Assessment of novel accessory enzymes to enhance biomass deconstruction

by

Daniela Alejandra Vargas Figueroa
B.Sc. in Chemical Engineering, University of Concepción, 2015

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF APPLIED SCIENCE

in

THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES (FORESTRY)

THE UNIVERSITY OF BRITISH COLUMBIA (Vancouver)

May 2018

© Daniela Alejandra Vargas Figueroa, 2018
The following individuals certify that they have read, and recommend to the Faculty of Graduate and Postdoctoral Studies for acceptance, a thesis/dissertation entitled:

Assessment of novel accessory enzymes to enhance biomass deconstruction

submitted by Daniela Alejandra Vargas Figueroa in partial fulfillment of the requirements for the degree of Master of Applied Science in Forestry

Examing Committee:

Dr. Jack Saddler (Forestry, UBC)
Supervisor

Dr. Jinguang Hu (Postdoc, Aalto University, Finland)
Supervisory Committee Member

Dr. Harry Brumer (Michael Smith Laboratories, UBC)
Supervisory Committee Member

Dr. Heather Trajano (Chemical & Biological engineering, UBC)
Additional Examiner

Additional Supervisory Committee Members:

Supervisory Committee Member

Supervisory Committee Member
Abstract

The production of monomeric sugars from lignocellulosic feedstocks is challenging, partly due to the high enzyme loadings that are typically required to effectively break down biomass substrates. One pretreatment approach is to try to keep most of the sugars associated with the solid fraction where cellulose and hemicellulose degrading enzymes will be needed to both open-up the lignocellulosic matrix and hydrolyse the polymeric matrix. Past work has shown that the addition of “accessory enzymes” to the “cellulase” cocktail can enhance the hydrolytic performance of enzyme mixture while reducing the protein/enzyme loading required to hydrolyse pretreated biomass substrates.

The potential of novel “hemicellulose-specific” enzymes to work synergistically with traditional (Celluclast) and more recent (CTec series) cellulase mixtures was assessed on a range of pretreated and “model” cellulosic substrates. Xyloglucanases showed a higher degree of cooperation than did mixed-linkage glucanases. However, although they generally enhanced cellulose hydrolysis, this synergistic cooperation was strongly influenced by the type of enzyme activity, substrate composition and enzyme and substrate concentration. The backbone-acting xyloglucanase from Bacteroides ovatus, BoGH5, demonstrated a broader specificity compared to other accessory enzymes, resulting in a higher degree of cooperation with cellulase enzymes and enhanced cellulose hydrolysis. Supplementing Celluclast with both backbone acting and debranching enzyme α-xylosidase, resulted in an over 10% improvement in cellulose hydrolysis for substrates with a higher hemicellulose content. This also correlated with an increase in the release of xyloglucan-derived oligomers. As a result of this enhanced synergism, it was possible to reduce the overall protein/enzyme loading by
35% when a goal of 80% cellulose hydrolysis of alkali pretreated corn stover within 72hrs was targeted.

The thesis work suggested that xyloglucan plays a role in limiting enzyme access to the cellulose and therefore the xyloglucan must be disrupted in order to facilitate effective cellulose hydrolysis. It is likely that xyloglucan acts as a physical barrier, possibly coating cellulose microfibrills and/or restraining cellulose fiber swelling.
Lay Summary

The production of cellulosic ethanol, a gasoline substitute, from forest and agricultural waste shows great potential as one way to reduce greenhouse gas emissions originating from transport. However, the processing of woody materials to ethanol is complicated. First, a pretreatment is required to “open up” the biomass structure while retaining as much of the original sugar based polymers as possible. Then, a “cocktail” of enzymes is required to breakdown the cellulose and hemicellulose to sugars. These sugars are then fermented to ethanol. Despite the potential of cellulosic ethanol as source of sustainable transport fuel, currently too high a concentration of enzymes is required to degrade woody materials. This translates into elevated production cost and limits its application at industrial scale. Thus, the main goal of this study was to assess the potential of adding novel enzymes to the cocktail, resulting in enhanced biomass deconstruction and reduced enzyme/protein-loading requirements.
Preface

All the work presented henceforth was conducted by Daniela Alejandra Vargas Figueroa in the Forest Products Biotechnology/Bioenergy Laboratories at the University of British Columbia, Point Grey campus.
Table of contents

Abstract........................................................................................................................................ iii
Lay Summary ...................................................................................................................................... v
Preface............................................................................................................................................... vi
Table of contents ................................................................................................................................. vii
List of tables ......................................................................................................................................... ix
List of figures ......................................................................................................................................... x
List of units and abbreviations ........................................................................................................... xii
Acknowledgements .............................................................................................................................. xiv

1 Introduction ....................................................................................................................................... 1
  1.1 Background .................................................................................................................................... 1
  1.2 Bioconversion process of lignocellulosic biomass ........................................................................... 4
  1.3 Recalcitrance structure of lignocellulosic biomass ......................................................................... 4
  1.3.1 Heterogeneity of hemicelluloses .............................................................................................. 6
  1.4 Biomass pretreatment .................................................................................................................... 9
  1.4.1 Physicochemical changes of biomass after pretreatment ............................................................ 12
  1.5 Enzymatic hydrolysis of pretreated lignocellulosic biomass ............................................................ 15
  1.6 Accessory enzymes to enhance biomass deconstruction ................................................................. 19
  1.6.1 Hemicellulases ......................................................................................................................... 20
  1.7 Synergism between cellulases and major accessory enzymes ......................................................... 26
  1.8 Fungal and bacterial enzymes: our targets for biomass deconstruction .......................................... 29
  1.9 Thesis objectives ............................................................................................................................ 33

2 Materials and methods ..................................................................................................................... 35
  2.1 Lignocellulosic feedstocks and pretreatment technologies ............................................................... 35
  2.2 Enzymatic hydrolysis ..................................................................................................................... 36
  2.3 Analytical methods ......................................................................................................................... 38
  2.4 Enzyme activity assay .................................................................................................................... 39
  2.5 Cellulose accessibility ..................................................................................................................... 40
  2.5.1 Simon’s staining technique ....................................................................................................... 40
  2.6 Fibre quality analyzer, FQA ........................................................................................................... 41
  2.7 Particle size distribution, Mastersizer 2000 .................................................................................... 41

3 Results and Discussion ..................................................................................................................... 42
  3.1 Assessment of the possible contribution of novel accessory enzymes to the deconstruction of pretreated aspen and corn stover substrates ................................................................. 42
    3.1.1 Background ............................................................................................................................. 42
    3.1.2 Target enzymes for biomass saccharification ........................................................................... 44
3.1.3 Specific activities of novel enzymes and commercial preparations ................................46
3.1.4 Screening of the possible contribution of novel, accessory enzymes when added to the “cellulase mixture” Celluclast .................................................................47
3.1.5 Combination between backbone acting novel enzymes and α-xyllosidase ............55
3.1.6 Release of xyloglucan derived oligomers using novel enzymes .........................59
3.1.7 Assessment of whether the addition of novel, accessory enzymes can enhance the hydrolytic potential of the CTec 3 “cellulase mixture” ...........................................63
3.1.8 Conclusions ....................................................................................................65
3.2 Understanding the mechanism behind cellulase and xyloglucanase synergism ..........66
3.2.1 Background ..................................................................................................66
3.2.2 Enzymatic performance of “cellulase” commercial mixtures .............................69
3.2.3 Synergism between Bacteroides ovatus enzymes and cellulases is increased at later stage of hydrolysis .................................................................72
3.2.4 Is β-galactosidase supplementation required to enhance xyloglucan decomposition? ....74
3.2.5 Influence of substrate concentration on enzyme synergism ............................76
3.2.6 Physicochemical changes after xyloglucanases treatment of pretreated corn stover ....80
3.2.7 Conclusions ..................................................................................................88

4 Final conclusions and future work ........................................................................90
4.1 Conclusions ....................................................................................................90
4.2 Future work ....................................................................................................91
  4.2.1 Confirm xyloglucan location and assess the addition of xyloglucanases ..........91
  4.2.2 Addition of other accessory enzymes in combination with novel xyloglucanases ....91
  4.2.3 Use of xyloglucanase enzymes for xyloglucan-oligosaccharides production ..........91

References ............................................................................................................93

Appendices ...........................................................................................................105

Appendix A. Xyloglucanase supplementation enhanced xylose release after 72h ..........105
Appendix B. Particle size distribution of SPCS after xyloglucanase treatment ..........106
List of tables

Table 1. Common hemicelluloses according to plant origin................................................................. 7
Table 2. Summary of various technologies of pretreatment in order to facility lignocellulistic biomass
deconstruction........................................................................................................................................ 9
Table 3. Novel accessory enzymes that were assessed for their possible contribution to enhancing biomass
deconstruction........................................................................................................................................ 31
Table 4. Pretreatment conditions for biomass substrates utilized in this study................................. 36
Table 5. Target enzymes for biomass saccharification, including origin, type of activity, optimum temperature
and pH.................................................................................................................................................... 45
Table 6. Protein concentration of accessory enzymes utilized in this study ...................................... 46
Table 7. Enzyme activities (U/mg) of commercial enzyme mixtures and purified enzymes in model substrates
............................................................................................................................................................... 47
Table 8. Chemical composition of aspen and corn stover substrates from different pre-treatment technologies,
presented as % ......................................................................................................................................... 48


List of figures

Figure 1. Structure of lignocellulosic biomass (Rubin, 2008) (Reproduced with permission from Springer Nature). ................................................................. 5
Figure 2. Key hemicellulose building units (Reproduced with permission from (Gröndahl et al., 2004). Copyright 2004, American Chemical Society). ................................................................. 6
Figure 3. Proposed pathway of xyloglucan degradation by novel accessory enzymes ............................................................................................................. 32
Figure 4. Xyloglucan acts as a physical barrier to cellulases and therefore, xyloglucanases are required to improve cellulose accessibility. Model substrates were prepared at incubating tamarind xyloglucan (10% w/w) with microfibrillated cellulose (MFC) for 72 h and enzymatic hydrolysis was carried out at 1% substrate concentration, cellulases loading of 7.5 mg protein/g cellulose and xyloglucanases loading of 2 mg protein/g cellulose ............................................................................................................. 50
Figure 5. Xyloglucanases-backbone acting enzymes enhance cellulose hydrolysis in corn stover (SPCS and AFEX CS) to a greater extent than it does aspen substrates (SPA and TMP-bio) at 2% substrate concentration and cellulase loading of 15 mg protein/g cellulose. Xyloglucanase loading supplemented was different for both tested biomass (mg protein/g cellulose): 2.5 (corn stover substrates) and 5 (aspen substrates). Data not shown for RMP ................................................................................................................................. 52
Figure 6. Synergistic interaction between best backbone acting xyloglucanases BoGH5, VvEG16, CjGH74 and PbGH5 and debranching enzyme α-Xyl31A (α-31A) in corn stover and aspen substrates at 2% substrate concentration and cellulases loading of 15 mg protein/g cellulose. Xyloglucanase loadings were different for both biomass (mg protein/g cellulose): 2.5 (corn stover substrates) and 5 (aspen substrates). Data not shown for RMP ................................................................................................................................. 56
Figure 7. Xylose and glucose release in monomer and oligomers form increased during enzymatic hydrolysis of corn stover and aspen substrates after xyloglucanases supplementation. Xyloglucanase protein loading were different for both biomasses (mg protein/g cellulose): 2.5 (corn stover substrates) and 5 (aspen substrates), including both backbone-acting and debranching enzymes. ................................................................................................................................. 61
Figure 8. Xyloglucan oligomers formation increases during enzymatic hydrolysis of corn stover and aspen substrates after xyloglucanases supplementation and it correlates with improvements in cellulose hydrolysis ................................................................................................................................. 62
Figure 9. Synergist interaction between novel accessory enzymes and commercial cellulase mixture CTec 3 in corn stover and aspen substrates. Total enzyme loading was 7 mg protein/g cellulose (5 mg/g of CTec 3 and 2 mg/g of each accessory enzyme candidate). ................................................................................................................................. 64
Figure 10. Cellulose hydrolysis performance of “cellulase” commercial mixtures, Celluclast and CTec 2 at increasing protein loading in both pretreated corn stover substrates, SPCS (■) and AFEX CS (▲). Enzymatic hydrolysis using Celluclast and CTec 2 were carried out at solid loadings of 2 and 10%, respectively along 72 hours. ................................................................................................................................. 70
Figure 11. Time course of hydrolysis of pretreated corn stover substrates at 2% solids loading utilizing Bacteroides ovatus accessory enzymes, BoGH5 and BoGH31A. “Cellulase” enzyme loading was 15 mg
Celluclast/g cellulose and xyloglucanase loading was 2.5 mg protein/g cellulose. BoGH31A supplementation was in a ratio of 1:1.5 respect to BoGH5. ................................................................. 73

**Figure 12.** Cellulose hydrolysis after 72 hours utilizing Bacteroides ovatus accessory enzymes in pretreated corn stover substrates at 2% solids loading. C: Celluclast/Novozyme188; GH5: BoGH5 (backbone acting enzyme); GH31: BoGH31 (α-xidosidase); GH2: BoGH2A (β-galactosidase). ................................................................. 75

**Figure 13.** Time course hydrolysis of pretreated corn stover substrates at 10% solids loading utilizing Bacteroides ovatus accessory enzymes, BoGH5 and BoGH31A. Simultaneous supplementation of BoGH31A to BoGH5 was compared to sequential addition after 24 hours. “Cellulase” enzyme loading was 30 mg protein/g cellulose and xyloglucanase loading was 2.5 mg protein/g cellulose. BoGH5 : BoGH31A supplementation ratio was 1.5 : 1. ......................................................................................... 77

**Figure 14.** Influence of substrate loading in synergistic interaction between backbone acting BoGH5 and debranching enzyme BoGH31A in pretreated corn stover substrates. SPCS: steam pretreated corn stover; AFEX CS: ammonia fiber expansion pretreated corn stover. ......................................................................................... 79

**Figure 15.** Cellulose hydrolysis of both pretreated corn stover substrates by various cellulase commercial enzymes mixtures at 10% solid loading after 72 h. Total protein loading of all mixtures is 32.5 mg/g cellulose. ......................................................................................... 80

**Figure 16.** Changes in cellulose accessibility, Simons’ staining, orange dye, mg/g, after xyloglucanases treatment with/without commercial “cellulase” mixture along 24 hours. C: Cellulases mixture of Celluclast and Novo188 (β-glucosidases); α-xyl: α-xidosidase, BoGH31A. Control: sodium acetate buffer ......................................................................................... 81

**Figure 17.** Fibre size distribution after xyloglucanases treatment with/without commercial “cellulase” mixture after 24 hours in SPCS and AFEX CS. BoGH5: backbone acting enzyme; α-xyl: α-xidosidase, BoGH31A. Control: sodium acetate buffer. ......................................................................................... 85

**Figure 18.** Change in mean fibre width after xyloglucanases treatment with/without commercial “cellulase” mixture after 24 hours in SPCS and AFEX CS. α-xyl: α-xidosidase, BoGH31A. Control: sodium acetate buffer. ......................................................................................... 87
### List of units and abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>⁰C</td>
<td>Celsius degree</td>
</tr>
<tr>
<td>AFEX</td>
<td>ammonia fibre expansion</td>
</tr>
<tr>
<td>AFEX CS</td>
<td>ammonia fibre expansion pretreated corn stover</td>
</tr>
<tr>
<td>AIL</td>
<td>acid insoluble lignin</td>
</tr>
<tr>
<td>Ara</td>
<td>arabinose</td>
</tr>
<tr>
<td>CBHs</td>
<td>cellobiohydrolases</td>
</tr>
<tr>
<td>CBM</td>
<td>carbohydrate-binding module</td>
</tr>
<tr>
<td>CMC</td>
<td>carboxymethyl cellulose</td>
</tr>
<tr>
<td>DNS</td>
<td>3,5-dinitrosalicylic acid</td>
</tr>
<tr>
<td>DO</td>
<td>direct orange dye</td>
</tr>
<tr>
<td>DW</td>
<td>dry weight</td>
</tr>
<tr>
<td>EGs</td>
<td>endoglucanases</td>
</tr>
<tr>
<td>EX</td>
<td>endo-1,4-xylanases</td>
</tr>
<tr>
<td>FQA</td>
<td>fibre quality analyser</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>Gal</td>
<td>galactose</td>
</tr>
<tr>
<td>GH</td>
<td>glycoside hydrolase</td>
</tr>
<tr>
<td>Glu</td>
<td>glucose</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>HEC</td>
<td>hydroxyethyl cellulose</td>
</tr>
<tr>
<td>HPLC</td>
<td>high-performance liquid chromatography</td>
</tr>
<tr>
<td>L</td>
<td>liter</td>
</tr>
<tr>
<td>LCC</td>
<td>lignin-carbohydrate complex</td>
</tr>
<tr>
<td>LPMOs</td>
<td>lytic polysaccharide monooxygenases</td>
</tr>
<tr>
<td>LW</td>
<td>length weighted</td>
</tr>
<tr>
<td>Man</td>
<td>mannose</td>
</tr>
<tr>
<td>MFC</td>
<td>microfibrillated cellulose</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>ml</td>
<td>milliliter</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>----------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>MLG</td>
<td>mixed linkage glucan or β-glucan</td>
</tr>
<tr>
<td>mm</td>
<td>millimetre</td>
</tr>
<tr>
<td>mM</td>
<td>micro molar</td>
</tr>
<tr>
<td>MPa</td>
<td>mega Pascal</td>
</tr>
<tr>
<td>MW</td>
<td>mean width</td>
</tr>
<tr>
<td>n/a</td>
<td>negligible activity detected</td>
</tr>
<tr>
<td>NH₃</td>
<td>ammonia</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>p-NPC</td>
<td>p-nitrophenyl-β-D-celllobioside</td>
</tr>
<tr>
<td>p-NPG</td>
<td>p-nitrophenyl-β-D-glucopyranoside</td>
</tr>
<tr>
<td>p-NPX</td>
<td>p-nitrophenyl-β-D-xylopyranoside</td>
</tr>
<tr>
<td>RMP</td>
<td>refine mechanical pulping</td>
</tr>
<tr>
<td>SO₂</td>
<td>sulfur dioxide</td>
</tr>
<tr>
<td>SP</td>
<td>steam explosion</td>
</tr>
<tr>
<td>SPA</td>
<td>steam pretreated aspen</td>
</tr>
<tr>
<td>SPCS</td>
<td>steam pretreated corn stover</td>
</tr>
<tr>
<td>TMP</td>
<td>thermomechanical pulping</td>
</tr>
<tr>
<td>w/v</td>
<td>weight/volume</td>
</tr>
<tr>
<td>w/w</td>
<td>weight/weight</td>
</tr>
<tr>
<td>XG</td>
<td>xyloglucan</td>
</tr>
<tr>
<td>XTH</td>
<td>xyloglucan transferase/hydrolase</td>
</tr>
<tr>
<td>Xyl</td>
<td>xylose</td>
</tr>
<tr>
<td>βG</td>
<td>β-glucosidase</td>
</tr>
<tr>
<td>μm</td>
<td>micrometre</td>
</tr>
</tbody>
</table>
Acknowledgements

I would like to express my sincere gratitude to my supervisor, Dr. Jack Saddler, for his support and guidance throughout my MASc program and for providing me with the opportunity to study as a part of the Forest Products Biotechnology/Bioenergy (FPB) research group at UBC. It would like to also thank my committee member Dr. Jinguang Hu and Dr. Harry Brumer for their valuable advice and contributions throughout my studies.

Special thanks also goes to previous and current members of the FPB/B group for the peer tutoring and friendship, which made every day a fun and memorable experience.

I am very grateful to my family for their constant love and support, thank you for your encouragement in any step of the way and always being there for me. Your support in all of my decisions including studying this far from home has meant the world to me. Finally, for not least, I would like to thank my boyfriend, Scott, for being so supportive while I was at graduate school. Thanks for your emotional support, for being so understanding and always pushing me to do my best.
1 Introduction

1.1 Background

The utilization of lignocellulosic biomass as a feedstock for monomeric sugar production continues to be challenging, partially due to the high enzyme loadings that are required to effectively break down lignocellulosic biomass. This need for high protein loadings is related to the restricted ability of the enzymes to reach the cellulose microfibrils as “cellulose accessibility” is limited, partially due to the presence of lignin and hemicellulose (Arantes and Saddler, 2010). Increasing cellulose accessibility through the utilization of a pretreatment step has been shown to be effective in enhancing enzyme accessibility to cellulose (Rollin et al., 2011). However, most of the pretreatments that have been assessed to date have different optimal conditions depending if the main goal is to maximize cellulose, hemicellulose or lignin recovery. In addition, hemicelluloses have proven to be susceptible to loses during many pretreatments, resulting in the loss of potential sugars and the production of degradation products (Chandra et al., 2007). This has proven to be a problem for several second-generation bioethanol production situations (Gírio et al., 2010). Therefore, the work within this thesis assessed a variety of pretreatments with the main goal of maximizing sugar recovery from both the cellulose and hemicellulose components of the biomass feedstock.

It was recognised that, by selecting a pretreatment approach that attempts to keep most of the sugars derived from cellulose and hemicellulose in the solid fraction, cellulose-degrading enzymes as well as enzymes able to disrupt and help open up the lignocellulosic matrix would be beneficial. Therefore, an assessment of the potential contribution of different “accessory enzymes”, such as hemicellulases, that could enhance biomass deconstruction should be
enlightening. Thus, one of the main objectives of the thesis work was to produce a range of pretreated substrates, under acid, alkali and mechanical conditions. The application of a range of pretreatments provided us with a good selection of substrates with different hemicellulose content and structural characteristics. These substrates were subsequently used to assess the potential of our various “hemicellulose” enzymes to enhance biomass deconstruction.

Although cellulases are the most important enzymes when trying to hydrolyse cellulose, they primarily target the cellulose microfibrills which tend to be “buried” within other polysaccharides such as hemicelluloses and lignin (Banerjee et al., 2010c). One way to try and overcome this issue, is to add hemicellulose-hydrolyzing enzymes to help ‘open up’ the biomass matrix by reducing the strong association of hemicelluloses with cellulose microfibrills (Van Den Brink and De Vries, 2011; Gao et al., 2011; Shi et al., 2011). Other workers have assessed this strategy by introducing “accessory enzymes” to the enzyme cocktail. This approach has been shown to enhance cellulases hydrolytic performance while reducing the protein/enzyme loading required to hydrolyse pretreated biomass substrates (Gao et al., 2011; Hu et al., 2011; Kumar and Wyman, 2009a).

However, hemicelluloses are highly heterogenous. For example, a variety of linkages including ester bonds, glycosidic bonds, removal of side chain have been the targets for the catalytic action of variety of “hemicellulases” including, carbohydrate esterases, glycoside hydrolases, polysaccharide lyases, xyloglucan hydrolases, endo-acting hemicellulases, among others (Van Den Brink and De Vries, 2011; Saritha et al., 2016; Sweeney and Xu, 2012). The application of these hemicellulases in combination with cellulase enzymes has shown promise, resulting in increased cellulose hydrolysis rates and conversions on a range of pretreated substrates (Gao et al., 2011; Gao et al., 2014; Hu et al., 2011; Kumar and Wyman 2009a; Zhang and Viikari,
2014). From these studies, it was suggested that hemicelluloses such as xylan act as a physical barrier to cellulolytic enzymes. Consequently, hemicellulose removal increases cellulose accessibility, fibre swelling and fibre porosity (Hu et al., 2013). To complement this earlier work the current study focussed on the influence of other hemicellulosic structures such as xyloglucan and mixed linkage glucan. These hemicellulose-structures have not been studied to the same extent that xylan-type hemicelluloses have been. Despite the limited information available, the potential of “loosening” xyloglucan has been shown to be beneficial if enhanced cellulose hydrolysis is targeted, as both structures seems to form a highly resistant complex that is more recalcitrant to enzymatic degradation (Kaida et al., 2009; Vincken et al., 1994).

Consequently, one of the main objectives of the thesis work was to elucidate the effectiveness of a variety of novel accessory enzymes active on xyloglucan and mixed linkage glucan activities within a range of industrially relevant pretreated substrates. The down-selected best candidates, which included backbone- and sidechain- hydrolyzing activities, were further studied to try to better understand their synergistic interaction with traditional (Celluclast) and more recent (CTec series) cellulase mixtures. Their possible synergistic interactions with cellulases resulting in enhanced hydrolysis rates and extent of hydrolysis were evaluated. We hoped that, by modifying the components in the enzyme cocktail, it might be possible to reduce the total protein loading required to achieve equal glucose yields. Changes in the physicochemical properties of the substrate that might influence the extent of cellulose hydrolysis (such as gross fibre characteristics and cellulose accessibility) were also assessed for each lignocellulosic substrates after applying “cocktails” that included the novel accessory enzymes.
1.2 Bioconversion process of lignocellulosic biomass

The bioconversion process of lignocellulosic biomass to monomeric sugar involves the following major steps: pretreatment, to disrupt the highly organized cell wall structure and increase cellulose substrate accessibility; enzymatic hydrolysis, to produce a variety of monomeric sugars; the subsequent fermentation of the sugars to a range of fuels or chemicals (Avanthi et al., 2017). In the following sections, the structure of lignocellulosic biomass, with special focus on hemicelluloses, is presented. It also includes a description of all of pretreatments that were applied and their effects on the subsequent biomass composition.

1.3 Recalcitrance structure of lignocellulosic biomass

The structure of lignocellulosic biomass consists of a highly organize network of cellulose fibers, embedded with an amorphous matrix of hemicellulose, pectins and polyphenolic lignin (Ebringerová and Thomas, 2005). The building unit of cellulose is cellobiose, \(\beta\-D\-glucopyranosyl\) (1\(\rightarrow\)4)-linked, which forms long and linear polymeric chains that aggregate in highly crystalline microfibrils (Ebringerová and Thomas, 2005). These microfibrils are at the same time arranged into macrofibrils to give structural stability to the plant cell wall (Figure 1). Because of this highly organized structure, lignocellulosic biomass is extremely resistant to chemical and biological hydrolysis.
Figure 1. Structure of lignocellulosic biomass (Rubin, 2008) (Reproduced with permission from Springer Nature).

Unlike cellulose, hemicellulose has a much more varied distribution and composition within the lignocellulosic complex. It is compromised of different hexoses and pentoses substituted with neutral sugars or acidic groups (Ebringerová, 2006). Lignin, that lends woody plants and trees their rigidity, is an extremely complex polyaromatic compound formed by the non-repetitive polymerization of aromatic alcohols known as monolignols (Figure 1) (Rubin, 2008).

Cellulose microfibrills are typically precisely arranged and strongly associated with hemicelluloses and lignin. This complicates the enzyme mediated saccharification process as it reduces the physical accessibility of cellulases to reach the cellulosic fibres and increases unproductive adsorption of cellulase enzymes onto lignin (Deutschmann and Dekker, 2012; Lynd et al., 2002). As mentioned earlier, hemicelluloses occur in structural variations differing in side-chain types, localization, types and distribution of substitutes groups to the main backbone. This heterogeneity in structure and distribution has contributed to difficulty in better understanding how hemicelluloses influence cellulose enzymatic hydrolysis. This one of the reasons why this thesis
work wanted to assess the role that mixed-linkage glucan (β-glucan) and xyloglucans might play in restricting effective cellulose hydrolysis in pretreated biomass substrates.

1.3.1 Heterogeneity of hemicelluloses

As mentioned earlier, in comparison to cellulose the molecular architecture of hemicellulose is quite complex as it includes different monomeric units, various substitution patterns and degree of branching. Hemicelluloses are heteropolysaccharides whose composition and amount vary between different plant species, as well as, between particular sources, depending on factors such as origin and plant growth stage (Hansen and Plackett, 2008). In most trees, the hemicellulose comprises between 20% and 30%, and the most common monosaccharides are D-xylose, D-glucose, L-arabinose, D-galactose, D-mannose, D-glucuronic acid, 4-O-methyl-D-glucuronic acid and D-galacturonic acid (Figure 2).

**Figure 2.** Key hemicellulose building units (Reproduced with permission from (Gröndahl et al., 2004). Copyright 2004, American Chemical Society).

Hemicelluloses are classified according to the predominant sugar in the main β-1,4-linked polymeric chain and, depending on the biomass source, different structure can be found, including xylans, mannans, xyloglucans or mixed linkage glucans (Table 1). In hardwoods, glucuronoxylans
are the most abundant type of hemicellulose. Xyloglucan is also present, comprising between a 20 to 25% of the primary cell wall (Fry, 1989). Small amounts of glucomannans can also be found (Álvarez et al., 2016). Glucoronoxylans consists of a linear backbone of β-1,4-ᴅ-xylopyranose with the presence of acetyl groups and uronic acid every 10ᵗʰ xylose residues (Pereira et al., 2003). In comparison, xyloglucans are comprised of a cellulosic backbone but with the presence of ᴅ-xylose, ᴅ-galactose and L-arabinose substitutes along the main backbone. Up to 75% of glucose units are substituted with xylose residues, which can carry additional L-arabinose and ᴅ-galactose forming branches with two or three sugar units (de Vries et al., 2001). As a result, backbone acting enzymes as well as debranching enzymes are required in order to remove side groups and enhance the performance of endo-acting enzymes.

<table>
<thead>
<tr>
<th>Biological origin</th>
<th>Polysaccharide type</th>
<th>Amount, %</th>
<th>Type of linkage between monomeric units</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Agricultural crops</strong></td>
<td>Arabino glucuronoxylan</td>
<td>5 - 10</td>
<td>β-(1,4)-Xyl</td>
</tr>
<tr>
<td></td>
<td>Arabinoxylan</td>
<td>0.15 - 30</td>
<td>β-(1,4)-Glc</td>
</tr>
<tr>
<td></td>
<td>Xyloglucan</td>
<td>2 - 5</td>
<td>β-(1,4)-Glc-Man</td>
</tr>
<tr>
<td><strong>Hardwoods</strong></td>
<td>Glucuronoxylan</td>
<td>15 - 35</td>
<td>β-(1,4)-Xyl</td>
</tr>
<tr>
<td></td>
<td>Xyloglucan</td>
<td>2 - 25</td>
<td>β-(1,4)-Glc</td>
</tr>
<tr>
<td></td>
<td>Glucomannan</td>
<td>2 - 5</td>
<td>β-(1,4)-Glc-Man</td>
</tr>
<tr>
<td><strong>Softwoods</strong></td>
<td>Galactoglucomannan</td>
<td>20 - 25</td>
<td>β-(1,4)-Glc-Man</td>
</tr>
<tr>
<td></td>
<td>Glucomannan</td>
<td>2 - 5</td>
<td>β-(1,4)-Glc-Man</td>
</tr>
</tbody>
</table>

(Information based on Alvarez et al. 2016; Girio et al. 2010; Saritha et al. 2016; Ebringerová et al. 2005)

In contrast to hardwoods, the main hemicellulose present in softwoods is galactoglucomannan, which consists of a linear backbone of β-ᴅ-glucopyranosyl and β-ᴅ-mannopyranosyl units linked together through β-1,4 glycosidic bonds (Álvarez et al., 2016).
Similar to other hemicelluloses, galactoglucomannans have substitute groups along their main polymeric chain including α-D-galactopyranosyl units and acetylation at C2 or C3.

Cereal grasses share a similar hemicellulose composition to hardwoods as both biomass sources have glucuronoxylan as their main hemicellulose structure. However, in cereal grasses such as corn stover, glucuronoxylans can also have arabinose substitute units present at the C2 and C3 positions. In addition, xyloglucan compromises between 2 and 5% in grasses and about 10% in the primary cell walls of softwoods (Fry, 1989). Mixed-linkage glucan or β-glucans are unique to the Poales, a taxonomic order that includes cereal grasses such as corn. They consist of β-D-glucopyranose units linked through β-1,4 and β-1,3 glycosidic bonds, in the typical proportions of 70 and 30% respectively (Ebringerová and Thomas, 2005).

Xyloglucan interactions with cellulose have been reported to predominate in three forms. Some portion that cross-links between the microfibrils and which are susceptible to enzymatic cleavage. A fraction that are tightly bound to the microfibril surface and, a third component trapped within the microfibril periphery (Pauly et al., 1999). This structural complexity indicates the importance of trying to better understand the role that xyloglucan might play in enzyme mediated deconstruction of biomass and the better characterization of the enzymes involved in hemicellulose modification and solubilisation plus their possible influence on enhancing cellulose hydrolysis (Pauly et al., 1999).

In the work reported here, corn stover and aspen were used, partly due to their likelihood as serving a biofuels feedstocks and due to their similarities in hemicellulose structure as well as the xyloglucan and β-glucan components of their hemicellulose fractions. Corn stover is currently being used at an industrial scale for second-generation bioethanol production (Sigoillot and Faulds,
Poplar is one of the most promising wood species that could provide a feedstock for future biorefineries due to its relatively rapid growth and its already commercial use in the forest products sector.

1.4 Biomass pretreatment

Because of the heterogeneous and complex structure of lignocellulosic feedstocks, a pretreatment step is typically required to increase biomass digestibility prior enzymatic hydrolysis. Over the past decade, several pretreatments have been assessed. These include mechanical (milling and grinding), chemical (alkali, acid, organic solvents), thermochemical, chemical and biological approaches (Maurya et al., 2015; Mosier et al., 2005; Silveira et al., 2015). Thermochemical pretreatments are often preferred over chemical and mechanical pretreatments due to a more favorable combination of performance, capital and operating costs (Maurya et al., 2015). However, as mentioned before, we are particularly interested in pretreatments that keep most of carbohydrates within the solid fraction. The main advantages and limitations of different pretreatment technologies are summarized in Table 2.

<table>
<thead>
<tr>
<th>Pretreatment Technology</th>
<th>Examples</th>
<th>Advantages</th>
<th>Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mechanical</td>
<td>Ball milling/grinding</td>
<td>Reduces crystallinity and particle size</td>
<td>High costs associated to energy consumption</td>
</tr>
<tr>
<td>Chemical</td>
<td>Acid treatments: Autohydrolysis, Steam explosion (SO₂) Alkaline treatment: Organosolv, Ammonia fiber expansion (AFEX)</td>
<td>Hemicellulose hydrolysis and lignin modifications; cost effective</td>
<td>Formation of degradation products</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lignin removal, increases accessible surface area</td>
<td>Requires solvent recycling process and not efficient for biomass with high lignin content (AFEX)</td>
</tr>
<tr>
<td>Biological</td>
<td>White-rot fungi</td>
<td>Degradates lignin and hemicelluloses, no chemicals required, low energy input</td>
<td>Low conversion rates (&lt; 20%)</td>
</tr>
</tbody>
</table>

Table 2. Summary of various technologies of pretreatment in order to facilitate lignocellulosic biomass deconstruction.
Of the pretreatments assessed, autocatalytic processes, including hot water extraction, auto-hydrolysis and hydrothermal pretreatments have been suggested to be among the most promising “front ends” for bioconversion processes (Harmsen et al., 2010; Kumar et al., 2009; Maurya et al., 2015). However, long residence times, high operating temperatures and high water consumptions are often required to compensate for the absence of chemicals. Therefore, the interest in combining both chemical and physical approaches as a more effective strategy of pretreatment. Thus, steam explosion (SP) is one of the most commonly used pretreatment to break down lignocellulosic biomass, by increasing cellulose accessibility while solubilising the hemicellulose (Chandra et al., 2007; De Lima et al., 2016). Autohydrolysis is the dominant reaction occurring during steam pretreatment. It has been shown to effectively enhance the hydrolysis of hardwood- and agricultural residues- based substrates, with the presence of a high volume of acetic groups associated with hemicellulose contributing to its effectiveness (Chandra et al., 2007; De Lima et al., 2016). The release of acetic groups during pretreatment act as an acidic catalyst facilitating biomass deconstruction (Nabarlatz et al., 2007). Despite these advantages, steam explosion pretreatment also has some limitations. The use of extreme conditions (high temperatures and residence times) results in a significant portion of the hemicelluloses being lost as degradation products. To try to at least partially alleviate this issue, small amounts of SO₂ have been added. The utilization of SO₂ has been shown to reduce pretreatment severity as well as reduce the formation of degradation products (Chandra et al., 2007). Despite the advantages of utilizing acid catalyzed steam pretreatment, hemicelluloses are partly removed and lost in the water-soluble fraction. Therefore, we wanted to compare this approach with other pretreatment technologies, such as alkaline and mechanical pretreatments. Alkaline technologies such as ammonia fiber explosion (AFEX), are often referred to as the alkaline version of steam explosion,
using high pressured ammonia instead of steam (De Lima et al., 2016; Silveira et al., 2015). In contrast to common alkaline treatments using sodium hydroxide or lime in which lignin is mostly removed, AFEX pretreatment solubilizes a smaller fraction by cleaving the lignin-carbohydrate complexes and keeping most of the cellulose and hemicellulose intact (Chundawat et al., 2010). The main contribution to the cost of this pretreatment is the use of ammonia, although it can be recovered and reused (Campbell et al., 2013; Gunawan et al., 2017). However, this style of pretreatment is only efficient for herbaceous crops and grasses due to their low lignin content (Garlock et al., 2009).

In contrast to thermochemical pretreatments, where the main goal is to increase cellulose accessibility to cellulolytic enzymes by partially removing hemicellulose and removing/modifying lignin, mechanical pretreatment generally preserve most of the original biomass structure. As more sugars in the solid fraction are available for bioconversion, mechanical pretreatments offer a high potential for industrial scale bioprocessing (Da Silva et al., 2010; Koo et al., 2011). However, even though most sugars are preserved, high concentrations of protein/enzyme loadings are still required to effective hydrolyse mechanically treated substrates due the restricted accessibility (Arantes and Saddler, 2010). Therefore, processes which can combine mild chemical and mechanical refining treatments should be of special interest if we are to develop effective pretreatments at a large-scale. With this approach in mind, TMP based processes have been developed to try to process lignocellulosic materials into different value-added bio products. This approach typically involves a mild alkaline chemical treatment and mechanical refining prior to enzymatic hydrolysis (Yuan and Browne, 2011).

In the current work described within this thesis, thermochemical pretreatments including steam explosion (SP) and ammonia fiber explosion (AFEX) were compared to mechanical refining
pretreatments that included refiner mechanical pulping (RMP) and thermomechanical pulping (TMP). It was hoped that the comparison between thermochemical and mechanical approaches would provide valuable insights with regard to the importance that the hemicellulose component might play. The structural changes within the lignocellulosic structure after pretreatment are discussed in the following sections.

1.4.1 Physicochemical changes of biomass after pretreatment

As mentioned earlier, the nature of the biomass substrate and the various pretreatments used influence the resulting distribution of cellulose, hemicellulose and lignin. At the same time this affects the possible degree of synergism between the cellulase enzyme mixture and accessory enzymes. In the following section, the main effects of pretreatment on biomass structure and composition are briefly summarized.

1.4.1.1 Steam explosion

During steam explosion pretreatment (SP), biomass is subjected to high-pressure saturated steam at temperatures ranging between 160 to 260 °C (0.7 to 4.8 MPa) and then suddenly exposed to atmospheric pressure, causing an explosive decompression (Menon and Rao, 2012). This process can last from seconds to a few minutes to promote hemicellulose hydrolysis and finishes with a violent discharge (explosion) (Xiao et al., 2017). The combined action between high-pressure steam and the explosion drastically modifies the plant cell wall structure, disrupting the linkages between hemicellulose, lignin and cellulose. Thus, increasing the potential of cellulose hydrolysis. It is known that hemicellulose acts as a physical barrier to cellulases, likely by coating the microfibrills surface. Therefore its removal should facilitate enzyme-mediated cellulose hydrolysis as accessibility is increased (Kumar and Wyman, 2009b). In addition, some lignin is
removed and another portion is redistributed on the fiber surface due to depolymerisation reactions. However, a critical disadvantage of steam explosion pretreatment is the loss of carbohydrates as degradation products, such as furfural and hydroxymethylfurfural and as soluble sugars. Thus, one of the goals of the thesis work was to see if other styles of pretreatment, such as mechanical pulping or AFEX, which retain most of the original cell wall components with the cellulose might be a better approach, in combination with an improved, hemicellulose containing enzyme cocktail.

1.4.1.2 Ammonia fiber expansion

The removal of lignin through the utilization of alkaline pretreatment technologies has shown a high potential due to relatively low operational cost, low inhibitor formation and a resulting substrate with a high cellulose content (Kim et al., 2016). However, the addition of alkaline solutions such as sodium hydroxide and lime during pretreatment elevates the pH. Therefore, an extensive washing step is required until suitable operational conditions are reached for the subsequent enzymatic hydrolysis and fermentation steps. The elevated costs associated with high water consumption have focus attention on another alkaline pretreatment, ammonia fiber explosion (AFEX). In this style of pretreatment, lignocellulosic biomass is subjected to high-pressure ammonia. Similar to steam explosion, the pressure is suddenly reduced causing an increase in biomass surface area but without a subsequent washing step (Campbell et al., 2013; Chundawat et al., 2010; Gollapalli et al., 2002).

It has been suggested that AFEX pretreatment enhances lignocellulosic biomass digestibility through disruption of the lignin-carbohydrate complex (LCC) ester linkages between the hemicellulose and lignin, resulting in a partial depolymerisation/solubilisation of the hemicellulose and lignin (Kumar et al., 2009; Menon and Rao, 2012). The partial solubilisation of
the hemicellulosic and lignin components in the exterior of the cell walls increases cellulose accessibility. This has been shown to be a key factor if efficient cellulose hydrolysis is to be achieved (Arantes and Saddler, 2010; Chundawat et al., 2010). In addition, the ammonization of active methoxyl sites of lignin during AFEX pretreatment has been shown to reduce enzyme unproductive binding. Thus, the hydrolytic efficiency of the whole enzyme cocktail is improved (Kumar et al., 2009; Sewalt et al., 1996). However, this style of pretreatment is only efficient for herbaceous crops and grasses due to their low lignin content (Garlock et al., 2009).

1.4.1.3 Mechanical and thermomechanical pulping

In contrast to chemical treatments, mechanical ones preserve most of the carbohydrates components within the solid fraction. This potentially results in a higher recovery of carbohydrates after hydrolysis. The hydrolysis of refiner mechanical pulps (RMP) is enhanced due to a reduction in particle size and crystallinity, increase in internal and external fibrillation and fibre shortening or cutting (Batalha et al., 2015; Gil et al., 2009). As a result, cellulose accessibility to hydrolytic enzymes is enhanced and therefore, refining has been shown to improve the hydrolyzability of lignocellulosic substrates (Batalha et al., 2015; Ertas et al., 2014; Koo et al., 2011; Zhu et al., 2009). However, high enzymes loadings are still required in order to effectively hydrolyze mechanical treated substrates (Chandra et al., 2016). This problem has encouraged the assessment of combining mechanical treatments with thermochemical ones such as steam explosion (Pschorn et al., 2008), sulphite (Zhu et al., 2009) or alkali ones (Yuan and Browne, 2011). A major goal is to partially modify or remove the lignin prior-to-or-post the refining process. An alkaline thermomechanical pulping (TMP-bio) process has been developed by FPInnovations to try to process lignocellulosic biomass to added value chemicals and fuels (Yuan and Browne, 2011). This treatment results in more readily enzyme hydrolysed carbohydrates, allowing both
hemicellulose separation and the recovery of a sulfur-free high-quality lignin (Yuan and Browne, 2011).

As discussed in this section, the structure and composition of the resulting substrates is influenced by the conditions applied during pretreatment (acid, alkali or neutral). Each of the pretreatments has different advantages and disadvantaged in their effectiveness and in their implementation regarding energy and chemical inputs. However, the main goal of an ideal pretreatment remains the same. Increasing the hydrolyzability of lignocellulosic substrates while preserving most of cellulose and hemicellulose carbohydrates in a useful form for further processing.

1.5 Enzymatic hydrolysis of pretreated lignocellulosic biomass

As a result of the complexity of lignocellulosic biomass, the enzymatic conversion to monomeric units is a complex process involving enzymes with diverse activities all cooperating together to effectively hydrolyze cellulose. Generally, enzyme-mediated cellulose hydrolysis can be divided into the three main steps of the primary slow “amorphogenesis” phase in which the lignocellulosic matrix is loosen up and more susceptible to further degradation by cellulase enzymes. A second phase, in which insoluble cellulose chains are reduced to shorter soluble oligosaccharides. Followed by the final hydrolysis of these soluble oligomers to monomeric sugars (Arantes and Saddler, 2010; Lynd et al., 2002).

According to the Carbohydrate-Active EnZYmes (http://www.cazy.org) data base, enzymes are classified into families according to similarities on the amino acid sequences (Henrissat et al., 1991). The lignocellulose-degrading enzymes that are involved in breaking down the glycosidic bond between two or more carbohydrates or between a carbohydrate and a non-
carbohydrate moiety are classified as glycoside hydrolase (GH). Currently, there is a total of 145 glycoside hydrolase families with close to 14 GH families including cellulases enzymes with huge diversity and different synergistic effects in order to completely hydrolyze cellulose (Sweeney and Xu, 2012). The cleavage of glycosidic bonds follows an acid/base reaction in which a proton donor and nucleophile/base are required and they can follow either a retaining or inverting mechanism of the anomeric centre (Davies and Henrissat, 1995).

Hydrolitic cellulase enzymes are the most studied enzymes involved in lignocellulosic biomass deconstruction. Their structure generally consists of two structurally and functionally different domains including a large catalytic domain linked to a much smaller carbohydrate-binding module (CBM), which are linked through a serine and threonine rich O-glycosylated hinge region (Lynd et al., 2002; Teeri and Reinikainen, 1992). The function of the CBM is to bring closer the catalytic domain to the cellulose surface by binding directly and therefore, facilitate cellulose hydrolysis. In addition, it has been shown that CBMs also promote disruption of the crystalline regions by reducing the strong hydrogen bonding between the cellulose fibrils and as result, cellulose surface area is increased (Gao et al., 2001; Wang et al., 2008).

In general, cellulase enzymes are produced by anaerobic/aerobic bacteria and fungi. Depending of the organism, cellulase enzyme may be produced as a complexed system called a cellulosome, which contains a scaffolding protein and many bound enzymes or as free enzymes (Lynd et al., 2002). Some anaerobic bacteria have been shown to mainly produce cellulosomes, whereas, aerobic bacteria and fungi typically release non-complexed systems of individual components including hydrolytic cellulases and accessory enzymes (Lynd et al., 2002).

The filamentous fungus, *Trichoderma reesei* is the most widely used fungus for industrial cellulolytic enzyme production and has shown to produce all of enzyme activities required for
efficient biodegradation of crystalline cellulose (Teeri and Reinikainen, 1992). These activities consist of (1) endoglucanases, which hydrolyse the amorphous or soluble derivatives of cellulose in a random manner, (2) exo-glucanases or cellobiohydrolases, which release cellobiose as the main product from reducing and non-reducing ends of the cellulose chains and (3) β-glucosidases, which break down cellobiose and low molecular weight cellulose-oligosaccharides into glucose (Lynd et al., 2002; Teeri and Reinikainen, 1992). *Trichoderma reesei* produces at least two exoglucanases/cellobiohydrolases (CBHI and CBHII), five endoglucanases (EGI, EGII, EGIII, EGIV and EGV) and two β-glucosidases (βG) (Lynd et al., 2002; Markov et al., 2005). Despite the relevance of *Trichoderma reesei* in the bioprocessing industry, its production of β-glucosidases is relatively low and not sufficient to efficiently convert cellulose to glucose (Chen et al., 1992; Sternberg et al., 1977) and therefore, cellobiose is accumulated as main product. Cellobiose has being defined as a strong inhibitor of cellobiohydrolase enzymes, thus negatively affecting cellulose hydrolysis (Zhao 2004). Because of this inhibitory effect, cellulase enzymes mixtures derived from *T. reesei* are generally supplemented with β-glucosidase enzymes from *Aspergillus niger*, in order to increase both cellulose hydrolysis rate and glucose release (Seidle et al., 2004; Sternberg et al., 1977).

Studies using purified cellulase enzymes derived from *Trichoderma reesei* have shown that the most important for cellulose degradation are CBH I (Cel7A), CBH II (Cel6A), EG I (Cel7B), EG II (Cel5A) and β-glucosidase (Gao et al., 2010b). Exo-cellobiohydrolase I (Cel7A) has shown to be the most abundant (30 – 50%) and probably the key enzyme required for the efficient hydrolysis of native crystalline cellulose (Divne et al., 1994; Markov et al., 2005). Cel7A cleaves β-1,4-glycosidic bonds from the reducing end of cellulosic chains, processively with retention of configuration and forming cellobiose as main product (Davies and Henrissat, 1995; Divne et al.,
1994). Exo-cellobiohydrolase II (Cel6A) is the second most abundant enzyme after Cel7A, constituting between 20 to 30% of the whole cocktail (Markov et al., 2005). Similar to Cel7A, Cel6A processively cleaves β-1,4-glycosidic bonds, forming cellobiose as main product, however, starts from the non-reducing end and inverts the configuration of the glycosidic bond (Rouvinen et al., 1990). As a result of their distinct preference for reducing and non-reducing ends of cellulose chains, both cellobiohydrolases are required for efficient cellulose degradation (Lynd et al., 2002). However, both enzymes are relatively slow in reducing the degree of polymerization of cellulose (Lynd et al., 2002) and therefore, endoglucanases are required. Endo-acting enzymes mainly control the reduction in degree of polymerization of cellulose chains, by randomly cleaving the β-1,4-glycosidic bonds within cellulose chains at amorphous regions and therefore, creating new cellulose chain ends that are susceptible to cellobiohydrolases activity (Béguin and Aubert, 1994; Rouvinen et al., 1990). Endoglucanase I (Cel7B) is the major endoglucanase produced by Trichoderma reesei constituting between 5 to 10% of the total amount produced (Markov et al., 2005). Structurally, Cel7B has an extended open substrate-binding cleft in contrast to tunnel-like structure of Cel7A and Cel6A, characteristic of their processive catalytic activity (Kleywegt et al., 1997). Cel7B have the broadest specificity of both endoglucanases, showing activity not only in cellulose, but also in hemicelluloses such as xylan (Biely, 1991), barley β-glucan and tamarind xyloglucan (Nakazawa et al., 2008). In contrast, Cel5A constitutes 1 to 10% of the total of enzymes produced and it is a highly specific endoglucanase, as only cleaves the unsubstituted β-1,4-glycosidic linkages present in cellulose and barley β-glucan (Nakazawa et al., 2008).

As mentioned before, production of β-glucosidases from Trichoderma reesei is relatively low and not sufficient to efficiently convert cellulose to glucose and therefore, cellulase enzymes mixtures are supplemented with β-glucosidases from Aspergillus niger, excellent source of βG
activity (Woodward and Wiseman, 1982). β-glucosidase from *Aspergillus niger* belongs to family GH3 β-glycosyl hydrolase and proceeds with retention of configuration and has at least five glucose subsites (-1 to +4) (Davies et al., 1997; Seidle et al., 2004).

### 1.6 Accessory enzymes to enhance biomass deconstruction

As described in section 1.3, cellulose is entangled and surrounded by hemicellulose, which consists of a group of heterogeneous polysaccharides with multiple units, and lignin. At the same time, hemicellulose and lignin are cross-linked through lignin-carbohydrate complexes (LCCs), that are believed to play an important role in the recalcitrance of lignocellulosic biomass (d’Errico et al., 2015; Du et al., 2013). Therefore, the removal of lignin, hemicelluloses and LCCs would be beneficial if we are to improve cellulose accessibility and facilitate cellulase enzymes adsorption onto the cellulose fibers (Mansfield et al., 1999). In addition, even though thermochemical approaches have shown to be effective in removing hemicellulose, the overall sugar recovery is compromised as hemicelluloses are partially degraded at high severities (Chandra et al., 2007). Therefore, the work described within the thesis work focussed on facilitating cellulose accessibility to cellulase enzymes by removing the hemicellulosic portion left in the solid fraction by adding accessory enzymes to the enzyme cocktail. It was hoped that, by utilizing this approach, hemicellulose can also be recovered and converted into valuable sugars for further processing.

Over the last few years, various accessory enzymes, including hemicellulases, lignin-modifying enzymes, lytic polysaccharide monooxygenases (LPMOs), esterases, among others, have been studied with a major goal of improving the hydrolytic potential of cellulase enzymes during the hydrolysis of lignocellulosic substrates. As an example, the evolution of Novozymes enzyme mixtures (Figure 4, Hu J. 2014) from Celluclast to CTec 3, shows the introduction of
accessory enzymes which has increased the effectiveness of the cocktail. As a result, the total protein loading required to effectively hydrolyze cellulose has been reduced significantly.

Among the different accessory enzymes, hemicellulases, lignin-modifying enzyme (laccases) and LPMO have been shown to be effective in enhancing the hydrolytic potential of cellulase when added to a range of lignocellulosic substrates (Goldbeck et al., 2014; Agger et al., 2014; Hu et al., 2013, 2015; Öhgren et al., 2007; Harris et al., 2010; Singh et al., 2017; d’Errico et al., 2016). Interestingly, Agger et al., (2014) showed that an LPMO enzyme can act on hemicellulose, cleaving the glucose backbone of xyloglucan. As only hydrolytic enzymes with focus on hemicellulose were utilized throughout this thesis work, the structure and catalytic mechanism of LPMO were not discussed, although good reviews are accessible here (Agger et al., 2014; Harris et al., 2010).

1.6.1 Hemicellulases

As described in section 1.3.1, hemicelluloses are highly substituted along the hemicellulosic backbone. Thus, their effective hydrolysis requires a larger diversity of enzymes. In general, hemicellulose acting enzymes can be divided into groups that cleaves along the main backbone, called core enzymes, and a second group that removes the substituents that are present along the main backbone, called debranching enzymes (Bhattacharya et al., 2015; Biely et al., 2016).

Common hemicelluloses are xylan, mannan, mixed linkage glucan and xyloglucan. However, their specific composition and degree of branching depend of the type of biomass (Ebringerová and Thomas, 2005). As a result, different hemicellulase enzymes combinations are required for the effective hydrolysis of different biomass feedstocks (Gao et al., 2011).
Glucuronoxylan and arabinoxylan are the most abundant hemicellulose in hardwoods and agricultural residues, which are target for xylanases enzymes with endo-activity (1,4-xylanases, EX) to cleave the internal glycosidic bonds along the backbone. β-xylosidases to cleave xylose monomers from the non-reducing end of xylo-oligosaccharides and debranching enzymes to hydrolyze side chains (Biely et al., 1997; Collins et al., 2005). Studies analysing the supplementation of xylanases to cellulase enzymes have shown their effectiveness in increasing cellulases hydrolytic potential during the hydrolysis of lignocellulosic substrates (Bura et al. 2009; Hu et al. 2011; Kumar and Wyman 2009c; Öhgren et al. 2007). However, the degree of synergism is highly dependent of the hemicellulose content in the substrate. During pretreatment, the structural features of hemicellulose are partially changed and a fraction is generally lost in the water-soluble stream of acid treatments (García-Aparicio et al., 2007; Hu et al., 2013). The main explanation for this synergistic interaction is that xylan blocks cellulase enzymes, as it is closely associated to cellulose. Therefore, when xylanases hydrolyse the residual hemicellulose, the access of endoglucanases/exoglucanases and β-glucosidases to cellulose microfibrils is improved (Hu et al., 2011; Öhgren et al., 2007).

Endo-1,4-xylanases (EX) hydrolyzes the main backbone of xylan and generally belong to glycosidase hydrolases families GH5, 8, 10, 11 and 43, with GH10EX and GH11EX being the dominant ones (Pollet et al., 2010; Sweeney and Xu, 2012). Both of these endo-xylanases follow the ‘retaining’ mechanism during hydrolysis (Törrönen et al., 1994). However, they differ in their substrate specificity. GH11EX is a ‘true’ xylanase as it cleaves internal β-1,4-xylosidic bonds and has a preference for unsubstituted regions. GH10EX has a broader specificity, as it cleaves linkages next to a single- or double- substituted xylose unit (Pollet et al., 2010). In addition, GH10 xylanases have shown catalytic activity towards aryl-cello oligosaccharides and β-1,4-linkages in mixed
linkage xylan. However, no preference towards cellulose or β-1,3-linkages was observed (Andrews et al., 2000; Biely et al., 1993; Biely et al., 1997). As a result of their different specificities, both families also have clear differences in the structure of their catalytic domain. The catalytic domain of GH10 has a (β/α)₈ barrel fold and binds the substrate in the active-site cleft, while GH11 has a β-jelly roll structure and binds the substrate in both the active site cleft and at the surface (Pollet et al., 2010).

As a result of the depolymerisation of xylan through the addition of endo-xylanases, this increases the formation of xylan-derived oligomers. β-xylosidases are also required to hydrolyse xylo-oligosaccharides to xylose (Qing and Wyman, 2011a). By utilizing β-xylosidase, total sugar recovery is increased and, more importantly, xylo-oligosaccharide inhibition effect over cellulase activity is reduced, allowing a more efficient hydrolysis of both xylan and cellulose (Polizeli et al., 2005; Qing and Wyman, 2011b).

Xyloglucanases and mixed linkage glucanases are another interesting type of hemicellulose accessory enzymes. However, they have not been studied to the same extent that xylanases have. This was major reason why we assessed their potential to enhance biomass deconstruction. Despite the limited information regarding both enzymes, it has been suggested that loosening xyloglucan will facilitate cellulose hydrolysis. It is likely that the hemicellulose fraction forms a highly resistant complex with cellulose microfibrills that is likely more recalcitrant to enzymatic degradation (Kaida et al., 2009; Vincken et al., 1994).

### 1.6.1.1 Xyloglucanases

Xyloglucan hydrolases or endo-xyloglucanases (XEG) are enzymes that cleave xyloglucan, which consists of a β-(1,4) glucan backbone. It also incorporates α-(1,6)-xylose
substitution groups that are double substituted by either $\alpha$-(1,2)-arabinose or $\beta$-(1,2)-galactose units (Hsieh et al., 2009; Vincken et al., 1995). As a result of substitution groups being present, both backbone acting and debranching enzymes are required for effective xyloglucan hydrolysis. Therefore, it is anticipated that, by removing the substitution groups through utilizing debranching enzymes, backbone-acting enzymes have more access to the cleavage of glycosidic bonds along the main backbone (Saritha et al., 2016). However, as discussed in the following section, xyloglucan backbone-acting enzymes or xyloglucan hydrolases have different hydrolytic activities depending if they are able or not to cleave the glycosyl bond next to substituted glucose units.

Xyloglucan hydrolases belong to the glycoside hydrolase (GH) families 5, 9, 12, 16, 44 and 74, with GH74 being the most abundant (Sweeney and Xu, 2012). Other members of GH74 are endoglucanases and oligoxyloglucan-reducing end-specific cellobiohydrolases (Damasio et al., 2017). Endo-xyloglucanase from family GH74 are inverting enzymes that primarily cleave the xyloglucan backbone at unsubstituted glucose units. They also hydrolyze at xylosyl-substituted residues in the -1 subsite (Damasio et al., 2017). In addition, the substrate-binding region of GH74 xyloglucanases lies in an open cleft, formed at the intersection of the N- and C-terminal domains, with a surface formed through the connection of the $\beta$-propeller blades of both domains (Martinez-Fleites et al., 2006). In contrast to GH74 xyloglucanase from *Trichoderma reesei*, GH12 enzymes from *Aspergillus niger* are not able to cleave at branched glucose residues. Instead, they prefer xyloglucan-derived oligomers with more than six glucose units and at least one without side groups (Van Den Brink and De Vries, 2011). In contrast to GH12 from *Aspergillus niger*, GH12 derived from *Bacillus licheniformis* has the capacity to tolerate xylose and galactose residues as well as unbranched glucans. This thought to be due to its $\beta$-jelly roll conformation (Gloster et al., 2007). Similarly, the GH5 xyloglucanase derived from *Paenibacillus pabuli* displays an open active
center with a (β/α)s barrel shape, in which the side chain groups of xyloglucan are accommodated (Gloster et al., 2007). Interestingly and similar to cellobiohydrolases, bacterial xyloglucanases from Paenibacillus have also shown processive catalytic action, in which both positive subsites W318 and W319 are needed to maintain the binding interaction with xyloglucan through a stacking effect (Matsuzawa et al., 2014).

As a result of the similar structural features between cellulose and xyloglucan structures, endoglucanase (EGIV) and β-glucosidase also shown catalytic capacity to cleave the xyloglucan backbone at unsubstituted glucose units (de Vries et al., 2001; Vincken et al., 1994). From these studies it was concluded that the removal of the xyloglucan coating from cellulose microfibrils by EGIV is beneficial if we are to enhance effective cellulose hydrolysis.

As previously discussed, all side chains attached to the main hemicellulosic backbone have to be released for a complete and fast degradation. For the specific case of xyloglucan, α-xylosidase, β-1,4-galactosidase, α-fucosidase and α-arabinofuranosidase are hypothetically required, however xyloglucan from dicotyledons and grasses have small amount of fucose and arabinose (Benko et al., 2008), thereby our analysis will only focus in two side groups: α-ᴅ-xylose and β-1,4-galactose.

Xylose substitution is a key functionality of xyloglucan in higher plants, with a lower presence in grasses and cereals (Scott-Craig et al., 2011). The removal of xylose groups is controlled by α-ᴅ-xylosidase enzyme, which is absent in most commercial enzymes mixtures derived from Trichoderma reesei as it is not existent in the genome of most filamentous fungi (Jabbour et al., 2013; Scott-Craig et al., 2011). Despite the lack of α-ᴅ-xylosidase activity in the commonly used enzyme mixtures, organisms such as Aspergillus niger and Aspergillus flavus have shown to be effective in secreting different types of α-xylosidase (GH31) (de Vries and Visser...
2001; Scott-Craig et al. 2011). Enzymes from both organisms have shown to be equally effective in removing the xylose residues from different type of polysaccharides derived from xyloglucan, including \( p \)-nitrophenyl-\( \alpha \)-D-xylanopyranoside, isoprimeverose and longer oligosaccharides (Matsushita et al., 1985; Matsushita et al., 1987; Yoshikawa et al., 1993a; Yoshikawa et al., 1993b).

As xylose groups along xyloglucan are partially substituted with galactose units, it has been suggested that \( \beta \)-galactosidases may also play a role in the efficient degradation of lignocellulosic biomass (de Vries et al. 2000). However, most of the studies concerning the characterization of catalytic activities involving \( \beta \)-galactosidase (GH2 and GH35) have mostly focused on their capacity to hydrolyze lactose and not lignocellulosic substrates (Gamauf et al. 2007; van den Brink and de Vries 2011). Despite the limited information regarding this enzyme, \( \beta \)-galactosidase derived from \textit{Aspergillus niger} has shown to work via a retaining mechanism and increase the release of galactose residues from wheat flour when combined with other accessory enzymes (de Vries and Visser, 2001; de Vries et al. 2000). In addition, previous work have confirmed the presence of galactose residues in xyloglucan from \textit{Poaceae}, family that includes cereal and grasses (Hsieh and Harris, 2009). The suggested composition of xyloglucan from cereal and grasses, follows the nomenclature system developed by Fry et al., (1993), in which: \( G \) = unsubstituted \( \beta \)-D-Glc; \( X \) = \( \alpha \)-D-Xyl-(1 \( \rightarrow \) 6)-\( \beta \)-D-Glc; \( S \) and \( L \) = \( X \) with \( \alpha \)-L-Araf-(1 \( \rightarrow \) 2) and \( \beta \)-D-Galp-(1 \( \rightarrow \) 2) attached, respectively; and \( F \) = \( L \) with \( \alpha \)-L-Fucp-(1 \( \rightarrow \) 2) attached. Utilizing this nomenclature, the suggested structure of xyloglucan in \textit{Poaceae} is XXLGG, with the possibilities of acetyl groups attached at unsubstituted glucose units (Fry et al., 1993; Hsieh and Harris, 2009).

In the work reported here, we tested a variety of xyloglucanases from different fungi and bacteria including endo-activity as well as debranching catalytic function such as \( \alpha \)-xylosidase and
β-galactosidase. Enzyme kinetic, structure and catalytic functionality of all tested enzymes are presented in section 1.8.

1.6.1.2 Mixed-linkage glucan or β-glucans

Mixed-linkage glucan or β-glucans are hemicelluloses unique to the Poales, taxonomic order that includes cereal grasses as corn (Scheller and Ulvskov, 2010). This type of hemicellulose consist of β-D-glucopyranose units linked through β-1,4 and β-1,3 glycosidic bonds with significantly more β-1,4 linkages (70%) than β-1,3 (30%) (Ebringerová and Thomas, 2005). The most frequently used enzyme to characterize β-glucans is licheninase (endo-β-1,3-1,4-glucanase), which cleaves the β-1,4 linkage of the glucose unit next to a β-1,3 linkage, yielding oligosaccharides with only 1 glucose unit linked through β-1,3 linkage next to a reducing end (Ajithkumar et al., 2006; Wood et al., 1991). In addition to licheninase, laminarinase have also shown activity towards β-glucan by acting in the β-1,3 linkage between 2 glucose units (Ajithkumar et al., 2006; McGregor et al., 2017). Similarly, endo-1,4-β-glucanase is also capable to hydrolyze β-glucan, but only at the internal β-1,4 linkages. In contrast to these enzymes, in this work, we have the opportunity to test the potential of a unique endo-β-glucanase derived from Vitis vinifera. VvEG16, with activity towards both xyloglucan and mixed linkage glucan structures (McGregor et al., 2017). More details of this enzyme are presented in section 1.8.

1.7 Synergism between cellulases and major accessory enzymes

In order to completely hydrolyze cellulose, three different enzymes activities are typically required, including endoglucanases, exo-glucanases or celllobiohydrolases and β-glucosidases (Lynd et al., 2002; Teeri and Reinikainen, 1992). When these enzymes cooperate synergistically, the combined action of all cellulase enzyme is higher than the sum of the individual action of each
enzyme (Henrissat et al. 1985). However, the resulting synergism between cellulolytic enzymes (exo/exo, endo/endo or exo/endo) is highly dependent of the structural features of the substrate and enzyme concentration utilized (Henrissat et al. 1985; Jalak et al. 2012). The more accepted interpretation of synergism between endoglucanases (EGs) and celllobiohydrolases (CBHs) is that the randomly cleavage by EG creates new chain ends on the cellulose surface, which are starting points for the processive CBHs (Henrissat et al., 1985; Jalak et al., 2012). In addition to this mechanism, it has been proposed that the removal of amorphous regions by EGs facilitates CBHs enzymatic performance, as the later are generally trapped after reaching amorphous regions (Igarashi et al., 2011; Jalak et al., 2012).

Synergism between exo-glucanases, CBHI (Cel7A) and CBHII (Cel6A) has been proposed to be a result of the different catalytic preferences of both exo-acting enzymes, in which Cel7A degrades the substrate from the reducing end, whereas Cel6A preferentially cleaves from the non-reducing end and less processively (Barr et al., 1996). In addition to their different catalytic activities, Cel7A increases internal fibrillation, as well as, creates new hydrolysis sites when combined with Cel6A and vice versa (Barr et al., 1996; Igarashi et al., 2011).

Although the synergism between cellulase enzymes has shown to be effective in enhancing the degradation of insoluble cellulose, its effect is limited when applied to real lignocellulosic substrates and as a result, high enzyme loadings are still required to reach relatively high sugar conversions (Andersen et al., 2008; Jalak et al., 2012). Therefore, one of the main challenges in the bioconversion process of lignocellulosic biomass is to reduce the cost associated to enzymes. Different strategies have been studied in order to overcome this issue, such as engineer enzymes to make them more tolerant to industrial conditions, a more cost effective enzyme production,
attempt to recycle enzymes, combination of “cellulase” enzyme mixture and accessory enzymes, among others (Banerjee et al., 2010c; Lynd et al., 2002; Pribowo et al., 2012).

Past work has shown that the addition of “accessory enzymes” to the “cellulase” cocktail can enhance the hydrolytic performance of enzyme mixture while reducing the protein/enzyme loading required to hydrolyse pretreated biomass substrates (Berlin et al., 2007; Hu et al., 2011; Hu et al., 2013; Hu, 2014; Selig et al., 2008). Therefore, the motivation for this research is to study the potential of accessory enzymes, specifically, hemicellulases in improving cellulases hydrolytic performance.

As a result of hemicelluloses physically blocking cellulase enzymes by covering the cellulose microfibrills, when cellulase and xylanases enzymes are combined, fibre swelling and cellulose accessibility are have been shown to increase due to the solubilisation of xylan (Berlin et al., 2007; Hu, 2014; Selig et al., 2008). Despite the effectiveness of hemicellulases in enhancing cellulase performance, the degree of synergism is highly dependent of the resulting substrate after pretreatment (Hu et al., 2013). Generally, enzymes with broader specificities such as GH10EX (xylanase) and backbone acting xyloglucanase GH5 have shown the best performance in enhancing cellulose hydrolysis over a range of substrates when supplemented to cellulases (Hu et al., 2013). Similar to the supplementation of backbone acting enzymes, the incorporation of debranching enzymes such as α-arabinofuranosidase and α-glucuronidase have also shown to enhance xylan enzymatic deconstruction and therefore, facilitate cellulose degradation (Gao et al. 2011; Huang et al. 2017). It has been shown that both glucose and xylose yields are enhanced when cellulose and hemicellulose are cooperatively hydrolyzed (Billard et al. 2012; Gao et al. 2011; Kumar and Wyman 2009c).
Even though xyloglucanases have not been studied to the same extent as xylanases, past work has shown their ability to enhance the hydrolytic performance of the commercial cellulase mixtures Celluclast and CTec2 (Benko et al., 2008; Hu et al., 2011; Jabbour et al., 2013; Scott-Craig et al., 2011). In addition, these studies separately evaluated the effects of backbone acting xyloglucanases (GH5 and GH74) and debranching enzyme α-xylosidase. However, to the best of our knowledge, no studies have assessed the interaction between backbone acting xyloglucanases and debranching enzyme on real lignocellulosic substrates. In addition, commercial cellulases preparations derived from *Trichoderma reesei* lack α-xylosidase activity (Jabbour et al., 2013). Therefore, we studied two different bacterial organisms, *Bacteroides ovatus* and *Cellvibrio japonicus*, with the capacity to secrete this enzyme.

### 1.8 Fungal and bacterial enzymes: our targets for biomass deconstruction

High production of cellulose acting enzymes including cellobiohydrolases and endoglucanases is a characteristic of fungal organisms such as *Trichoderma reesei* (Markov et al., 2005). In comparison to other cellulolytic enzymes, cellobiohydrolases account to over 70% of the total enzymes secreted and endoglucanases close to 20%, whereas hemicellulases typically account for less than 1% (Sweeney and Xu, 2012). As a result of the limitations of *Trichoderma reesei* producing high concentration of hemicellulases, there is great interest in studying other fungal organisms, as well as, bacterial sources. What makes bacteria attractive as a suitable candidate for enzyme production is their ability to resist more extreme environmental conditions (Bhattacharya et al., 2015; Maki et al., 2009; Sweeney and Xu, 2012). Strains that are resistant to elevated temperatures, high or low pH, are of the high interest, as they are expected to be more resilient to operational conditions that may be present in the bioconversion process (Maki et al., 2009). Therefore, it would be of interest to assess if the addition of bacterial hemicellulases to
fungal commercial “cellulase” enzymes mixtures exhibit synergism in the deconstruction of cellulose. Past work has reported exo/exo and exo/endo synergism in the hydrolysis of microcrystalline cellulose when bacterial cellulolytic enzymes were combined with fungal enzymes (Baker et al., 1995; Baker et al., 1998). More importantly, the combination of bacterial hemicellulases and fungal cellulases enhanced the hydrolysis of lignocellulosic substrates by increasing both glucose and xylose yields as well as reducing the total protein loading needed for equivalent sugar conversions (Gao et al., 2010a; Gao et al., 2011).

In the work reported in this thesis, eleven bacterial accessory enzymes derived from five different organisms including *Cellvibrio japonicus*, *Bacteroides ovatus*, *Paenibacillus pabuli*, *Prevotella bryantii* and *Vitis vinifera* were assessed. Four enzymes representing backbone- and sidechain-hydrolyzing activities toward xyloglucan from GH5, GH74 and GH31 families were selected from *Cellvibrio japonicus* (Attia et al., 2016; Attia et al., 2018; Larsbrink et al., 2011; Larsbrink et al., 2014b). In parallel, four target enzymes from the recently identified complex gene locus of a common human gut microorganism, *Bacteroides ovatus* (Larsbrink et al., 2014a), were selected based on their efficiency in hydrolysis of xyloglucan (BoGH5A, BoGH31A and BoGH2) and mixed linkage glucan (BoGH16) (Tamura et al., 2017). Additional novel enzymes were selected from the GH5 family, including bacterial enzymes from *Paenibacillus pabuli* (PpXG5) and *Prevotella bryantii* (PbGH5A) (McGregor et al., 2016) and a plant enzyme from GH16 family derived from *Vitis vinifera* (VvEG16). This extended the variety of hemicellulases from xyloglucanases to mixed linkage glucanases (McGregor et al., 2017). Catalytic affinities and relevant information for the target enzymes studied in this thesis work are presented in Table 3. In addition, figure 3 describes the proposed pathway of xyloglucan degradation through the addition of the different accessory enzymes.
### Table 3. Novel accessory enzymes that were assessed for their possible contribution to enhancing biomass deconstruction

<table>
<thead>
<tr>
<th>Origin</th>
<th>Type</th>
<th>Enzyme</th>
<th>Substrate</th>
<th>Enzymes affinities</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacteroides ovatus</em></td>
<td>Backbone acting</td>
<td>BoGH5A</td>
<td>XG</td>
<td>Hydrolyzes xyloglucan at C&lt;sub&gt;1&lt;/sub&gt; of unbranched glucose unit. Broad active-site cleft to equally recognize distinct side chain branching of xyloglucans: shows equivalent hydrolysis kinetics in galacto-xyloglucan, fucogalacto-xyloglucan and arabinogalacto-xyloglucan.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BoGH16</td>
<td>MLG</td>
<td>Endo-glucanase that cleaves the (1→4)-glycosidic linkage bond next to a (1→3)-glucose residue.</td>
</tr>
<tr>
<td></td>
<td>Debranching enzymes</td>
<td>BoGH31A</td>
<td>XG</td>
<td>Debranching enzyme, α-xyllosidase, essential role by removing α-1,6-xylene residues from the non-reducing end of xyloglucan-oligomers.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BoGH2</td>
<td>XG</td>
<td>Debranching enzyme, β-galactosidase, essential role by removing the β-1,2- galactose residues attached to α-xylene residues.</td>
</tr>
<tr>
<td><em>Cellvibrio japonicus</em></td>
<td>Backbone acting</td>
<td>CjGH74</td>
<td>XG</td>
<td>Highly specific endo-xyloglucanase, able to hydrolyze at unbranched backbone glucose residues, but unable to hydrolyze shorter substrates formed by two or three glucose units. No endo-xylanase activity. Fundamental importance of the presence of xylene residues along the glucan backbone, as is unable to hydrolyze cellulose substrates as CMC and HEC.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CjGH5D</td>
<td>XG</td>
<td>Specific endo-xyloglucanase that catalyze hydrolysis at C1 of unbranched glucose residues of XG.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CjGH5F</td>
<td>XG</td>
<td>Specific endo-xyloglucanase that catalyze hydrolysis at C1 of unbranched glucose residues of XG.</td>
</tr>
<tr>
<td></td>
<td>Debranching enzymes</td>
<td>CjXyl31A</td>
<td>XG</td>
<td>Debranching enzyme, α-xyllosidase, which hydrolyzes the xylene present at the non reducing end terminal from extensively branched xyloglucan-oligomers. Preference for longer chains, poor activity on disaccharides.</td>
</tr>
<tr>
<td><em>Paenibacillus pabuli</em></td>
<td>Backbone acting</td>
<td>PpXG5</td>
<td>XG</td>
<td>Highly specific endo-xyloglucanase, with similar affinity as BoGH5. Binding of the β (1→4)-glucan backbone of both enzymes is essentially identical.</td>
</tr>
<tr>
<td><em>Prevotella bryantii</em></td>
<td>Backbone acting</td>
<td>PbGH5</td>
<td>XG - MLG</td>
<td>Predominant activity as endo-glucanase in MLG, but also competent endo-xyloglucanase. Preference for unbranched glucose units in xyloglucan (similar to BoGH5), but also activity over highly branched units as secondary sites of attack. On MLG, PbGH5 has preference over β (1→3) linkages, but it is also able to cleavage β (1→4).</td>
</tr>
<tr>
<td><em>Vitis vinifera</em></td>
<td>Backbone acting</td>
<td>VvEG16</td>
<td>XG - MLG</td>
<td>Endo-glucanase that hydrolyzes the backbone of both XG and MLG. On MLG, VvEG16 has preference for β (1→4) linkages over β (1→3). Specifically, it cleaves the β (1→4)-glycosidic linkage bond to a β (1→3)-glucose residue.</td>
</tr>
</tbody>
</table>

Information for each enzyme was took from: BoGH3B, BoGH5A and BoGH31A: (Larsbrink et al., 2014a); BoGH16: (Tamura et al., 2017); CjGH74: (Attia et al., 2016; Larsbrink et al., 2014b); CjXyl31A: (Larsbrink et al., 2011); VvEG16: (McGregor et al., 2017); CjGH5D and CjGH5F: (Attia et al., 2018); PbGH5A: (McGregor et al., 2016). CMC: Carboxymethyl cellulose; HEC: Hydroxyethyl cellulose; MLG: Mixed linkage glucan; XG: Xyloglucan.
As mentioned before, the thesis work studied two different types of xyloglucanases: 1) endo-acting and 2) debranching enzymes, α-xylosidase and β-galactosidase. The proposed pathway of xyloglucan degradation by these enzymes is presented in Figure 3. First, endo-xyloglucanases hydrolyze xyloglucan at C1 position of unbranched glucose unit, freeing different oligosaccharides. Then, debranching enzymes, α-xylosidase and β-galactosidase, are able to hydrolyze the xylose and galactose residues, respectively.

Figure 3. Proposed pathway of xyloglucan degradation by novel accessory enzymes.
1.9 **Thesis objectives**

The main focus of the thesis work was to assess the possible contribution that novel “accessory enzymes” might have in enhancing the enzyme mediated deconstruction of pretreated lignocellulosic substrates. There was a major focus on xyloglucan and mixed linkage-glucan structures as they were expected to be relevant to the effective hydrolysis of pretreated agricultural and forest biomasses. As xyloglucanases and mixed linkage glucanases have not been studied to the same extent as other hemicellulases, we hoped that this work would provide some insights with regard to their relevance to biomass deconstruction and potentially increase cellulase hydrolytic performance while maximizing sugar production at low enzyme loadings. The thesis work is divided in two chapters. The specific objectives for each chapter are summarized below.

Chapter 3.1, the main objective was to identify and quantify the effectiveness of novel xyloglucanases and mixed linkage glucanases in enhancing enzyme-mediated cellulose hydrolysis on a range of lignocellulosic substrates. Eleven enzymes derived from five different organisms, including bacterial accessory enzymes were added to mechanically- and chemically-treated substrates at increasing concentrations from 1 to 5 mg/g. The interactions of a commercial cellulase mixture (Celluclast 1.5) and backbone acting enzymes was assessed on pretreated aspen and corn stover substrates. Subsequently, backbone-acting enzymes were combined with debranching enzymes derived from the same organism to evaluate possibilities of synergism and facilitate cellulose degradation. To measure the specificity of the enzymes toward xyloglucan and mixed linkage glucan, the activities on both substrates were tested as well as their performances in model substrates based on microfibrillated cellulose. The best candidates were selected according to the observed increase in cellulose hydrolysis when supplemented to the “base” cellulase mixture, Novozymes Celluclast mixture. Finally, these potentially useful enzyme additions were tested in
combination with a more complex cellulase enzyme mixture (CTec 3) to assess if the inclusion of these accessory enzymes might improve xyloglucanases performances and the overall hydrolytic performance of the mixture.

Chapter 3.2, the main objective was to try to better understand the mechanism behind the observed synergistic interaction between backbone-acting and debranching enzymes. The hope was to reduce the overall protein loading required for effective hydrolysis. The best candidates, including backbone- and sidechain-hydrolyzing activities, were assessed on steam pretreated corn stover (SPCS) and ammonia fibre expansion pretreated corn stover (AFEX CS). The comparison between acid and alkali pretreatments used substrates with different hemicellulose composition and content with the aim of better understanding how the enzymatic removal of xyloglucan might influence cellulose hydrolysis. The supplementation of the debranching enzyme, α-xylosidase with backbone acting enzymes was assessed. Strategies included simultaneous and sequential addition of enzymes. The influence of cellulase/xyloglucanases synergistic interaction on hydrolysis rates and extent of hydrolysis was evaluated. Finally, we tried to better understand how xyloglucan removal influenced substrate physicochemical properties, gross fibre characteristics and cellulose accessibility. These properties were assessed after applying a xyloglucanase treatment to the substrates. As summarised in the thesis, this helped us better elucidate the observed synergism between cellulolytic enzymes and novel accessory xyloglucanase enzymes.
2 Materials and methods

2.1 Lignocellulosic feedstocks and pretreatment technologies

Corn stover and aspen were selected as raw materials as representatives of agricultural residues and hardwood biomass. Mechanical pretreatments, including refined mechanical pulping (RMP) and thermomechanical pretreatment (TMP-bio) were applied and compared to chemical pretreatments. These included steam explosion (SP) and ammonia fibre expansion pretreatment (AFEX). Steam pretreated aspen and corn stover were prepared as described earlier (Bura et al., 2009) with only corn stover incorporating acid impregnation with SO$_2$. Prior to steam pretreatment, corn stover (DW, dry weight of 300g) was impregnated with 4.0% anhydrous SO$_2$ (w/w) overnight in sealable plastic bags. Similarly, aspen wood chips (DW, dry weight of 200 g) were mixed with water in a ratio 1:1 and left overnight at room temperature. After impregnation, the procedure was the same for both biomass substrates. Batches of 50 g (DW) were separately loaded into a preheated 2 L Stake Tech III (Stake Technologies, Norvall ON, Canada) steam gun at pretreatment conditions described in Table 4. After pretreatment, the water insoluble fractions were separated using a vacuum system, extensively washed and kept at -20 °C for further analysis.

Ammonia fiber expansion pretreatment was carried out at Michigan State University as described by Balan et al., (2009). Corn stover (DW, 60% moisture) was loaded into a pressurized AFEX reactor at conditions described in Table 4.

RMP was prepared from aspen as described earlier by Chandra et al., (2016). Aspen wood chips were fed into a 12” Sprout Waldron Laboratory Refiner with D2A507 plates. The refining was carried out in multiple steps, reducing the disc clearance from 0.5 mm to 0.1 mm. Finally, FPInnovations kindly provided TMP-bio aspen substrate.
In addition to lignocellulosic substrates, Microfibrillated cellulose (MFC) provided by Biofilaments was utilized as model substrate in combination with xyloglucan (XG) from Tamarind seed (Megazyme).

### 2.2 Enzymatic hydrolysis

Four different commercial preparations from Novozymes were used in combination with the novel accessory enzymes. A cellulase cocktail (Celluclast 1.5 L; protein content 130 mg/ml) derived from *Trichoderma reesei*, a β-glucosidase preparation (Novozyme 188, protein content 233 mg/ml) derived from *Aspergillus niger* and two more complex enzyme mixtures (Cellic CTec2, protein content 152 mg/ml; Cellic CTec3, protein content 183 mg/ml). In all of the hydrolysis assays, β-glucosidase (Novozyme 188) was supplemented at half loading (in g per gram of cellulose) of Celluclast to avoid any end-product inhibition. As the objective of this thesis is to maximize sugar recovery, all enzymes were tested at the optimum conditions of cellulase enzyme mixtures derived from *Trichoderma reesei*, i.e. pH: 5.0 and T: 50 °C as they are the main component of the whole enzyme cocktail.

**Table 4. Pretreatment conditions for biomass substrates utilized in this study**

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Biomass</th>
<th>Temperature, °C</th>
<th>Time, min</th>
<th>SO2 % w/w</th>
<th>NH3 : biomass</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Steam pretreatment</td>
<td>Corn stover</td>
<td>190°C</td>
<td>5</td>
<td>4</td>
<td>-</td>
<td>SPCS</td>
</tr>
<tr>
<td></td>
<td>Aspen</td>
<td>210°C</td>
<td>5</td>
<td>-</td>
<td>-</td>
<td>SPA</td>
</tr>
<tr>
<td>Ammonia fiber expansion</td>
<td>Corn stover</td>
<td>120°C</td>
<td>30</td>
<td>-</td>
<td>1:1</td>
<td>AFEX CS</td>
</tr>
<tr>
<td>Refined mechanical pulping</td>
<td>Aspen</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>RMP</td>
</tr>
<tr>
<td>Thermomechanical pulping</td>
<td>Aspen</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>TMP-bio</td>
</tr>
</tbody>
</table>
The Carbohydrate Enzymology Research Group, UBC lead by Professor Harry Brumer, provided all of the novel accessory enzymes. The strategy utilized to rapidly screen the effectiveness of accessory enzymes was to supplement them to cellulase enzyme mixture, Celluclast. Cellulase enzyme loading was determined as the minimum required to reach a 60% of cellulose hydrolysis (15 mg protein per gram of cellulose), while accessory enzymes were individually assessed at increasing loadings of 1, 2.5 and 5 mg/g cellulose, with the objective of achieve a final 70% cellulose hydrolysis.

First, batch hydrolysis of pretreated corn stover and aspen were carried out at 2% (w/v) solids loading in sodium acetate buffer 50 mM pH 4.8 at 5 ml total volume, respectively. Reactions mixtures were mechanically shaken at 150 rpm in a horizontal shaker incubator (IST-4075, GMI) at 50°C for 72 h. First only backbone acting were tested and then debranching enzymes were incorporated in a ratio of 1.5: 1, respectively. The possibilities of synergism were assessed through combining backbone-acting enzymes to debranching enzymes, CjXyl31A and BoGH31A, by keeping the enzyme loading constant.

Similarly, batch hydrolysis using the model MFC – XG substrates were carried out at 1% (w/v) solids loading in sodium acetate buffer 50mM pH 4.8 at 1 ml total volume in an orbital shaker incubator (Combi H12; hybridization incubation) at 50°C for 72 h. All analyses were performed in duplicate and standard deviation are presented. Hydrolysis was ended by heating up the system to 100°C for 10 minutes to inactive the enzymes. Supernatants were centrifuged at 4200 rpm for 15 minutes and stored at -20°C for subsequent carbohydrate analysis. Hydrolysis yields, % were calculated based on the theoretical cellulose and xylan available in the substrates.

In Chapter II of this thesis, the main objective was to understand the synergistic interaction found in Chapter 1 with the aim of reducing the total enzyme loading. For this objective, only
Bacteroides ovatus enzymes - BoGH5, BoGH31A and BoGH2 – and pretreated corn stover substrates were utilized. Batch hydrolysis were carried out at the same experimental conditions previously described, but incorporating time measurements throughout the course of hydrolysis and comparing 2% vs 10% solid loadings. In addition, different strategies were utilized to assess synergism along 72 h: simultaneous supplementation of debranching enzyme BoGH31A and backbone acting enzyme BoGH5 at time zero of hydrolysis or separate supplementation of debranching enzymes after 24 h. During this final strategy, the first 24 hours only incorporated backbone acting enzymes and after addition of debranching enzyme, hydrolysis was continued for 48 h. With the objective of compare the effectiveness of xyloglucanases in increasing cellulase enzyme hydrolytic potential, a separate analysis was completed through adding only cellulase enzymes (Celluclast and CTec 2) at increasing concentrations to pretreated corn stover substrates. This analysis allows us to compare the hydrolytic performance of cellulase enzymes with the combination of cellulases and xyloglucanases at equal enzyme loadings and therefore, evaluate possibilities if any to reduce total protein loading.

In order to assess the changes on physicochemical properties of the substrates, batch hydrolysis of corn stover substrates were carried out at 2% (w/v) solids loading in sodium acetate buffer 50mM pH 4.8 at 50 ml total volume reaction. In addition, accessory enzymes loading was the equivalent as previous experiments and analyzed in presence or absence of cellulase enzymes (Celluclast 1.5 L).

2.3 Analytical methods

The protein content of all enzymes was measured using Ninhydrin assay with bovine serum albumin (BSA) as protein standard (Starcher, 2001). The quantitative analysis of the chemical compositions of all pretreated substrates was determined according to Klasson standard method
Glucose and xylose concentration in the hydrolysate were analyzed utilizing high-performance liquid chromatography (HPLC) using fucose as the internal sugar standard. Sulfuric acid hydrolysis was performed in order to break down all oligosaccharides into monomeric form, which are easily measured using HPLC. Therefore, oligomer content was calculated as the difference in monosaccharide concentration between original hydrolysate and hydrolysate after acid hydrolysis.

### 2.4 Enzyme activity assay

The specific activities and product analysis of all novel accessory enzymes available on literature, were compared with the specific activities assessed on model substrates. Cellbiohydrolase, β-glucosidase and β-xylosidase activities were determined by using p-nitrophenyl-β-D-cellobioside (p-NPC), p-nitrophenyl-β-D-glucopyranoside (p-NPG), and p-nitrophenyl-β-D-xylopyranoside (p-NPX) as substrates, respectively, according to Saha and Bothast, (1996). Xylanase, endoglucanase, xyloglucanase and mixed linkage glucanases activities were determined utilizing a modified version of the method described from Lin and Thomson, (1991). Briefly, birch wood xylan, tamarind seed xyloglucan, carboxymethylcellulose (CMC) and barley β-glucan were dissolved in 50 mM sodium acetate buffer (pH 4.8) by stirring overnight at room temperature. Samples with 70 μl of the substrates dissolved in sodium acetate were mixed with 30 μl of appropriately diluted enzymes in microplates and they were incubated at 400 rpm for 10, 20 and 30 minutes at 50°C. The enzymatic reaction was stopped by adding 200 μl of 3,5-dinitrosalicylic acid (DNS) reagent followed by 30 min at 105°C in an oven. Evaporation was limited by covering the plate with a lid and extra aluminum. Reducing sugar content of the samples were analyzed by measuring the absorbance at 540 nm using xylose and glucose as standards for calibration. The amount of reducing sugar (μmol) released during assay was plotted at different
hydrolysis times and the enzyme activity (μmol/min) was equal to the slope of the linear section of the curve. All analyses were performed in triplicate.

2.5 Cellulose accessibility

2.5.1 Simon’s staining technique

Cellulose accessibility to cellulases was estimated by the Simons’ stain technique according to the modified procedure by Chandra et al., (2008) using orange dye (DO-Pontamine Fast Orange 6RN) obtained from Pylam Products Co. Inc. (Garden City, NY, U.S.) and fractionated via an Amicon filtration system. Approximately 10 mg dry weight (DW) of non-dried samples was weighed into each of six 1 ml micro-centrifuge tubes (Thermo Fisher Scientific Inc). The solution in each tube was composed by 0.1 ml of phosphate-buffered saline (PBS, pH 6) and DO dye solutions (10 mg/ml), in a series of increasing volumes (0.25, 0.50, 0.75, 1.0, 1.5, and 2.0 ml). Then, nanopure water was added to complete the final volume of 1 ml in each tube. The tubes were incubated overnight at 70°C in an orbital shaker at 300 rpm. After incubation, the tubes were centrifuged at 5,000 rpm for 10 min, and an aliquot of the supernatant was placed in a disposable cuvette for absorbance reading on a Cary 50 UV-Vis spectrophotometer at 455 nm. The amount of dye adsorbed on the fibre was calculated by subtracting the dye concentration in the supernatant (leftover) to the initial concentration of the dye, according to the Beer-Lambert law. The measurement of increasing concentration of DO and its absorbance at 455 nm allows calculating the extinction coefficient, which is equal to the slope of obtained linear curve. All analyses were performed in triplicate.
2.6 Fibre quality analyzer, FQA

Fibre length and width were determined using a high-resolution fibre quality analyser (FQA) (LDA02; OpTest Equipment, Inc., Hawkesbury, ON, Canada) as described by Robertson et al., (1999). The total amount of fibres counter per sample were 10,000 and all samples were run in duplicate. The ranges of mean length and mean width were 0.07 to 10 mm and 7 to 60 μm, respectively. However, only fibres over 0.50 mm in length are incorporated in the mean width calculation.

2.7 Particle size distribution, Mastersizer 2000

The fibre mean width calculation using FQA is limited to the bigger fibres over 0.50 mm, which only includes a 10% of the total distribution of fibres lengths within the substrate. In order to have a better representation of all fibres including fines present in both corn stover, particle sizes distribution after xyloglucanase treatment were determined using a Mastersizer 2000 (Malvern Instruments Ltd., Worcestershire, UK). Pretreated samples were dispersed in water using 6 min ultrasonic treatment to measure the particle size distribution in μm.
3 Results and Discussion

3.1 Assessment of the possible contribution of novel accessory enzymes to the deconstruction of pretreated aspen and corn stover substrates

3.1.1 Background

Although cellulases are the major enzymes for saccharification of lignocellulosic biomass, they predominantly target the cellulosic polysaccharide, with little focus on the hemicellulosic polysaccharides such as xylan, mannan, xyloglucan and mixed linkage glucan. However, it has been shown that the strong association of hemicelluloses with cellulose microfibrills restricts the accessibility of cellulases to the cellulose (Banerjee et al., 2010c). Therefore, multi-component “enzyme cocktails” (including both cellulose and hemicellulose hydrolysis/disrupting activities) are often required to achieve a fast and efficient deconstruction of lignocellulosic biomass. This strategy of introducing “accessory enzymes” to the cellulase enzymes has shown to be an efficient strategy to further reduce the cost associated with high enzyme loading by enhancing the hydrolytic performance of cellulase by a synergistic interaction (Gao et al., 2011; Gao et al., 2014; Hu et al., 2011; Kumar and Wyman 2009a; Zhang and Viikari, 2014). However, depending on the physicochemical characteristics of pretreated lignocellulosic substrates, the degree of synergism between cellulases and accessory enzymes can vary significantly (Hu et al., 2011; Li et al., 2011). As described in detail in the thesis introduction, an effective biomass pretreatment tries to increase the hydrolyzability of the substrates while maintaining good carbohydrate recovery. Thus, a large amount of hemicellulosic sugars remain associated with cellulose in these pretreated substrates. Therefore, cellulose-degrading enzymes, as well as different types of hemicellulase enzymes are often required to break down lignocellulosic substrates and enhance cellulose accessibility. By
utilizing both type of enzymes, total sugar recovery should be maximised while biomass
deconstruction is greatly enhanced.

As described in section 1.3.1, hemicellulose has a complex and heterogenous structure,
which requires a range of enzymes to break down various ester bonds (side chain groups) and
glycosidic bonds (backbone) during hydrolysis (Van Den Brink and De Vries, 2011; Saritha et al.,
2016; Sweeney and Xu, 2012).

However, most of the previous studies have only focused on the importance of removing
glycogen by xylanases. The removal of other hemicellulosic components such as xyloglucan and
mixed linkage glucan have not yet received much attention. Despite limited research, loosening
xyloglucan has been shown to help in the effective hydrolysis of cellulose as it forms a highly
resistant complex with cellulose microfibrills, limiting hydrolysis of the cellulose component
(Kaida et al., 2009; Vincken et al., 1994). The use of family 5 xyloglucanase from Paenibacillus
has been shown to enhance cellulase hydrolytic performance during hydrolysis of pretreated cereal
derived substrates (Benko et al., 2008; Hu et al., 2013). In addition, previous studies also concluded
that the removal of the xyloglucan coating from cellulose microfibrills aids in the effective
hydrolysis of cellulose (de Vries et al., 2001; Vincken et al., 1994). However, although xyloglucan
has a highly substituted structure (Section 1.6.1.1), to our knowledge, no study has assessed the
potential synergism between xyloglucan backbone acting enzymes and debranching enzymes such
as α-xylosidase. Similarly, no study has evaluated the potential of mixed linkage glucanases to
enhance biomass deconstruction, despite having a favourable structural conformation composed
of only glucose units (Section 1.6.1.2).

Therefore, one of the main objectives of section 3.1 was to assess the potential synergistic
interaction among cellulases and a variety of novel accessory enzymes with xyloglucan and mixed
linkage glucan activities during hydrolysis of a range of industrially relevant pretreated lignocellulosic substrates. Various backbone-acting xyloglucanases and mixed linkage glucanases were, initially, individually assessed, to evaluate their potential to work synergistically with traditional (Celluclast) and more recent (CTec series) cellulase mixtures. The best candidates were subsequently selected according to the higher increments obtained in glucose release after the supplementation of the novel “hemicellulose-specific” enzymes. These best candidates were then further studied when added with the debranching enzyme, α-xylosidase. Enzyme activity analysis of the purified enzymes were performed to evaluate the specificity of the enzymes towards xyloglucan and mixed linkage glucan. They were then compared with the hydrolytic performance of cellulase commercial mixtures during the hydrolysis of optimally pretreated lignocellulosic substrates.

3.1.2 Target enzymes for biomass saccharification

The Carbohydrate Enzymology Research Group, UBC lead by Professor Harry Brumer, provided all of the hemicellulases enzymes with specificities for xyloglucan (XG) and mixed linkage glucan (MLG) (Table 5). Briefly, four enzymes representing backbone- and sidechain-hydrolyzing activities toward xyloglucan were selected from Cellvibrio japonicus. In parallel, four target enzymes from the recently identified complex gene locus of a common human gut microorganism, Bacteroides ovatus were selected for their efficiency in XG metabolism (BoGH5A, BoGH31A and BoGH2) and MLG (BoGH16). One additional enzyme specific for xyloglucan was selected from the GH5 family, bacterial enzyme from Paenibacillus pabuli (PpXG5). In addition, another GH5 family enzyme derived from Prevotella bryantii (PbGH5A) and a plant enzyme from Vitis vinifera (VvEG16) were selected, to extend the variety of hemicellulose substrate specificities from xyloglucan to β-glucans.
As expected, all enzymes shown substantial differences in their optimum temperature and pH, as well as protein concentrations (Table 6). Despite these differences, all of the enzymes were tested at conditions as close as possible to the optimum conditions of the cellulolytic commercial enzyme mixtures derived from *Trichoderma reesei*, i.e. pH: 5.0 and T: 50°C.

<table>
<thead>
<tr>
<th>Origin</th>
<th>Enzymes</th>
<th>Type</th>
<th>Optimum $T^\circ$[°C]</th>
<th>Opt pH</th>
<th>Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Cellvibrio japonicus</em></td>
<td>CjGH74</td>
<td>endo-xyloglucanase</td>
<td>65</td>
<td>6.5</td>
<td>XG</td>
</tr>
<tr>
<td></td>
<td>CjGH5F</td>
<td>endo-xyloglucanase</td>
<td>50</td>
<td>7.0</td>
<td>XG</td>
</tr>
<tr>
<td></td>
<td>CjGH5D</td>
<td>endo-xyloglucanase</td>
<td>50</td>
<td>7.5</td>
<td>XG</td>
</tr>
<tr>
<td></td>
<td>CjXyl31A</td>
<td>α-xyllosidase</td>
<td>45</td>
<td>6.5</td>
<td>XG</td>
</tr>
<tr>
<td><em>Bacteroides ovatus</em></td>
<td>BoGH5A</td>
<td>endo-xyloglucanase</td>
<td>37*</td>
<td>6.5</td>
<td>XG</td>
</tr>
<tr>
<td></td>
<td>BoGH31A</td>
<td>α-xyllosidase</td>
<td>37*</td>
<td>6.0</td>
<td>XG</td>
</tr>
<tr>
<td></td>
<td>BoGH16</td>
<td>endo-β-glucanase</td>
<td>55</td>
<td>6.5</td>
<td>MLG</td>
</tr>
<tr>
<td></td>
<td>BoGH2</td>
<td>β-galactosidase</td>
<td>37*</td>
<td>7.0</td>
<td>XG</td>
</tr>
<tr>
<td><em>Paenibacillus pabuli</em></td>
<td>PpXG5</td>
<td>xyloglucanase</td>
<td>50</td>
<td>5.5</td>
<td>XG</td>
</tr>
<tr>
<td><em>Prevotella bryantii</em></td>
<td>PbGH5</td>
<td>β-glucanase/endo-xyloglucanase</td>
<td>37</td>
<td>5.0</td>
<td>XG - MLG</td>
</tr>
<tr>
<td><em>Vitis vinifera</em></td>
<td>VvEG16</td>
<td>endo-β-glucanase</td>
<td>40</td>
<td>5 → 6.5</td>
<td>XG - MLG</td>
</tr>
</tbody>
</table>

Xyloglucan (XG), Mixed-linkage glucan (MLG), (*) Tested at 37 °C
Specific activities of novel enzymes and commercial preparations

The specific enzyme activities of the commercial cellulase and beta-glucosidase enzyme preparations (Celluclast 1.5L and Novozyme 188) and various purified xyloglucanases (XG) and mix-linkage glucanases (MLG) were firstly assessed as described in section 2.4. As expected, the commercial cellulase enzyme mixture, Celluclast, showed the highest cellobiohydrolase (CBH) and endoglucanase (EG) activities, with significantly lower β-glucosidase and β-xylosidase activities. As expected, the Novozyme 188, a commercial considerable β-glucosidase preparation, showed high β-glucosidase activity (263 U/mg) with slight CBH activity, as shown in Table 7. In contrast, all of the tested purified cellulase “accessory” enzymes showed negligible CBH, endoglucanase, β-glucosidase and β-xylosidase activities, but high activities towards their specific substrates xyloglucan and mixed linkage glucan, respectively. It is worth noting that these cellulase accessory activities varied significantly according to the different enzymes, where PbGH5 showed the highest capacity towards xyloglucan (440 U/mg). As previously described by (McGregor et al., 2016), PbGH5 also shares comparable activity towards MLG (408 U/mg), with BoGH16 being the dominant mixed linkage glucanase (994 U/mg).

<table>
<thead>
<tr>
<th>Origin</th>
<th>Enzyme</th>
<th>Protein concentration, mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cellvibrio japonicus</strong></td>
<td>CjGH74</td>
<td>5.5</td>
</tr>
<tr>
<td></td>
<td>CjGH5F</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>CjGH5D</td>
<td>8.9</td>
</tr>
<tr>
<td></td>
<td>CjXyl31A</td>
<td>2.6</td>
</tr>
<tr>
<td><strong>Bacteroides ovatus</strong></td>
<td>BoGH5</td>
<td>14.9</td>
</tr>
<tr>
<td></td>
<td>BoGH31A</td>
<td>4.1</td>
</tr>
<tr>
<td></td>
<td>BoGH2</td>
<td>17.2</td>
</tr>
<tr>
<td></td>
<td>BoGH16</td>
<td>4.1</td>
</tr>
<tr>
<td><strong>Prevotella bryantii</strong></td>
<td>PbGH5</td>
<td>10.9</td>
</tr>
<tr>
<td><strong>Paenibacillus pabuli</strong></td>
<td>PpXGH5</td>
<td>4.9</td>
</tr>
<tr>
<td><strong>Vitis vinifera</strong></td>
<td>VvEG16</td>
<td>9.4</td>
</tr>
</tbody>
</table>

3.1.3 Specific activities of novel enzymes and commercial preparations

The specific enzyme activities of the commercial cellulase and beta-glucosidase enzyme preparations (Celluclast 1.5L and Novozyme 188) and various purified xyloglucanases (XG) and mix-linkage glucanases (MLG) were firstly assessed as described in section 2.4. As expected, the commercial cellulase enzyme mixture, Celluclast, showed the highest cellobiohydrolase (CBH) and endoglucanase (EG) activities, with significantly lower β-glucosidase and β-xylosidase activities. As expected, the Novozyme 188, a commercial considerable β-glucosidase preparation, showed high β-glucosidase activity (263 U/mg) with slight CBH activity, as shown in Table 7. In contrast, all of the tested purified cellulase “accessory” enzymes showed negligible CBH, endoglucanase, β-glucosidase and β-xylosidase activities, but high activities towards their specific substrates xyloglucan and mixed linkage glucan, respectively. It is worth noting that these cellulase accessory activities varied significantly according to the different enzymes, where PbGH5 showed the highest capacity towards xyloglucan (440 U/mg). As previously described by (McGregor et al., 2016), PbGH5 also shares comparable activity towards MLG (408 U/mg), with BoGH16 being the dominant mixed linkage glucanase (994 U/mg).
In addition, CjGH74 and BoGH5 xyloglucanases showed relatively low capacity towards xyloglucan. However, both of these enzymes were expected to increase the release of glucose molecules, once combined with the debranching enzyme, α-xylosidase.

3.1.4 Screening of the possible contribution of novel, accessory enzymes when added to the “cellulase mixture” Celluclast

In order to evaluate the effectiveness of novel accessory enzymes, substrates with different physicochemical characteristics were selected. Briefly, aspen and corn stover were selected as representatives of hardwood and agricultural residues, respectively. Mechanical pulping and alkali based pretreatments such as TMP-bio, RMP, and AFEX were employed to minimize the structure and distribution of hemicelluloses within the biomass, while the acid catalyzed steam explosion was used to remove hemicellulose during pretreatment. As expected for hardwood- and agricultural- based substrates, the main hemicellulosic component was xylan. Its content ranged from 3.6 to 22.7% (Table 8). Refiner mechanical pretreated aspen (RMP) and ammonia fibre expansion pretreated corn stover (AFEX CS) contained large amounts of hemicellulose which had

<table>
<thead>
<tr>
<th>Enzyme mixture</th>
<th>CBH</th>
<th>Endoglucanase</th>
<th>β-glucosidase</th>
<th>β-xylosidase</th>
<th>XG</th>
<th>MLG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Celluclast 1.5L</td>
<td>137.5</td>
<td>413.0</td>
<td>14.8</td>
<td>32.9</td>
<td>2.8</td>
<td>6.1</td>
</tr>
<tr>
<td>Novozyme 188</td>
<td>28.8</td>
<td>16.5</td>
<td>262.9</td>
<td>4.3</td>
<td>n/a</td>
<td>n/a</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Purified enzymes</th>
<th>XG</th>
<th>MLG</th>
</tr>
</thead>
<tbody>
<tr>
<td>BoGH5</td>
<td>7.5</td>
<td>n/a</td>
</tr>
<tr>
<td>BoGH16</td>
<td>n/a</td>
<td>993.8</td>
</tr>
<tr>
<td>CjGH74</td>
<td>8.5</td>
<td>n/a</td>
</tr>
<tr>
<td>VvEG16</td>
<td>0.01</td>
<td>0.05</td>
</tr>
<tr>
<td>PbGH5</td>
<td>440.4</td>
<td>408.3</td>
</tr>
</tbody>
</table>

CBH: Cellbiohydrolases; XG: Xyloglucanase, MLG: Mixed linkage glucanase; n/a: negligible activity detected
hemicellulosic structures closer to the original structure of the biomasses (Balan et al., 2009; Chundawat et al., 2010; Gunawan et al., 2017) (Table 8).

Table 8. Chemical composition of aspen and corn stover substrates from different pre-treatment technologies, presented as %

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Pretreatment</th>
<th>Ara, %</th>
<th>Gal, %</th>
<th>Glu, %</th>
<th>Xyl, %</th>
<th>Man, %</th>
<th>AIL, %</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspen</td>
<td>Thermomechanical pulpning</td>
<td>3.5</td>
<td>3.3</td>
<td>46.9</td>
<td>12.4</td>
<td>2.2</td>
<td>19.8</td>
<td>TMP-bio</td>
</tr>
<tr>
<td></td>
<td>Refiner mechanical pulpning</td>
<td>3.7</td>
<td>4.0</td>
<td>42.2</td>
<td>13.3</td>
<td>2.3</td>
<td>28.9</td>
<td>RMP</td>
</tr>
<tr>
<td></td>
<td>Steam pretreatment</td>
<td>0.3</td>
<td>0.1</td>
<td>54.5</td>
<td>3.6</td>
<td>2.1</td>
<td>35.5</td>
<td>SPA</td>
</tr>
<tr>
<td>Corn stover</td>
<td>Steam pretreatment</td>
<td>1.3</td>
<td>0.4</td>
<td>49.3</td>
<td>16.7</td>
<td>1.2</td>
<td>23.1</td>
<td>SPCS</td>
</tr>
<tr>
<td></td>
<td>Ammonia fiber expansion</td>
<td>3.8</td>
<td>1.3</td>
<td>35.7</td>
<td>22.7</td>
<td>1.7</td>
<td>15.0</td>
<td>AFEX CS</td>
</tr>
</tbody>
</table>

Ara Arabinan, Gal Galactan, Glu Glucan, Xyl Xylan, Man Mannan, AIL Acid Insoluble Lignin

By comparing the relative amounts of the hemicellulosic sugars xylose, arabinose, galactose and mannose (Table 8), it was clear that the steam explosion pretreatment of both types of biomasses differently affected the solubilisation of the hemicellulosic component. Specifically, steam explosion pretreatment was highly effective in removing hemicelluloses from hardwoods, probably due to the high level of acetylation and high presence of glucuronic acid groups along the hemicellulosic backbone. This was apparent as the steam-pretreated aspen had a significantly lower percent of arabinose (0.3%), galactose (0.1%) and xylose (3.6%) in comparison to mechanical treatments. In contrast, SO₂ catalyzed steam pretreated corn stover (SPCS) retained a greater portion of the hemicellulosic component, resulting in a significant higher portion of xylose (16.7%) in comparison to the steam pretreated aspen. This is probably due to the lower level of acetylation characteristic of xylans from agricultural residues, as xylose units are double substituted by arabinose residues and not acetyl groups (section 1.3.1).
Both mechanically treated aspen substrates have shown clear differences in their lignin content, but slight differences in their hemicellulose compositions. As observed, the hemicellulose fractions only differed by 2% whereas the lignin content of thermomechanical-pretreated aspen (TMP-bio) was reduced by 10% by the mild alkali treatment. The utilization of substrates with these structural differences allows us to further assess how the presence of the remaining hemicellulose influence the addition of accessory enzymes, such as xyloglucanases and mixed linkage glucanases. Thus, this range of pretreated substrates should provide ideal candidates for evaluating the roles and functions of these novel hemicellulase enzymes (Table 8). (The production and characterization of these substrates is presented in Materials and methods, section 2.1).

The cellulose hydrolysis boosting effects of these potential hemicellulose backbone attacking enzymes (endo-xyloglucanases and endo-β-glucanases) and debranching enzyme (α-xylosidase) were initially assessed on a model substrate where microfibrillated cellulose (MFC) was mixed with a highly substituted xyloglucan from tamarind seed (Figure 3). As expected, the addition of xyloglucan to MFC clearly reduced the cellulose hydrolysis by cellulase acting enzymes (Celluclast), while the addition of xyloglucanase could increase the cellulose hydrolysis back to the initial level (Figure 3). Therefore, it seems apparent that the xyloglucan acts as a physical barrier, covering the cellulose microfibrills, and reducing the efficacy of the cellulases. Consequently, the supplementation of xyloglucanases would help break down this barrier and improve cellulases accessibility to cellulose microfibrills. This was the main objective of this thesis work. As mentioned several times before, xyloglucan is strongly associated with cellulose microfibrills (Pauly et al., 1999; Vincken et al., 1995) and therefore, its removal is expected to facilitate cellulose hydrolysis (Kaida et al., 2009).
The potential beneficial influence of these accessory enzymes were next assessed on a “library” pretreated substrates, as described in section 2.2. First, the cellulase enzyme loading was defined as the minimum loading required to achieve 60% cellulose hydrolysis after 72 hrs hydrolysis. Accessory enzymes were incorporated at 3 different protein loadings, 1, 2.5 and 5 mg/g in order to define the minimum amount required to achieve over a 10% increasing in cellulose hydrolysis and a final cellulose hydrolysis yield of 70%. In the following figures, only the selected loading defined as 2.5 and 5 mg/g for corn stover and aspen substrates are shown. It appeared that hemicellulases cooperated synergistically with Celluclast to enhance cellulose hydrolysis. However, the improvement was both substrate and enzyme dependent. The xyloglucanases appeared to cooperate with the cellulases to a greater extent than did the mixed linkage glucanases (Figure 5). Despite the documented higher xyloglucan content in hardwoods (Fry, 1989), xyloglucanases were less effective in hydrolyzing the aspen substrates and showed better

**Figure 4.** Xyloglucan acts as a physical barrier to cellulases and therefore, xyloglucanases are required to improve cellulose accessibility. Model substrates were prepared at incubating tamarind xyloglucan (10% w/w) with microfibrillated cellulose (MFC) for 72 h and enzymatic hydrolysis was carried out at 1% substrate concentration, cellulases loading (Celluclast plus β-glucosidase) of 7.5 mg protein/g cellulose and xyloglucanases loading of 2 mg protein/g cellulose.
performance on corn stover, as it required half of the enzyme loading (5 vs 2.5 mg/g) to reach the 10% increment in cellulose hydrolysis.
Figure 5. Xyloglucanases-backbone acting enzymes enhance cellulose hydrolysis in corn stover (SPCS and AFEX CS) to a greater extent than it does aspen substrates (SPA and TMP-bio) at 2% substrate concentration and cellulase loading of 15 mg protein/g cellulose. Xyloglucanase loading supplemented was different for both tested biomass (mg protein/g cellulose): 2.5 (corn stover substrates) and 5 (aspen substrates). Data not shown for RMP.
The supplementation of only backbone acting enzymes did not seem to act synergistically with cellulases in both mechanically treated biomass. This was probably due to the limited accessibility of these accessory enzymes to their desired targets. Of the backbone-acting enzymes, CjGH74, BoGH5 and PpXGH5 were shown to be the most effective by enhancing cellulose hydrolysis to a greater extent. However, this confirmed past observations that the enhancement in cellulase hydrolytic potential varied with pretreatment and type of biomass (Benko et al., 2008; Gao et al., 2011; Hu et al., 2011).

Among the different enzymes assessed on corn stover, CjGH74 endo-xyloglucanase from *Cellvibrio japonicus* achieved a 10% improvement in AFEX CS. This implied that the xyloglucan remained substituted after pretreatment, but with some unbranched glucosyl residues regions, as CjGH74 only cleaves next to an unbranched glucosyl unit (Attia et al., 2016). In addition to cleaving at unsubstituted glucose units, CjGH74 is unable to hydrolyze shorter xyloglucan-oligomers and requires the presence of xylose residues on the β (1→4)-glucan backbone to effectively hydrolyze xyloglucan (Attia et al., 2016). Consequently, the reduced hydrolytic effect of CjGH74 on SPCS in comparison to AFEX CS might be due to the length reduction of the xyloglucan chains and loss of xylose residues during steam pretreatment.

In comparison to other tested xyloglucanases, the PbGH5A was able to hydrolyze at both unbranched and branched glucosyl residues (Larsbrink et al., 2014b). Therefore, because of this broader activity, it was expected that the PbGH5 might show greater synergism with cellulase enzymes, resulting in increased cellulose hydrolysis. However, both of the xyloglucanases, CjGH74 and BoGH5, were shown to be more impactful during the hydrolysis of the pretreated corn stover substrates (Figure 4). Interestingly, regardless of the lower xylose content of the steam-pretreated aspen, the addition of α-xylosidase CjXyl31A seemed to facilitate cellulose hydrolysis.
Mixed linkage glucanases (BoGH16 and VvEG16) were also assessed for their possible beneficial influence and compared to the xyloglucan acting enzymes. As mentioned before, β-glucan (MLG) are unique to the group that includes cereal and grasses such as corn stover (Ebringerová and Thomas, 2005), therefore it was expected that the mixed linkage glucanases such as VvEG16 and BoGH16 would have some impact on improving biomass deconstruction. In contrast to the specific mixed linkage glucanase BoGH16, VvEG16 has endo-glucanase activity not only in MLG, but also in XG. It was apparent that the supplementation of VvEG16 to cellulase enzyme mixtures resulted in a 10% increase in the glucose yield. However, the addition of the BoGH16 only resulted in a slight improvement. This indicated that the removal of xyloglucan is more relevant to enhanced cellulase hydrolytic potential than mixed linkage glucan removal. Slight improvements of up to 5% xylose release after xyloglucanases supplementation were also observed. However, similar to increased glucose release, the influence was substrate and enzyme dependent (Appendix A). However, it was anticipated that these values would increase once α-xylosidase is incorporated into the enzyme cocktail.

In the following section, the novel accessory enzymes that showed the best performance in increasing cellulose hydrolysis when combined with cellulases were next added with the debranching enzyme, α-xylosidase. The candidate enzymes selected were BoGH5 and CjGH74, both specific xyloglucanases, and VvEG16 and PbGH5, both endo-β-glucanases with shared activity for xyloglucan and mixed linkage glucan. In addition, two α-xylosidases (GH31) derived from different organisms such as Cellvibrio japonicus (CjXyl31A) and Bacteroides ovatus (BoGH31A) were assessed. Backbone-acting enzymes were paired with the α-xylosidase from the same organism, i.e., BoGH5 – BoGH31 and CjGH74 – CjXyl31A. It was speculated that, by using enzymes from a single organism is more likely that they will be compatible with each other because
they are co-evaluated in the same genome (Banerjee et al., 2010a) and thereby potentially achieve the greatest degree of synergism.

3.1.5 Combination between backbone acting novel enzymes and α-xylosidase

Our interest in studying the α-xylosidase was due to its absence in most cellulase commercial mixtures as *Trichoderma reesei* is unable to secrete this enzyme (Scott-Craig et al., 2011). Therefore, the potential of α-xylosidase to enhance lignocellulosic biomass deconstruction was assessed by combining this enzyme with commercial cellulase mixtures. In addition, as shown in the previous section, xyloglucanases have shown to be effective in enhancing cellulase hydrolytic potential. Thus we also wanted to evaluate the possible synergism between backbone-acting and debranching enzymes in order to maximize both glucose and xylose recoveries.

In this section, hemicellulose backbone attacking enzymes were assessed in combination with the debranching enzyme, α-xylosidase, from both *Cellvibrio japonicus* and *Bacteroides ovatus* (Figure 5). It appeared that the hemicellulases cooperated synergistically with α-xylosidase in enhancing cellulose hydrolysis. However, the improvements were only significant for specific substrates and enzyme combinations. The greatest improvements in cellulose hydrolysis were observed for both the alkaline treated substrates, TMP-bio and AFEX CS, where the addition of xyloglucan debranching enzymes further increased cellulose hydrolysis by 15 and 13%, respectively. These greater increments in cellulose hydrolysis of both substrates was likely related to the more intact hemicellulose structure after the alkali based pretreatments (Table 8).
Figure 6. Synergistic interaction between best backbone acting xyloglucanases BoGH5, VvEG16, CjGH74 and PbGH5 and debranching enzyme α-Xyl31A (α-31A) in corn stover and aspen substrates at 2% substrate concentration and cellulases loading of 15 mg protein/g cellulose (Celluclast plus β-glucosidase). Xyloglucanase loadings were different for both biomass (mg protein/g cellulose): 2.5 (corn stover substrates) and 5 (aspen substrates). Data not shown for RMP.
However, to achieve similar improvements in cellulose hydrolysis, the pretreated aspen substrates required double the amount of accessory enzymes than did the pretreated corn stover (Figure 6). It appeared that the addition of novel accessory enzymes is more effective in grass derived substrates.

As a result of the variety of enzymes and substrates tested, we next assessed the backbone-acting novel enzymes and α-xylosidase (Xyl31A) on pretreated aspen followed by pretreated corn stover substrates. Good synergistic interaction was apparent between both xyloglucanases BoGH5A and CjGH74 with their respective Xyl31A enzymes on the pretreated aspen substrate. This suggested the occurrence of a relatively high level of substitution – characteristic for xyloglucan in hardwoods – and lack of unbranched glucose residues prior to α-xylosidase supplementation (Attia et al., 2016; Scott-Craig et al., 2011). In addition, as α-xylosidase reduces the degree of xylose substitution of short xyloglucan-oligomers, it is likely that these are now easier to digest by the endoglucanases and β-glucosidases present in the cellulase mixture, increasing both glucose and xylose yields.

An increase of 11% in cellulose hydrolysis once α-xylosidase was supplemented to CjGH74 during the hydrolysis of TMP-bio was observed (Figure 6). Our previous results (section 3.1.4) suggested that xyloglucanases were not very impactful during the hydrolysis of aspen substrates. However, the effective cooperation with α-xylosidase suggested that both activities are required for effective xyloglucan degradation due to the presence of xylose residues. Endo-acting xyloglucanases help to release xyloglucan-oligosaccharides into the liquor phase, and then the α-xylosidase was able to remove the side groups to facilitate further digestion. Therefore, the utilization of both enzymes enhanced xyloglucan hydrolysis, further enhancing cellulose hydrolysis. This increase in cellulose hydrolysis after the combined addition of both types of

57
xyloglucanases was also likely related to the hydrolysis of the different xyloglucan oligosaccharides to monomers, which reduced their possible inhibitory effect on cellulases (Qing et al., 2010). In contrast, PbGH5A does not show a boosting effect after CjXyl31A addition in all pretreated substrates. This was probably due to the known catalytic flexibility of this enzyme that allows it to hydrolyze at both branch and unbranched glucose units (McGregor et al., 2016).

Similar to what was observed with the pretreated aspen substrates, a clear synergistic interaction was achieved by combining backbone-acting xyloglucanase BoGH5 with its respective α-xylosidase during the hydrolysis of corn stover substrates. Through combining BoGH5 – BoGH31A, cellulose hydrolysis was increased from 7 to 13% during the hydrolysis of AFEX CS. These results are in line with past work that shown an up to 10% increase in glucose and xylose yields once α-xylosidase derived from Aspergillus niger was included in the whole enzyme cocktail during the hydrolysis of alkali treated corn stover (Jabbour et al., 2013; Walton et al., 2016). As mentioned before, in addition to xyloglucan acting enzymes, mixed linkage glucanases were also studied. Specifically, endo-β-glucanase VvEG16 with shared activity for XG and MLG also showed a synergistic effect when combined with α-xylosidase. This is likely due to the xyloglucanase capacity of this enzyme, as previous results showed that strict mixed linkage glucanases did not enhance cellulose hydrolysis.

Similar to the observed synergistic interaction on the aspen substrates, the synergistic interaction of CjGH74 - CjXyl31A during hydrolysis of corn stover substrates increased cellulose hydrolysis. However, the increase in the degree of cellulose hydrolysis was lower than that observed with the aspen substrates. This was probably a consequence of the lower xylose degree of substitution of cereal and grasses in comparison to higher plants such as hardwoods (Scott-Craig et al., 2011). In addition, CjGH74 was unable to hydrolyze the glucan chains and showed remarkably
low hydrolytic activity towards hydroxyethyl cellulose and carboxymethyl cellulose in comparison to tamarind xyloglucan. Therefore, to achieve an effective xyloglucan hydrolysis using this endo-xyloglucanase, it is likely that the presence of xylose residues is required.

Interestingly, when comparing both xyloglucanases, BoGH5 and CjGH74, they both showed similarities in their degree of synergism with cellulolytic enzymes. However, once α-xylosidase was added, the enzymes derived from Cellvibrio japonicus were able to enhance hydrolysis to a much greater extent than Bacteroides ovatus, especially on substrates with higher hemicellulose content such as TMP. This difference in catalytic behavior may be related to the different specificities of both enzymes in removing xyloglucan from the xyloglucan-cellulose complex (section 1.3.1). Xyloglucanases has been shown to disrupt the cross-linked fraction as well as the tightly bound surface portion (Chanliaud et al., 2004). It is likely that the combination of CjGH74 – CjXyl31A is able to hydrolyze xyloglucan to a greater extent, including both the cross-linked and the superficial fractions. Thus, by exposing more cellulose fibrils, cellulose hydrolysis is enhanced.

3.1.6 Release of xyloglucan derived oligomers using novel enzymes

To try to better understand the beneficial action of adding xyloglucanases to cellulases during hydrolysis of various biomasses, we next assessed if the release of sugar in an oligomeric form was also increased when xyloglucan acting enzymes were supplemented to cellulases (Figure 7). Similar to endoglucanase, xyloglucanase addition also resulted in the release of short oligomeric chains. For the thesis work, we are interested in glucose release as well as production of xyloglucan-derived oligomers. The total amount of oligomers was measured by using acid hydrolysis to produce their component monosaccharides as described in section 2.3
The assessment involved the use of BoGH5A and CjGH74 (xyloglucanases) in addition to VvEG16 and PbGH5A (endo-β-glucanases) in the presence and absence of α-xylosidase. The amount of glucose and xylose released in oligomer form was increased for most of the enzymes on all tested substrates, but to different degrees (Figure 6). The corn stover substrates showed a slightly higher increase in oligomer sugars released from AFEX CS in comparison to SPCS, which is probably a consequence of a more intact and abundant hemicellulosic fraction within the AFEX CS substrate. For the AFEX CS substrate, the addition of the most active xyloglucanase, CjGH74, increased both glucose and xylose release in an oligomeric form by 17 and 5% respectively. Similarly, the combined action of BoGH5A and α-xylosidase increased both, glucose and xylose release in an oligomer form 12 and 7%, respectively, while also increasing cellulose hydrolysis by 13%. 
Figure 7. Xylose and glucose release in monomer and oligomers form increased during enzymatic hydrolysis of corn stover and aspen substrates after xyloglucanases supplementation. Xyloglucanase protein loading were different for both biomasses (mg protein/g cellulose): 2.5 (corn stover substrates) and 5 (aspen substrates), including both backbone-acting and debranching enzymes.
For TMP-bio, the amount of glucose in oligomer form was slightly increased by 3% for both xyloglucanases (BoGH5 and CjGH74), whereas glucose in monomer form was increased to a greater extent. When both backbone-acting enzymes are supplemented with their respective α-xylosidase, glucose release in monomer form is highly increased as well as xylose release in oligomer form. These results suggest that a big portion of xyloglucan oligomers was broken down to monomeric sugars. However, after hydrolysis a significant fraction remains untouched. From this analysis, it appeared that the synergism between xyloglucanase enzymes and cellulase enzymes resulted in an increase glucose and xylose release in monomeric form while also increasing oligosaccharide release.

![Figure 8](image)

**Figure 8.** Xyloglucan oligomers formation increases during enzymatic hydrolysis of corn stover and aspen substrates after xyloglucanases supplementation and it correlates with improvements in cellulose hydrolysis

In addition, xyloglucan-derived oligomer formation increased with the improvement in cellulose hydrolysis for all of the tested substrates (Figure 8). This seemed to confirm that the removal of xyloglucan as oligosaccharides facilitates cellulose degradation by exposing more cellulose fibres therefore, enhancing the effectiveness of cellulases in hydrolyzing lignocellulosic substrates.
3.1.7 Assessment of whether the addition of novel, accessory enzymes can enhance the hydrolytic potential of the CTec 3 “cellulase mixture”

The best candidates assessed with the traditional cellulase enzyme mixture (Celluclast) – BoGH5A, PbGH5, CjGH74 and VvEG16 – were next added to a recently developed cellulase preparation CTec 3. The complex commercial cellulase mixture CTec 3, includes a large variety of activities active on cellulose, hemicellulose and lignin components while also showing more tolerance to inhibitors, in comparison to the previous commercial enzymes mixture, Celluclast (Zhai et al., 2016). In addition, previous work had shown that the presence of xyloglucan reduces cellulase hydrolytic potential. Thus, its removal should enhance cellulose hydrolysis. This was why we though CTec 3 might act synergistically with the novel hemicellulase enzymes and enhance cellulose hydrolysis (Figure 9).

As expected, the new commercial enzymatic cocktail CTec 3 hydrolyzes lignocellulosic substrates in a more efficient matter as only a third of Celluclast/Novozyme188 enzyme loading was required to achieve similar cellulose hydrolysis yields. The addition of equal protein loadings of CTec 3 was more effective in hydrolyzing SPCS in comparison to alkali treated corn stover (AFEX). The higher cellulose hydrolysis yields observed for SPCS were likely due to the increased digestibility/hydrolyzability after steam explosion pretreatment.

Similar to previous results, improvements in cellulose hydrolysis after xyloglucanases supplementation were both enzyme and substrate dependent. The greatest improvements were observed for alkali treated aspen, TMP-bio. Cellulose hydrolysis was increased from 56 to 65% when CTec 3 was supplemented with endo-xyloglucanase, BoGH5.
In addition, BoGH5 enzyme also slightly increased the cellulose hydrolysis of both steam-pretreated corn stover and aspen substrates by 5 and 3%, respectively. Similarly, slight improvements in glucose release were observed for PbGH5 and VvEG16 enzymes. By comparing the performances of both enzyme cocktails once xyloglucanases are supplemented, there is a slight difference where xyloglucanase has a greater impact on improving cellulose hydrolysis. For example, despite the great influence of CjGH74 in increasing cellulase hydrolytic potential during the hydrolysis of AFEX CS (Section 3.1.4), no positive impact was observed when CjGH74 was combined with CTec 3. Cellulose hydrolysis remained unchanged. Similarly, the combined addition of BoGH5 and Celluclast resulted in a modest increase in cellulose hydrolysis, whereas a 9% increase was observed when added to CTec 3.

![Figure 9](image-url) Synergist interaction between novel accessory enzymes and commercial cellulase mixture CTec 3 in corn stover and aspen substrates. Total enzyme loading was 7 mg protein/g cellulose (5 mg/g of CTec 3 and 2 mg/g of each accessory enzyme candidate).

As a result of its complex composition, it was interesting to see the level of synergism between CTec 3 and BoGH5. However, the reasons behind this synergist interaction were difficult
to elucidate. However, it might be partially influenced by the broad active-site cleft of BoGH5, which might allow this enzyme to equally accommodate xyloglucans with difference degree of substitution (Larsbrink et al., 2014a).

3.1.8 Conclusions

The model substrate results showed that xyloglucans could act as a physical barrier to cellulase enzymes. Therefore, xyloglucanases can disrupt this “barrier” and improve cellulase enzyme action. Xyloglucanases were shown to be more effective in hydrolyzing both aspen and corn stover substrates than mixed linkage glucanases. However, the degree of interaction was highly dependent of the substrate. Both backbone-acting and debranching enzyme α-xylosidase were required for substrates with a higher hemicellulose content, such as TMP-bio and AFEX CS. An over 10% increase in cellulose hydrolysis resulted from this synergistic cooperation. Xyloglucanases were more effective in hydrolyzing corn stover than aspen substrates (2.5 vs 5 mg/g).

For this reason, further analysis in section 3.2 only included corn stover substrates.
3.2 Understanding the mechanism behind cellulase and xyloglucanase synergism

3.2.1 Background

Earlier work presented in Chapter 3.1 showed that the supplementation of xyloglucanase enzymes (backbone acting and α-xylosidase) derived from Bacteroides ovatus to commercial cellulase mixtures resulted in a synergistic interaction which considerably increased cellulose hydrolysis over a range of cellulosic substrates. However, the exact mechanism behind this synergistic interaction was not clear. It was apparent that the xyloglucanase BoGH5, which has a broader specificity in comparison with other xyloglucanases assessed here, showed a higher degree of synergism with cellulase enzymes. In addition, α-xylosidase (the enzyme used to remove xylose branched on xyloglucan backbone) was shown to be a key debranching enzyme for xyloglucan deconstruction. It increased cellulose hydrolysis significantly when combined with BoGH5. Due to the relevance of this debranching enzyme, we wanted to further assess the mechanism behind the observed synergistic interaction of cellulase and xyloglucanases. Thus we used a time course analysis of hydrolysis to evaluate each substrates physicochemical characteristics, employing both acid and alkaline pretreated substrates. As the xylose units are typically substituted with galactose units, which may greatly impede the access to xylose units and xyloglucan backbone (section 1.6.1.1), we also assessed the possible role and function of both xyloglucan debranching enzyme – α-xylosidase and β-galactosidase, in the efficient degradation of lignocellulosic substrates.

Our previous results in Chapter 3.1 suggested that xyloglucan acts as a physical barrier, blocking the access of cellulases enzymes and therefore, its removal by xyloglucanase is needed to achieve an efficient cellulose hydrolyze at low cellulase loading. To understand the reasons behind the observed synergism and the role of xyloglucan in the deconstruction of lignocellulosic biomass, time course of hydrolysis would be extremely valuable (Van Dyk and Pletschke, 2012; Malgas et
Past work analysing the impact of time on the degree of synergism, suggested that two different behaviors are generally observed: 1) a reduction or 2) an increase in the degree of synergism throughout the course of hydrolysis (Van Dyk and Pletschke, 2012; Malgas et al., 2017). The first behavior suggests that the greatest synergism is achieved at the early stage of hydrolysis and decreases overtime (Billard et al., 2012). The reasons behind this behavior are related to the high level of interlinkages within the lignocellulosic matrix at the beginning of hydrolysis, meaning that good cooperation is required at the early stage, resulting in substrate loosing and thereby creation of more binding sites overtime (Malgas et al., 2017). Consequently, at later stages of hydrolysis, enzyme synergism is no longer necessary. Alternatively, it has also been suggested that synergism increases during time course of cellulose hydrolysis, reaching its highest point at the final phase (Banerjee et al., 2010b; Jung et al., 2008; Song et al., 2016). The second behavior suggests that the number of cleavage sites available for the enzymes are limited and that the removal of one component exposes another that was previously inaccessible. Thereby, the enzymatic removal of the first component allows the synergistic cooperation between both enzymes, which extends overtime. Consequently, an analysis of enzyme synergism throughout the course of hydrolysis would be particularly valuable to help us understand the role of xyloglucan in the deconstruction of lignocellulosic biomass. It might explain why xyloglucanases are synergistically acting with cellulases.

In addition to the influence of time on degree of synergism, it is important to assess any changes on the substrate physicochemical properties after xyloglucanase treatment to confirm if the observed synergism is a result of an increase in cellulose accessibility after removing xyloglucan.
cellulolytic action. 2) the removal of the hemicellulosic tissue within the microfibrils loosens the intimate contact and allows fibers to swell (Bura et al., 2009; Hu et al., 2011; Hu et al., 2013; Jia et al., 2015). This resulting swelling effect enhances cellulose deconstruction as the accessibility of cellulase acting enzymes to the microfibrils is increased through the incorporation of accessory enzymes (Hu, 2014). In the work reported below we assessed changes in the physicochemical properties of the substrates after the addition of xyloglucan acting enzymes, including cellulose accessibility and gross fibre characteristics. We hoped that these analysis would help us elucidate the reasons behind the observed synergism between cellulolytic enzymes and novel xyloglucanases.

As previously discussed, the observed synergism was highly dependent on the pretreatment utilized as well as type of biomass, resulting in higher increments in the hydrolysis of corn stover substrates than hardwood ones. As mentioned earlier, during steam pretreatment a big portion of hemicellulose is lost as oligosaccharides in the water-soluble fraction, whereas by utilizing an alkali approach, most of the hemicelluloses are kept within the substrate. Therefore, it was anticipated that the supplementation of xyloglucanases would be of greater importance in the hydrolysis of AFEX CS than SPCS. By comparing both substrates, a key objective of this chapter was to assess if the utilization of the synergistic interaction between xyloglucanase and cellulase enzymes could result in a reduction of the overall protein/enzyme loading required to achieve over 70% cellulose hydrolysis.

This part of the thesis work assessed possible synergism throughout the course of hydrolysis and evaluating substrates physicochemical characteristics by employing both acid and alkaline pretreated substrates. All of these analyses will provide us with valuable insights about the synergistic interaction between cellulase and xyloglucanase enzymes and helps us evaluate if by
utilizing this synergism, it is possible to reduce the overall protein loading required to achieve over 70% cellulose conversion.

3.2.2 Enzymatic performance of “cellulase” commercial mixtures

As mentioned before, the addition of xyloglucanases was more efficient in hydrolyzing corn stover than aspen substrates, and hence this chapter will only focus in comparing two differently pretreated corn stover substrates: steam vs alkali. The reasons behind the selection of both substrates is related to our previous results, which has shown that the effectiveness of xyloglucanases in enhancing cellulose hydrolysis is highly dependent of the hemicellulosic composition after pretreatment (section 3.1). During steam pretreatment, a big portion of hemicellulose is lost as oligosaccharides in the water-soluble fraction, whereas by utilizing an alkali approach, most of the hemicelluloses are kept within the substrate while lignin is partially removed. Therefore, by selecting two different pretreatment approaches, we are comparing substrates with also contrasting hemicellulose distribution and composition (AFEX CS: 30%; SPCS: 20%). Therefore, it was anticipated that the supplementation of xyloglucanases would be of greater importance in the hydrolysis of alkali treated corn stover due to its higher hemicellulose content.

In order to better understand the reasons behind the synergism between cellulase and xyloglucanases, both corn stover substrates were first hydrolyzed using increased loadings of the two commercial “cellulase” mixtures, Celluclast and CTec 2. Celluclast is the traditional cellulase preparation which is mostly composed of canonical cellulose hydrolytic enzymes such as endo- and exo-glucanases, whereas CTec 2, a relatively new cellulase preparation, also contains accessory activities in addition to cellulase enzymes. The utilization of Celluclast allows us to define the efficiency of just cellulases in hydrolyzing the cellulose component of both substrates whereas the
utilization of CTec 2 could help us to better understand to what extent accessory activities contribute to enhancing the effectiveness of cellulase enzymes during cellulose deconstruction.

As expected, the use of different enzyme mixtures resulted in different hydrolysis behaviours in both corn stover substrates, with steam pretreated corn stover (SPCS) being more readily hydrolysed than ammonia pretreated corn stover (AFEX CS) (Figure 10). Despite a continuous increasing in the enzyme loading, both corn stover substrates reached a maximum point of cellulose hydrolysis, with CTec 2 being more effective than Celluclast in enhancing cellulose degradation. Higher cellulose hydrolysis values were achieved at equal enzyme concentrations.

This result highlights the importance supplementing cellulases with accessory enzymes to increase both sugar release and hydrolysis rate. By utilizing just Celluclast, maximum values of cellulose hydrolysis of 80% and 72% were achieved for SPCS and AFEX CS, respectively. Similarly, even at high doses of CTec 2, only an 80% of the glucose present in AFEX CS was

![Figure 10. Cellulose hydrolysis performance of “cellulase” commercial mixtures, Celluclast and CTec 2 at increasing protein loading in both pretreated corn stover substrates, SPCS (■) and AFEX CS (▲). Enzymatic hydrolysis using Celluclast (plus β-glucosidase) and CTec 2 were carried out at solid loadings of 2 and 10%, respectively along 72 hours.](image)
hydrolysed, whereas the cellulose within SPCS is almost completely hydrolysed. This indicated that other types of accessory activities might be needed for effective hydrolysis of AFEX CS. As indicated before, the alkaline pretreatment condition of AFEX CS results in more hemicellulose being retained within a more complex structure, thus other types of activities such as xyloglucanases might be required to achieve effective hydrolysis. Despite the increase in hydrolysis after steam pretreatment, the addition of accessory enzymes is still required for a more efficient and complete cellulose hydrolysis.

As shown in Figure 9, CTec 2 does not perform equally well in both pretreated corn stover substrates. Twenty percent of the cellulose within AFEX CS remains non-hydrolyzed. It is important to remember that AFEX CS has a chemical composition comparable to untreated corn stover, suggesting that the theoretical amount of glucose might not only be part of cellulose, but also from other hemicellulosic components, such as xyloglucan and mixed linkage glucan. This cannot be resolved using the Klason procedure, the commonly use method for measuring the chemical composition of lignocellulosic substrates, as it does not differentiate if the glucose comes from cellulose or hemicelluloses. The hypothesis that the glucose in AFEX CS might not all be part of cellulose is supported by our previous results in Chapter 3.1, where the addition of accessory enzymes, specifically xyloglucanases, had a greater impact in increasing the glucose release of the AFEX CS substrate than the SPCS substrate. Therefore, in order to have a more efficient and complete cellulose hydrolysis, it is likely that xyloglucanases are required for the hydrolysis of substrates with a high hemicellulose content. This also means that the increasing glucose release after xyloglucanases supplementation is likely due to cellulose hydrolysis as well as xyloglucan deconstruction.
3.2.3 Synergism between *Bacteroides ovatus* enzymes and cellulases is increased at later stage of hydrolysis

As shown in section 3.1.5, the combined addition of backbone acting enzyme BoGH5 and debranching enzyme α-xylosidase (BoGH31A) was shown to act synergistically with cellulase acting enzymes. However, this analysis was performed after 72 hours, without taking in consideration the influence of time, which has been shown to have a significant impact on the degree of synergism (Billard et al., 2012; Van Dyk and Pletschke, 2012; Malgas et al., 2017). Therefore, time point analysis throughout the course of hydrolysis would provide valuable insights to help us better understand the synergistic interaction between cellulase, backbone acting and α-xylosidase.

This analysis was performed by simultaneously adding both type of xyloglucanases to cellulase enzymes at the beginning of hydrolysis and utilizing both acid and alkali pretreated corn stover substrates at low consistency (2% w/v) (Figure 11). Similar to our previous results from Chapter 3.1, the cooperation between both backbone acting and debranching enzymes was more favourable in enhancing cellulose hydrolysis than adding only BoGH5. Interestingly, for both pretreated corn stover substrates the synergistic interaction between BoGH5 and BoGH31A showed the highest improvement at the latter stages of hydrolysis, with only slight differences observed between both substrates at the initial stages of hydrolysis.
The supplementation of xyloglucanases resulted in an increase in cellulose hydrolysis for AFEX CS in the initial hours, whereas no distinguishable changes were observed for the SPCS substrate. This distinction between both substrates is likely due to the relatively intact hemicellulosic compositions after AFEX pretreatment, suggesting that the more accessible portion of xyloglucan remains untouched during ammonia explosion pretreatment. These results are in line with past work which showed an increase in the degree of synergism at the later stage of hydrolysis for alkali treated corn stover (Banerjee et al., 2010b; Song et al., 2016; Zhang and Viikari, 2014). An increase in the degree of synergism throughout the course of hydrolysis suggested that, at the beginning, xyloglucan is not easily accessible and therefore does not necessarily impede cellulose hydrolysis. However, as cellulose hydrolysis proceeds a portion of the cellulose that was not accessible earlier is now more accessible. This suggests that the xyloglucan is part of the more recalcitrant biomass fraction and is intimately associated with cellulose microfibrills, as previously reported (Pauly et al., 1999; Vincken et al., 1995).

Figure 11. Time course of hydrolysis of pretreated corn stover substrates at 2% solids loading utilizing Bacteroides ovatus accessory enzymes, BoGH5 and BoGH31A. “Cellulase” enzyme loading was 15 mg/g cellulose (Celluclast plus β-glucosidase) and xyloglucanase loading was 2.5 mg protein/g cellulose. BoGH31A supplementation was in a ratio of 1:1.5 respect to BoGH5.
3.2.4 Is β-galactosidase supplementation required to enhance xyloglucan decomposition?

As described earlier, complete degradation of hemicelluloses requires the supplementation of backbone acting enzyme. However, debranching enzymes are also needed to hydrolyze side group units (Collins et al., 2005; Huang et al., 2017). As previous work had shown that the backbone-acting enzyme acts synergistically with debranching enzyme α-xylosidase we wanted to assess if the removal of the galactose residues through β-galactosidase supplementation would facilitate the removal of xylose units, and subsequently, xyloglucan hydrolysis (de Vries et al. 2000). For cereal and grasses, past work has shown the occurrence of galactose residues in xyloglucan (section 1.6.1.1). Furthermore, the location of galactose residues at the non-reducing end of xyloglucan chains has been shown to inhibit the hydrolytic performance of endoglucanases and β-glucosidases (Spier et al., 2015). Therefore, we next wanted to assess if the removal of galactose residues through the inclusion of β-galactosidase would facilitate xyloglucan deconstruction. To accomplish this objective, the supplementation β-galactosidase to cellulase enzymes was assessed by three supplementations. First, only β-galactosidase, then in combination with backbone acting BoGH5 and finally, including the three components BoGH5, α-xylosidase (BoGH31) and β-galactosidase (BoGH2A). It is important to note that all three enzymes were derived from the same organism Bacteroides ovatus and that all experimental conditions applied were the same as reported in the previous section. As the enzyme loading was kept constant (2.5 mg/g), the β-galactosidase loading was defined as 20% of the total with equal loadings of BoGH5 and BoGH31.

It appeared that the incorporation of BoGH2A to the cellulolytic enzyme cocktail enhanced cellulose hydrolysis after 72 hours for both pretreated corn stover substrates. However, to a lower extent than the individual supplementation of BoGH5 and BoGH31 (Figure 12).
After supplementing with BoGH2A, cellulose hydrolysis in AFEX CS was increased by 5%, whereas, no changes were observed during the hydrolysis of SPCS. When a BoGH5 – β-galactosidase mixture was tested, a different behavior was observed. The slight decrease in cellulose hydrolysis during the hydrolysis of SPCS suggested that the backbone acting enzyme, BoGH5, was more relevant for efficient deconstruction. However, a synergistic interaction was observed when BoGH5 and BoGH2 were added to cellulase enzymes during the hydrolysis of AFEX CS as cellulose hydrolysis increased by 8%. However, the observed synergism is clearly lower in comparison to the synergism observed between backbone acting the debranching enzyme, α-xylosidase. It appears that the supplementation of both BoGH31 and BoGH5 is required to increase cellulose enzymatic deconstruction through xyloglucan removal, whereas the addition of BoGH2 negatively impacted the hydrolysis. This reduction in cellulose hydrolysis is likely related to the structural changes that xyloglucan undergoes after removing the galactose residues. As suggested by previous literature, removing the galactose residues enhances the self-association and further precipitation of xyloglucan oligosaccharides (Sims et al., 1998). Thus, it is highly likely that
BoGH5 is not able to effectively break down these strongly associated oligosaccharides, which negatively influenced cellulose hydrolysis.

3.2.5 Influence of substrate concentration on enzyme synergism

In order to achieve commercial feasibility of the bioconversion process of lignocellulosic biomass to sugars, high sugar concentrations are required. One of the approaches utilized to meet this target is to increase substrate concentration during hydrolysis, using a 20-25% (DW) concentration/consistency to achieve high sugar concentrations (>100 g/L). However, by utilizing this consistency is also possible to encounter significant limitations, such as inefficient mass transfer, increased levels of enzyme inhibitors from both sugars and degradation products, reduced enzyme adsorption, among others (Cara et al., 2007; Modenbach and Nokes, 2013; Zhang et al., 2009; Lu et al., 2010). With the aim of reduce these limitations at high solids loadings, the removal of hydrophilic polymers such as xylan and pectins have being suggested as one way to facilitate substrate liquefaction during hydrolysis as water availability is increased in the hydrolysis media (Viamajala et al., 2009). Similarly, enzyme synergism between cellulase and xylanases has been shown to significantly improve the hydrolysis of pretreated lignocellulosic substrates at increasing substrate concentrations (Hu, 2014). Despite our interest in applying conditions as close as possible to industry standards, we only analyzed the supplementation of accessory enzymes at 10% consistency, as enzyme stock was not sufficient to test a 20% substrate concentration. In addition to testing a higher consistency, the synergistic effect between both types of enzymes, BoGH31 was tested at two different conditions. Either by simultaneously addition with BoGH5 at the start of hydrolysis, or sequentially added after 24 h (Figure 13).
At higher consistencies, the improvement in cellulose hydrolysis after xyloglucanases supplementation was increased in both substrates for both backbone acting and debranching enzyme, but to different degrees (Figure 13). The synergistic interaction between BoGH5 and α-xylosidase clearly enhanced cellulose hydrolysis to a greater extent that just adding BoGH5. This highlighted the importance that both activities are required to effectively hydrolyse xyloglucan. No significant differences were observed between simultaneous and sequential supplementations.

However, the addition of BoGH31 after 24 h clearly enhanced the hydrolytic potential of BoGH5 as cellulose hydrolysis was greatly increased.

![Graphs showing cellulose hydrolysis over time for AFEX CS and SPCS substrates.]  

**Figure 13.** Time course hydrolysis of pretreated corn stover substrates at 10% solids loading utilizing *Bacteroides ovatus* accessory enzymes, BoGH5 and BoGH31A. Simultaneous supplementation of BoGH31A to BoGH5 was compared to sequential addition after 24 hours. “Cellulase” enzyme loading was 30 mg protein/g cellulose (Celluclast plus β-glucosidase) and xyloglucanase loading was 2.5 mg protein/g cellulose. BoGH5 : BoGH31A supplementation ratio was 1.5 : 1

At high consistencies, the synergistic cooperation between xyloglucanases and cellulases increased cellulose hydrolysis by 18% during AFEX CS hydrolysis, whereas an 8% was observed for SPCS. As mentioned before, AFEX CS has a higher hemicellulose content and xyloglucan seems to be highly branched, hence both xyloglucanase activities are required to break down
xyloglucan. The addition of xyloglucanases also increased the reaction rate during the hydrolysis of AFEX CS, resulting in 60% cellulose hydrolysis after 24 hours and over 81% after 48 hours.

In addition, similar to our previous results presented in section 3.2.3, the synergistic cooperation between BoGH5 and α-xylosidase increased throughout the course of hydrolysis in AFEX CS, reaching a maximum point after 48 hours. This again suggested that the xyloglucan is part of the more recalcitrant fraction and that it is closely associated with cellulose microfibrils. As more sites are available for cellulose and xyloglucan hydrolysis, the degree of synergism is increased at the later stage of hydrolysis.

The lower synergistic interaction between novel xyloglucanases and cellulase enzymes in SPCS in comparison to AFEX CS, may be related to the ability of endoglucanase and β-glucosidases to also hydrolyze xyloglucan (Spier et al., 2015; de Vries and Visser, 2001). Both type of enzymes are components of the cellulase commercial enzyme mixture that were used. They are also able to degrade xyloglucan with a low degree of substitution, which is likely comparable to the xyloglucan present in steam pretreated corn stover. Therefore, extra supplementation of xyloglucanases is not required for the effective hydrolysis of SPCS to the same extent that is needed with the AFEX pretreated substrate.

As expected, a higher hemicellulose concentration through increasing substrate consistency from 2 to 10% had a positive impact in the synergistic interaction between both xyloglucanases as well as the individual effect of BoGH5 (Figure 14). In addition, the increase in cellulose hydrolysis was more apparent for the AFEX CS substrate, because of its higher hemicellulose content in comparison to SPCS.
The comparison between cellulose hydrolysis achieved by different cellulase commercial enzymes mixtures at 10% solid loading proves how effective both xyloglucan-acting enzymes are in enhancing cellulase hydrolytic potential in the hydrolysis of alkali pretreated corn stover (Figure 15). When comparing equal enzyme loading between different enzyme cocktails, the mixture between Celluclast and xyloglucanases mixture reached an 87% cellulose hydrolysis in the AFEX CS substrate, which was not achieved when only using cellulase enzymes or even a more complex enzyme mixture as CTec 2. A similar cellulose hydrolysis yield (83%) of the AFEX CS substrate resulted after adding 50 mg/gram of cellulose of CTec 2. It was apparent that there was the synergism between cellulase and xyloglucanases, reducing the enzyme loading required to achieve 80% cellulose hydrolysis. Lowering the enzyme loading by approximately 35% (from 50 to 32.5 mg/g). In addition, CTec 2 is more effective in hydrolyzing SPCS, reaching nearly complete cellulose hydrolysis, whereas the combination with Bacteroides ovatus accessory enzymes only hydrolyzed 80% of the available cellulose.

Figure 14. Influence of substrate loading in synergistic interaction between backbone acting BoGH5 and debranching enzyme BoGH31A in pretreated corn stover substrates. SPCS: steam pretreated corn stover; AFEX CS: ammonia fiber expansion pretreated corn stover.
3.2.6 Physicochemical changes after xyloglucanases treatment of pretreated corn stover

As discussed throughout this thesis, the combined mixture of xyloglucanase and cellulase enzymes results in synergism, increasing cellulose hydrolysis to a greater extent than when using just the cellulolytic acting enzymes. However, the exact mechanism behind this synergism is still unclear as the interaction is dependent on several factors including pretreatment conditions, type of accessory enzymes and substrate concentration. Substrates properties such as gross fibre characteristics and cellulose accessibility have been shown to influence the extent of cellulose hydrolysis.
3.2.6.1 Does xyloglucan removal increase cellulose accessibility?

As mentioned earlier, cellulose accessibility has been defined as one of the most important parameters influencing effective enzyme mediated cellulose hydrolysis (Arantes and Saddler, 2010; Rollin et al., 2011). Among the different techniques currently used to assess the specific surface area of cellulose, the use of orange dye in the Simons’ staining (SS) technique has provided valuable information of the hydrolyzability of a substrate. The direct orange dye is highly specific in binding cellulose fibres rather than hemicellulose and/or lignin (Chandra et al., 2008; Chandra et al., 2009). Previous studies have shown that the total amount of direct orange dye adsorbed correlates well with the easier hydrolyzability of substrates as higher enzymatic hydrolysis yields are achieved (Chandra and Saddler, 2012). Thus, with the goal of better understanding the effects of removing xyloglucan, we used the SS technique to assess if cellulose accessibility (or total orange dye adsorbed) for both corn stover substrates varied after 24 hours of xyloglucanases treatment with/without cellulases (Figure 16).

![Bar chart](image)

**Figure 16.** Changes in “cellulose accessibility” or adsorbed, Simons’ staining, orange dye, mg/g, after xyloglucanases treatment with/without commercial “cellulase” mixture along 24 hours. C: Cellulases mixture of Celluclast and Novo188 (β-glucosidases); α-xyl: α-xylosidase, BoGH31A. Control: sodium acetate buffer.
It was apparent that there were differences in the amount of dye adsorbed when the xyloglucanases were individually supplemented or when combined with cellulase enzymes. Previous results had shown that the amount and type of xyloglucan between both corn stover substrates seems to differ. Our previous analysis suggest that the xyloglucan present in AFEX CS had a higher degree of substitution while a significant portion seemed to be trapped between cellulose microfibrils. In contrast, the xyloglucan in the SPCS substrate had less influence, probably because most of the xylose residues were lost during pretreatment. The data presented in Figure 15 seems to support these conclusions, as the amount of orange dye adsorbed in AFEX CS substrate increased when the xyloglucanases were supplemented to the cellulases. This increase in dye adsorption may be caused by two reasons. The removal of xyloglucan exposes an untouched fraction of cellulose microfibrils and/or the removal of xyloglucan within the microfibrils loses the intimate contact between them allowing to swell and consequently, adsorb more dye. This is consistent with a proposed model that tried to elucidate the role of xyloglucan within the cell wall structure where a significant portion of xyloglucan is located at inaccessible junctions between microfibrils (Park and Cosgrove, 2012). In addition, previous studies which have attempted to measure the capacity of xyloglucan to bind cellulose using $^{13}$C – NMR have shown that less than a 10% of the cellulose surface is covered by xyloglucan. It has been suggested that the rest is trapped within the microfibrills (Cosgrove and Jarvis, 2012). This is also consistent with our previous findings which reported that the performance of xyloglucanases increased over time. Thus, the combined interaction between cellulases and xyloglucanases is required to effectively hydrolyze cellulose chains as well as the portion of xyloglucan trapped within them.

It was apparent that the use of different type of xyloglucanases in combination with cellulases resulted in different responses in the measurement of cellulose accessibility. As expected
for both substrates, there is a reduction in cellulose accessibility when cellulases were added, as less cellulose is available during the course of hydrolysis. However, changes in the total dye adsorbed were observed when xyloglucanases were added to cellulases. However, these changes were both substrate and enzyme dependent. Although xyloglucan depolymerisation through backbone acting BoGH5 did not change cellulose accessibility in the AFEX CS substrate, when the xylose residues were removed through α-xylosidase supplementation, there was a clear enhancement in the amount of adsorbed dye. It was even higher once both accessory enzymes were combined. This higher adsorption was likely due to the exposure of more cellulose fibrils as the xyloglucan is hydrolyzed.

Particularly for the SPCS substrate, it seemed to be a result of a reduction in cellulose availability as the amount of adsorbed orange dye was reduced when xyloglucanases were supplemented to cellulases. This was even more apparent when the xylose residues were removed. In the presence of both the BoGH5 and BoGH31, there was a slight reduction in cellulose accessibility when the xyloglucan was removed. It is possible that the xyloglucan acts as a spacer between the microfibrils. Therefore, its removal increases the interlinking between the cellulose microfibrills causing a reduction in cellulose accessibility and total dye adsorbed.

As discussed earlier, each of the corn stover substrates were hydrolysed differently after xyloglucanases supplementation. The AFEX CS substrate had improved cellulose accessibility whereas the opposite trend was observed for the SPCS substrate. At the same time, xyloglucanases were more effective in enhancing cellulose hydrolysis of AFEX CS than steam pretreated corn stover. As mentioned before, the increase in cellulose accessibility suggested that the removal of xyloglucan exposes an untouched fraction of cellulose microfibrils. Thus, the removal of xyloglucan within the microfibrills loosens the intimate contact between them allowing the fibers
to swell and consequently, adsorbed more dye. Hu et al., (2011) reported that the observed synergism between xylanase and cellulase enzymes is related to increased cellulose accessibility as well as fibre swelling as fibre width is higher after hemicellulose removal. Therefore, in the following section, microfibrills characteristics including fibre width and length were assessed for both corn stover substrates. We hoped to better understand whether the increase in cellulose accessibility after removing xyloglucan was due to a swelling effect.

3.2.6.2 Changes in gross fibre characteristics after xyloglucanase treatment

It is important to note the difference in fibre lengths between both of the substrates following their respective pretreatments (Figure 17). For steam-pretreated substrates, it has been shown that a reduction in particle size during pretreatment is related to higher hydrolysis yields and this increases with severity (Mansfield et al., 1999). In the work reported here, steam pretreated fibres were 18.6 μm in mean width and 0.516 mm in length, whereas the AFEX CS fibres were almost double in width (30.6 μm) and considerable longer (0.796 mm). This difference in gross fiber characteristics might help explain the more readily hydrolyzability of SPCS substrate in comparison to the AFEX CS substrate as smaller fibres are easier to digest.

As expected, fibre fragmentation increased after the addition of cellulase enzymes as the length weighted in both substrates was reduced (SPCS: from 0.516 to 0.449 mm and AFEX: from 0.796 to 0.581 mm). In addition, the distribution of fibre size reflects the reduction in particle size following steam explosion pretreatment. Close to 50% of the fibres were between 0.05 and 0.25 mm, whereas for the alkali treated corn stover, only 34% were below this range.
Figure 17. Fibre size distribution after xyloglucanases treatment with/without commercial “cellulase” mixture after 24 hours in SPCS and AFEX CS. BoGH5: backbone acting enzyme; α-xyl: α-xylosidase, BoGH31A. Control: sodium acetate buffer. Cellulases: Celluclast enzyme mixture plus β-glucosidase.
When individual xyloglucanases were added to the SPCS substrate, the fibre lengths remained relatively constant (~ 0.52 mm) and no differences were observed between backbone acting and debranching enzymes derived from *Bacteroides ovatus* (Figure 16). These results were confirmed through the comparison of particle size distribution utilizing both FQA and Mastersizer 2000 (results in Appendix B). For both techniques, insignificant changes were observed. The addition of cellulase enzymes increased the level of fibrillation in both substrates, as the number of smaller fibres between 0.05 mm to 0.12 mm in length increased. However, when accessory enzymes were combined with cellulases, no changes in particle size distribution were observed (Figure 16).

As mentioned earlier, not only fibre length is reduced during steam pretreatment, but also mean fibre width. The cellulosic fibres after steam explosion pretreatment have a mean width of 18.6 μm, which is considerably lower to the 30.6 μm width of the alkali treated ones. These values remained relatively constant after xyloglucanases treatment in comparison to the control (Figure 18). However, once cellulases were added, the fibre mean width varied depending on the substrate. For the AFEX CS substrate there was a significant reduction in width from 30.6 to 27.6 μm whereas for the SPCS substrate the mean width slightly increased from 18.6 to 20.9 μm. The reduction in fibre width was likely related to the enzymatic action of cellulase enzymes, which progressively peel off the surface of the cellulosic fibrils. Similarly, the combination of cellulase and xyloglucanase enzymes have different effects on fibre mean width, resulting in a decrease in mean width for the AFEX CS substrate but an increase for the SPCS substrate.
Given the variability of these results, we hypothesized that they are probably related to the technical limitations of using FQA to determine fibre width, in which only fibres over 0.50 mm in length are included in the calculation. By only incorporating fibres over 0.50 mm in length, only 23 and 30% of the total amount of fibres present in SPCS and AFEX CS, respectively, were included in the final calculation. The exclusion of shorter fibres means that the values reported might not represent the true nature of all the fibres present in both substrates.

However, it was apparent that xyloglucan removal increased cellulose accessibility. It is also likely that xyloglucan acts as a physical barrier, possibly coating cellulose microfibrills and/or restraining cellulose fiber swelling.

**Figure 18.** Change in mean fibre width after xyloglucanases treatment with/without commercial “cellulase” mixture after 24 hours in SPCS and AFEX CS. α-xyl: α-xylosidase, BoGH31A. Control: sodium acetate buffer. Cellulases: Celluclast enzyme mixture plus β-glucosidase.
3.2.7 Conclusions

The focus of this part of the thesis was to assess the degree of synergism between cellulase enzymes and xyloglucanase accessory enzymes. As discussed earlier, the improvement in cellulose hydrolysis after the addition of both backbone-acting and debranching enzyme α-xylosidase was substrate dependent. As expected, greatest synergism was observed for alkali pretreated corn stover as the hemicellulosic fraction remains mostly intact during pretreatment. In contrast, steam pretreatment seems to decreases xyloglucan branching, losing xylose residues. This reduction in xylose residues resulted in a lower degree of synergism, as the addition of debranching enzymes α-xylosidase did not enhance cellulose deconstruction. The analysis of the hydrolytic performances of commercial enzymes mixtures, Celluclast and CTec 2, showed the effectiveness of acid catalyzed steam pretreatment in enhancing substrates digestibility, as nearly complete cellulose hydrolysis was achieved at high enzyme concentrations. The same analysis shown that only an 80% of the cellulose present in the AFEX pretreated substrate was hydrolyzed when only cellulase are utilized. This supported earlier results where the inclusion of accessory enzymes such as xyloglucanases were required to enhance cellulose hydrolyze. Time point analysis provided us with valuable information in which xyloglucan seems to be part of the most recalcitrant fraction of biomass, as the degree synergism increased through the course of hydrolysis.

By combining cellulase with xyloglucanase enzymes, BoGH5 and BoGH31, it was possible to reduce protein/enzyme loading by 35% of the required cellulase dosage needed to achieve 80% cellulose hydrolysis of the AFEX CS substrate. However, supplementation was less effective for the SPCS substrate as with most of the hemicellulose already solubilised during pretreatment, commercial cellulase mixtures were already effective. These results
seemed to support previous studies, which indicated that a significant portion of xyloglucan is trapped within the microfibrils. It appears that much of the xyloglucan is highly interconnected with cellulose and its removal can increase cellulose accessibility by exposing new cellulosic fibres, consequently facilitating enzyme mediated lignocellulosic substrate deconstruction.
4 Final conclusions and future work

4.1 Conclusions

As mentioned at the start of the thesis, there is no previous literature which describes possible synergism between cellulolytic enzymes and xyloglucan enzymes such as backbone-acting and debranching enzymes. The thesis work looked at how enzymatic removal of xyloglucan might influence the hydrolytic performance of cellulase enzymes when applied to lignocellulosic substrates. It is likely that xyloglucan acts as a physical barrier restricting access of some of the cellulose to cellulase enzymes. Therefore, xyloglucanases contribute to the disruption of this “barrier”, improving overall cellulose hydrolysis. However, the degree of synergism with cellulase enzymes is highly substrate dependent, with xyloglucanases being more effective in hydrolyzing corn stover than aspen substrates. The combination of backbone-acting and debranching enzyme α-xyllosidase resulted in about a 10% improvement in cellulose hydrolysis in substrates with high hemicellulose content, such as TMP-bio and AFEX CS. However, by using this enzyme synergism, it was possible to reduce enzyme loading by 35% to achieve 80% cellulose hydrolysis after 72h.

Following synergism throughout the course of hydrolysis also provided us valuable information which suggested that the xyloglucan was part of the more recalcitrant biomass fraction as enzyme synergism increased over time. In addition, our work supported previous studies that suggested that a significant portion of the xyloglucan is trapped within the microfibrils, implying that xyloglucan removal exposes new cellulosic fibres. This resulting increase in accessibility enhances enzyme mediated biomass deconstruction. The xyloglucanases – backbone-acting + debranching enzyme - from Bacteroides ovatus showed
good potential as a suitable candidate for supplementing current commercial cellulase preparations, likely enhancing the hydrolysis of many pretreated biomass substrates. Effective synergism was reflected in the amount of sugars released, both monomers and oligomers, and in increased cellulose accessibility.

4.2 Future work

4.2.1 Confirm xyloglucan location and assess the addition of xyloglucanases

It appeared that the synergism between cellulase and xyloglucanase enzymes was maximum at the later stage of hydrolysis. This suggested that a significant portion of the xyloglucan was trapped between the microfibrills, making cellulose more resistant to enzymatic attack. Further assessing xyloglucan location by using microscopy techniques might provide invaluable insights in better understanding the observed synergism.

4.2.2 Addition of other accessory enzymes in combination with novel xyloglucanases

By combining xyloglucanase enzymes with other accessory enzymes, such as xylanases, would be interesting to evaluate, to see if a further reduction in protein loading can be achieved. In addition, we should evaluate the optimum ratio of each component to reach maximum degrees of synergism.

4.2.3 Use of xyloglucanase enzymes for xyloglucan-oligosaccharides production

Although the main objective of this study was to maximize sugar recovery in monomer form, xyloglucan-oligosaccharide production by the novel xyloglucanases is also of interest to other sectors. For example, they have been used in applications such as sizing agents for textiles, wet-
end additives in papermaking and as a bounding agent to modify cellulosic surfaces. They could also be evaluated for the potential production of probiotics
References


Hu J. 2014. The role of accessory enzymes in enhancing the effective hydrolysis of the cellulosic component of pretreated biomass:1–209.


Hu J, Arantes V, Saddler JN. 2011. The enhancement of enzymatic hydrolysis of lignocellulosic substrates by the addition of accessory enzymes such as xylanase: is it an additive or synergistic effect? *Biotechnology and Biofuels* **4**:36.


Appendices

Appendix A. Xyloglucanase supplementation enhanced xylose release after 72h

Hydrolysis was carried out at 2% substrate concentration and cellulases loading of 15 mg protein/g cellulose (Celluclast: Novozyme 188, 1:2). Xyloglucanase loadings were different for both biomass (mg protein/g cellulose): 2.5 (corn stover substrates) and 5 (aspen substrates).
Appendix B. Particle size distribution of SPCS after xyloglucanase treatment

Analysis was performed in presence and absence of cellulase enzymes, along 24 hours.

Cellulase: commercial “cellulase” mixture Celluclast and Novo188 (β-glucosidases).

Control: Hydrolysis with only sodium acetate buffer.