th particle [µm]  
r\( k \) is radius of the \( k \)’th particle [µm]  
\( N_{b,k} \) is the number of particles in the \( k \)’th size sample  
n\( N_{SA} \) is the number of particles in the \( k \)’th size sample |
Table 6: Estimations of number of substrate particles, substrate surface area, and enzyme loading capacities of different particle sizes. Estimations according to equations in table 5. *Enzyme loading as in enzymatic hydrolysis experiments: 6 mg enzyme protein/g DM.

<table>
<thead>
<tr>
<th>Particle size (µm)</th>
<th>Radius (m)</th>
<th>Unit cell volume (ml)</th>
<th>Number of particles (#/sample)</th>
<th>Substrate surface area (m²/sample)</th>
<th>Maximal number of enzymes (pmol/sample)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000</td>
<td>5.00·10⁻⁴</td>
<td>2.83·10⁻³</td>
<td>28</td>
<td>8.88·10⁻³</td>
<td>16</td>
</tr>
<tr>
<td>710</td>
<td>3.55·10⁻⁴</td>
<td>1.01·10⁻³</td>
<td>79</td>
<td>12.5·10⁻⁵</td>
<td>23</td>
</tr>
<tr>
<td>355</td>
<td>1.78·10⁻⁴</td>
<td>1.27·10⁻⁴</td>
<td>632</td>
<td>25.0·10⁻⁵</td>
<td>46</td>
</tr>
<tr>
<td>250</td>
<td>1.25·10⁻⁴</td>
<td>4.42·10⁻³</td>
<td>1810</td>
<td>35.6·10⁻⁵</td>
<td>66</td>
</tr>
<tr>
<td>150</td>
<td>0.75·10⁻⁴</td>
<td>9.55·10⁻⁶</td>
<td>8380</td>
<td>59.2·10⁻⁵</td>
<td>109</td>
</tr>
<tr>
<td><strong>Total enzyme load</strong></td>
<td>0.12</td>
<td></td>
<td><strong>1.5·10³</strong></td>
<td>1.5·10³</td>
<td><strong>1.5·10³</strong></td>
</tr>
</tbody>
</table>

The apparent physical changes occurring when the substrate is milled and sieved into different particle size fractions are that the total surface area and the total number of particles increase with decreasing particle size. Table 6 contains an estimate of how the total substrate surface area changes with changing particle size all other things being equal (equations in Table 5) as well as an estimate of the maximal number of enzymes that might occupy the entire particle surface area. The latter estimate was done by considering the maximal number of enzyme molecules that might be positioned next to each other without overlapping on the outer surface of the substrate particles. Obviously; both this particular heterogeneous substrate and the fact that this enzyme blend encompassed both processive and endo-acting enzyme activities form a particular complex and unfortunate model case. Nevertheless, a consideration of successful collisions does provide some learning points. For the estimations a few basic
assumptions were made, namely that the substrate particles could be represented by completely dense spherical shaped structures that would pack as close as possible in a cubic closest packed face centered cell [34] defining a unit cell of 4 particles (eq. 1, Table 5) and that reaction conditions in general were equal to those applied in the bench mark enzymatic hydrolysis. Furthermore, it was assumed that the substrate density regardless of particle size was close to 1 g/ml when fully water saturated in the reaction. With respect to the enzymes, it was assumed that they had an average molecular weight 80 kDa. Even though the molecular size of glycosyl hydrolases varies greatly, the molecular masses of the minimal mixture range from ~42-100 kDa [26], and generally glycosyl hydrolases lay within the range of 25 to 125 kDa [25]. Furthermore, it was assumed that each enzyme could be represented by cubic structures of 3x3x3 nm, where a surface area of 9 nm$^2$ could interact with the substrate.

As expected, the number of enzymes that might interact with the substrate increased for increasing surface area (Table 6, in this case from 16 to 109 pmol). However, when compared to the total enzyme load in each sample it is evident that the number of free enzymes in solution was far greater than the available substrate surface (Table 6). Similar considerations have been described by Axelrod and Wang (1994) [35]. They introduced the “reaction-limited” receptor concept leading to low reaction probability as the rate limiting process rather than limitations in reactant collisions.

It can fairly easy be recognized that the number of reactant collisions are not limiting in this scenario either, since the number of individual substrate particles ranges from approx. 28 to 8400 and should be compared to an enzyme loading of $9 \times 10^{14}$ individual enzyme molecules (equivalent to $1.5 \times 10^3$ pmol, Table 6). From the apparent difference in order of magnitude it seems obvious that the probability of substrate particles
constantly colliding with enzyme must be very high. However, as described in a general receptor concept by Axelrod and Wang (1994) [35] the successful reactions are strongly dependent on thermodynamics on a micro-scale level possibly due to Brownian motions of the enzymes that may influence whether an effective binding occurs. Specifically for these kind of enzymatically catalysed reactions it also becomes crucial how the kinetics and affinities are for each enzyme, especially in the case where a certain coordination between activities occur, i.e. in the case of endo-β-xylanase and β-xylosidase activities and endo-glucanase and β-glucosidase activities.

3.4.2 Successful enzyme hits; theoretical versus experimental conversion

Despite all the different mechanisms affecting an enzymatic reaction and in particular complex non-Michaelis Menten reactions, it is intriguing to somehow try to describe the events of successful reactions. If the enzymatic reactions are simplified to a system of single reacting, independent reactions where it is assumed that every successful hit leads to the release of one monomeric component regardless of the actual mechanism (eventhough neither celllobiohydrolases, endo-glucanases or endo-xylanases release monomeric constituents), then certain estimations can be made that will provide insight to the reaction dependency of particle size. It is possible to estimate a total number of successful hits in every sample together with a total number of successful hits per substrate surface area. The latter can be interpreted as a measure of the hydrolysis efficiency.
Figure 1: A: Successful hits/substrate surface area when assuming full conversion in all particle size fractions as a function of total substrate surface area. B: Conversion of biomass as a function of particle size comparing extrapolated and experimental data.

If full conversion of all dry matter is assumed, a correlation between total substrate surface area and the number of successful hits per substrate surface area can be deduced.
by use of equation 2, 3, 7, and 8 in Table 5 and this correlation is visualized in Figure 1A. As can be realized from equation 7 (Table 5), the total number of successful hits is independent of particle size and in the example of total conversion it is furthermore constant between particle sizes. The plot in Figure 1A therefore shows that when the total substrate surface area decreases the efficiency of the hydrolysis needs to increase in order to still convert the same amount of substrate. The full conversion example provides little insight into how the hydrolysis depends on particle size, exactly because it assumes the same degree of hydrolysis for all particles. However, it is valuable for deriving an extrapolation expression for estimating the conversion in substrate with different particle sizes, if and only if these are solely dependent on particle size.

Based on such an extrapolation expression (equation 9 in Table 5) it is possible to estimate the conversion in all particle sizes if the conversion in one particle size is known. Table 7 shows such extrapolations together with the real observed conversion from experiments on DCB in Table 4 and these are plotted in Figure 1B as a function of particle size. From here it is apparent that the hydrolysis of the second largest particles (710 µm) follows the extrapolation based on particle size, whereas particles of 355 and 250 µm in diameter are somewhat more efficient in the hydrolysis (Figure 1B). The conversions in these two particular particle sizes were higher than what could be expected if these were only dependent on the corresponding increase in surface area. Interestingly, the smallest particles of 150 µm seem to fall behind in the conversion as compared to the extrapolated example, meaning that the enzymatic hydrolysis in these particles was apparently hindered compared to what could be expected if surface area was the only parameter determining the degree of hydrolysis. This was not directly realized by comparing the apparent conversion from the experiments, as the conversion in the smallest particles in absolute amounts was the most efficient (Table 7).
Table 7: Estimations of number of enzyme/substrate interactions leading to a successful reaction, e.i. number of successful hits ($N_S$) per substrate particle and number of successful hits ($N_{SA}$) per substrate surface area when conversion is extrapolated based on changes in substrate surface area (upper part) and experimentally observed conversion from experiments in table 4 (lower part). In the extrapolated example conversion in the largest particles (1000 µm) is set to be the same as the experimentally observed. All estimations based on the reaction volume, substrate and enzyme concentrations as those applied in table 4. Estimated according to equations given in table 5.\textsuperscript{a} Experimental conversion of substrate particle sizes smaller than 150 µm was not included in experiments in table 4.

<table>
<thead>
<tr>
<th>Extrapolation of conversion</th>
<th>Particle size (µm)</th>
<th>Conversion X (g/kg DM)</th>
<th>$N_S$</th>
<th>$N_{SA}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1000</td>
<td>24.5</td>
<td>1.75\times10^{18}</td>
<td>1.97\times10^{22}</td>
</tr>
<tr>
<td></td>
<td>710</td>
<td>34.5</td>
<td>2.47\times10^{18}</td>
<td>1.97\times10^{22}</td>
</tr>
<tr>
<td></td>
<td>355</td>
<td>68.9</td>
<td>4.94\times10^{18}</td>
<td>1.97\times10^{22}</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>97.8</td>
<td>7.02\times10^{18}</td>
<td>1.97\times10^{22}</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>163.1</td>
<td>11.7\times10^{18}</td>
<td>1.97\times10^{22}</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Experimentally observed conversion</th>
<th>Particle size (µm)</th>
<th>Conversion X (g/kg DM)</th>
<th>$N_S$</th>
<th>$N_{SA}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1000</td>
<td>24.5</td>
<td>1.75\times10^{18}</td>
<td>1.97\times10^{22}</td>
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<tr>
<td></td>
<td>710</td>
<td>33.1</td>
<td>2.38\times10^{18}</td>
<td>1.90\times10^{22}</td>
</tr>
<tr>
<td></td>
<td>355</td>
<td>104.6</td>
<td>7.50\times10^{18}</td>
<td>3.00\times10^{22}</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>115.2</td>
<td>8.26\times10^{18}</td>
<td>2.32\times10^{22}</td>
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<tr>
<td></td>
<td>150\textsuperscript{a}</td>
<td>122.6</td>
<td>8.79\times10^{18}</td>
<td>1.48\times10^{22}</td>
</tr>
</tbody>
</table>
The immediate question why some particles are hydrolyzed better than what could be expected based on surface area might have several possible answers. Firstly, this could be related to differences in biomass composition. For simplicity reasons these estimations are based on an assumption of uniform biomass composition between particle sizes but as already discussed this was not the case and could very well be part of the reason for the observed differences in conversion between the particles. With the enzyme blend used in these experiments (table 1) some biomass compositions were simply better hydrolysed than others.

Other possible reasons for accelerated hydrolysis in the mid-range particles might be that the surface area of the particles change during hydrolysis with respect to physical appearance and crystallinity for cellulose in particular, thus promoting binding to some parts of the substrate. Similar considerations were described in the erosion model by Väljamäe et al. 1998 [36]. Furthermore, the enzymes might be able to migrate from a non-productive binding site to a productive one without dissociating away from the substrate surface, as proposed by Axelrod and Wang (1994) [35], reducing the dimensionality of the reaction from 3D to 2D. The latter would greatly enhance the efficiency of the enzymatic reaction and whether this kind of phenomenon is possible might again be linked to biomass composition and surface appearance.

The fact that the hydrolysis in the smallest particle size samples was less effective might also be related to the biomass composition. As the particles became smaller, protein and possibly lignin tended to accumulate, which could have hindered the hydrolysis. Previous results of milling and sieving wheat straw also found that the content of ashes and minerals tended to build up in the smallest particle size fraction and that might also be contributing to lower conversion [17].
These estimations are based on very simplified assumptions and it is therefore important only to evaluate these in relation to each other and not the exact numerical size, as they provide no real quantification of the actual reactions. Unfortunately, the enzyme system in these examples would not obey the assumptions of being independent and releasing a monosaccharide from each successful reaction. Furthermore, several dynamics like molecular velocity and movement, changing reaction conditions as a result of hydrolysis, biomass heterogeneity, particle surface structure and porosity were not accounted for in these estimations. Also all enzymes were treated as if there were no differences in catalytic mode of action. Without a doubt these factors would influence the enzymatic process but despite the simplifications these estimations provide a new method of understanding the enzymatic reactions dependency on a physical property like changing substrate particle size. More of such simplified illustrations might give clues to how enhanced yields of reaction are achieved.

4. Conclusions

The data demonstrated that milling and sieving of corn bran created different particle size fractions that varied in monosaccharide composition and arabinoxylan substitution. Enzymatic hydrolysis of different substrate particle sizes gave different yields. The data suggested that these differences were caused by differences in particle surface area and biomass composition. Theoretical estimations of substrate particle-enzyme collisions supported that other parameters than surface area, e.g. biomass composition, apparently also affected the enzymatic hydrolysis. Hence, particle size reduction increases enzymatic hydrolysis yields due to increased substrate surface area, but the influence of other parameters such as biomass composition is equally vital to recognize.
5. Acknowledgement

Tomas Fernqvist, Risø DTU and Ove Skov Nielsen, Novozymes A/S are thankfully acknowledged for assisting in the pretreatment and destarching of corn bran, respectively. The work presented here has been co-financed by Novozymes A/S and the Food Graduate School Denmark to whom we are very thankful.
6. References


7 Conclusion

The overall aim of this work has been to obtain high and controlled enzymatic hydrolysis by applying relevant mono-component enzymes carefully selected to fit the polysaccharide composition and thereby obtain the minimal enzymatic requirements for better hydrolysis and understanding of corn bran. This strategy has proven efficient only to a certain extent, as complete hydrolysis is not obtained even with a careful and detailed iteration between envisioned structural makeup and enzymes selected for degradation. According to the currently known and accepted composition of arabinoxylan, which is also proven in this thesis, the relevant enzyme activities have been applied. The enzymes are active on the polysaccharide structures, because hydrolysis is actually achieved after pretreatment. However, at the moment there seems to be a gap between, which enzyme activities are necessary to degrade corn bran and the practical yields obtained when those are actually applied exactly because hydrolysis is not complete, even after pretreatment. This gap seems to stem from lack of understanding of the arabinoxylan heterogeneity, but most certainly also from a lack of understanding of the entire cell wall composition and how this determines the physical properties of the insoluble substrate. Hence the data obtained lead to the conclusion that corn bran has an exceptionally rigid and tight structure of the cell wall, which gives corn bran its unique physical properties that repel most enzymatic attacks.

With respect to the overall hypotheses in this thesis, the first major hypothesis that diferulic acid cross-linking between arabinoxylan molecules represents an intrinsic barrier to enzymatic hydrolysis seems less likely based on the work presented here. Paper 2 evaluates the effects of chemically removing diferulic acids from the raw corn bran prior to enzymatic hydrolysis, and the effects of this on the subsequent enzymatic degradation of arabinoxylan are negligible. Also, from the solubilized corn bran no effects on the overall hydrolysis are observed, when more or less full enzymatically catalyzed release of diferulic acids occurs. This informs that even though diferulic acids are present and particularly abundant in corn bran compared to other substrates, they do not impose a significant hindrance to hydrolysis. Still, the impact of efficient enzymatically catalyzed release of diferulic acids from the native corn bran has not been proven and it is therefore difficult to firmly dismiss an influence from these compounds on the overall structure and recalcitrance of the cell wall. Most studies, including those presented here, obtain enzymatically catalyzed release on pretreated or otherwise modified substrates making it difficult to exclude other influencing factors. At present it is therefore not possible to entirely dismiss the hypothesis, but the data achieved during this work do not confirm, that opening of diferulate cross-linkages are the key barrier to overcome in order to achieve enzymatic hydrolysis of corn bran.

Instead the second hypothesis that targeting different substituents on arabinoxylan can lead to increased degradation of corn bran seems to be true, especially when focusing on those kinds of substituents that are dominating in the overall structure. In that respect, it has been proven that acetylations are important substituents to consider and target by hydrolysis, as they on a molar basis represent a major contributor to arabinoxylan diversity, also observed by MALDI-TOF in this work. Moreover, the data verify that removal of feruloyl substituents should still receive appropriate attention when increased hydrolysis is
the objective, even though extended release of ferulic acid is not necessarily directly followed by extended release of arabinose. The latter realization sets a question mark to the position of feruloyl substitutions and indeed also arabinosyl positioning in the overall structure. Linkage analysis also suggests that arabinoxyls may be present in other configurations than the terminal substitutions on arabinoxylan and possibly even in heterogeneous side chains or arabinan structures.

The third major hypothesis that physical occurrence of the substrate is important has been demonstrated by different means. Solubilization is achieved as a result of different kinds of pretreatment and changes to substrate availability by increasing the specific surface area as a result of reducing particle size. Pretreatment of corn bran is at present indispensable if significant enzymatic hydrolysis is the goal so reducing the costs are important. Definitely, introducing a pH catalyst to the pretreatment reduce the need for heat input, especially in the low pH range. Optimal conditions for enzymatically catalyzed release of xylose and glucose can be compromised to lie in the range of pH 2-1.5, 150 °C for 45 min. However, the same pretreatment conditions completely eradicate enzymatically available arabinosyls from the substrate and generally induce large losses of valuable monosaccharides. Depending on further use of the pretreated material, it may be acceptable that certain amounts of the substrate is lost, but from an academic point of view the random chemical hydrolysis affects the substrate to an unwanted extent leaving very little room for understanding and exploring the effects of pretreatment.

Enlightened by the pH catalysed pretreatment experiments the impact of increased substrate availability on enzymatic hydrolysis was investigated by decreasing the substrate particle size and dividing it into similarly composed fractions of biomass. This cause an enhanced enzymatic hydrolysis which is related to the changed surface area conditions, but most likely also to the compositional differences observed between fractions. The most significant results from this study are that corn bran from a global point of view, is also very heterogeneous as it is on a micro-scale level. The composition of particularly arabinoxylan is not uniform through out the cell wall and this alone may create challenges for the enzymatic hydrolysis. Sorting the biomass by milling and sieving into similarly composed fractions can from a generic point of view create the basis for better understanding and directing the enzymatic hydrolysis when fewer structures are present.

It is also speculated that some of the resistance to enzymatic degradation is related to a network of polysaccharides interacting with structural proteins and even that some of the carbohydrate content originate from glycosylations of these proteins. However, it has not been possible to confirm or dismiss this idea, since pre-digestion of corn bran with certain proteases does not have an affect and neither does extraction of significant amounts of proteins. On the other hand, it is established that at least some monosaccharides are associated with the protein fraction, especially galactose and arabinose. Furthermore, amino acid profiling of the protein content shows presence of hydroxyproline stemming from structural proteins. In addition, linkage analysis confirms the presence of galactan structures possibly related to arabinogalactan type II, which again may originate from AGPs. Otherwise the amino acid profile is mostly dominated by residues typical for storage proteins. It is still a possibility that the
proteins obstruct the enzymatic hydrolysis, as the lack of effects from pre-digestion may simply be a result of incomplete hydrolysis.

This project has focused on corn bran and diferulic acids and I believe that the novelty of the work lies within the acknowledgement that the enzymes already known and applied are generally the right ones. What are still missing are the right physical conditions for these enzymes to react under. Working with corn bran in comparison to other cereal residues, which may degrade easier have illustrated the importance of considering physical properties of the cell wall, which in the end is of course governed by the polysaccharides and the matrix as such. But it is important to acknowledge that unknown physical traits on a global level retain the enzymes from catalyzing the desired reactions. I still believe that enzyme based methods for effective hydrolysis of corn bran can be further developed and that results obtained on corn bran as substrate will be also widely effective on other cereal residues. Therefore corn bran continues to be an interesting and important substrate in itself as well as a significant model substrate.
8 Future perspectives

Corn bran appears to be an attractive biomass stream for exploitation especially in the biofuels industry because it is abundant and readily available from corn starch processing. In the United States where corn production is massive the logistical requirements for using corn bran for biofuels or in biorefineries will be limited thereby making it an even more interesting resource. On the other hand, as verified in this thesis work the apparent limitations of immediate exploitation of corn bran are its inherent resistance to degradation and if this is not solved, corn bran may not be as suitable.

Working with corn bran as a substrate for enzymatic hydrolysis has been a challenging task and understanding the structural composition of the individual polysaccharides especially arabinoxylan is of utmost importance to enhance the yields of hydrolysis. Continued investigation of the structure is important, and will in time become one of the keys to obtaining satisfying hydrolysis. However, diferulic acid cross-linkings are probably of lower importance to address than other constraints and it seems yet vital to understand the overall distribution and/or compartmentalization of the cell wall components to effectively overcome the barriers of hydrolysis. Corn bran represents a substrate where nature during millions of years has done its utmost to evolve a protective shield for the germ. This shield is nature’s initial barrier and is defacto repelling enzymatic hydrolysis. Substrate availability in the broadest possible sense is therefore the primary obstacle. Not until this has been overcome will the enzymes be able to effectively catalyse the hydrolysis and efforts to understand the heterogeneity of arabinoxylan structures is therefore of secondary importance.

The questions are therefore what causes this exceptionally rigid exterior and how it is to be attacked? Corn bran repels water and does not swell in an aqueous solution, which in itself speaks of an environment hostile to hydrolysis. In that sense, it may also be interesting to consider another solvent system for the reactions than strictly aqueous buffers. However, there may be several reasons for this impenetrable exterior, but probably xylan interactions with cellulose microfibrils either by hydrogen bonding, or by actual incorporation into the microfibrils will significantly tighten the cell wall structure and leave the xylan inaccessible to hydrolysis. SEM visualization of the cell wall network of certain hemicellulose/cellulose matrices (Whitney et al., 1998) establish the impression of a very tight and entangled corporation between cellulose and hemicellulose and in that respect extended hemicellulose hydrolysis is closely coupled to cellulose hydrolysis. Other polymer interactions that most probably comprise the repelling exterior of corn bran are lignin/arabinoxylan networks that may form as a result of ether linkages between feruloyl groups and monolignols. Such interactions will both limit the effect of feruloyl esterases and the overall degradation of arabinoxylan, but it will most certainly also contribute to a hydrophobic surface of corn bran. Finally, interactions between structural proteins and arabinoxylan may also occur, especially through linkages between tyrosine residues in the proteins and feruloyl groups in arabinoxylan. All in all, these are examples of networks that will contribute to a strong and repellant exterior.
Nature’s own decompositional organisms for such matter namely fungi. It may be an interesting approach to study the methods and the micro-environmental conditions of fungal growth on corn bran (Shin et al., 2006). If in fact they are able to grow on corn bran they must possess means to overcome the physical barriers presented by the substrate. However, solid state fermentations are not the solution to optimal exploitation of corn bran, but the fungal strategy for progressive degradation may provide exceptional learning points. In that sense, new enzymes may emerge but more importantly also other non-catalysing metabolites or even acids may be part of the fungal machinery and thereby reveal conditions that can be mimicked in the lab.

Yet extremely convincing and interesting results are those obtained by Vaaje-Kolstad et al., 2010 where a protein of the CBM33 family, CBP21 is found to have a remarkable effect on enzymatic hydrolysis of β-linked chitin-oligomers via an oxidative activity. It was initially believed that this protein did not have any catalytic activity, but merely served as a carbohydrate binding domain. The implications of these results are tremendous since a homolog of this protein is found in the GH61 family of proteins, specific for enhancing cellulose hydrolysis and such enzymes specific for individual types of polysaccharides may exist.

Once the exterior of corn bran has been penetrated contribution of arabinoxyls to the arabinoxylan structure may need extra careful evaluation, since release of arabinose is generally lower than expected. Structural configuration of arabinose moieties have been a central issue for debate and speculations during this work and I believe that increased hydrolysis of arabinoxylan is strongly related to a higher arabinose release. Untraditional clues as to the configuration of arabinosyls in arabinoxylan are actually presented by Kormelink et al., 1991 where an unusual arabinofuranhydrolyase activity from A. awamori was first described. The enzyme is unusual because it has no activity towards the typical assay substrates, either representing different linear structures of arabinan or terminal substitution. But still it has a remarkable effect on particularly arabinoxylan hydrolysis from certain cereals. This speaks of arabinosyl configurations of a special character in cereal arabinoxylan, but it is also an interesting case because a specific enzyme activity has facilitated and possibly can reform the understanding of the substrate configuration. Enzymes of this kind comprise dual attractive properties both by catalyzing the hydrolysis and by providing new tools for elucidation of structures.

Another option for exploitation of corn bran is to only partially degrade it to defined oligomeric structures used for instance in the prebiotic area, but it will again be linked to the overall opening of the cell wall matrix.

A limitation to this field of research is the technical challenges of analysis. This is mostly related to acquiring more detailed understanding of the substrates, hydrolysis reactions, products and residuals. It is also a key limitation that analysis on insoluble plant materials is difficult. Most characterizing approaches are based on some sort of solubilization of the components of interest, which in effect disrupts the very construction we want to understand. Higher insight to the native substrates for instance by visualization
techniques like microscopy or spectroscopy may improve our comprehension of complex and interacting structures. Such approaches have been taken during this project but have not turned out successful; however advanced visualization techniques may provide some of the structural understanding on a global scale which is currently lacking.

Lastly, as also implied by the results in the manuscript for paper 3, physical parameters and thermodynamic conditions in an enzymatic hydrolysis must be considered when evaluating and understanding the performance of the reactions. Especially in the case of enzyme systems like the multi-acting cellulases and hemicellulases the reactions dependency on interrelated effects between enzymes, physical appearance of the substrate, liquid/solid barriers and other thermodynamic parameters may give important clues to reaction boundaries. An entire research area focuses on describing and modeling such conditions, namely statistical physics that applies probability theory, statistics and mathematical modeling in describing large populations of inherent stochastic nature. Description of enzymatic reactions by such methods may provide information on how the enzymes affect each other in the reaction, but also if parameters of more physical character influence the reaction. In turn this may give clues on how to enhance enzymatic reactions of complex plant materials.
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